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Correlation of Iron Metabolism Genes Haplotypes with Beta Thalassemia Major patients in Palestine

By

Hilal Saleh Odeh

Supervisor

Prof. Hisham Darwish

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Members of the Thesis Committee:

1. Prof. Hisham Darwish .....
2. Dr. Fekri Samara .....
3. Dr. Saad Al-Lahham .....

Declaration

Here I declare that my MSc thesis entitled "Correlation of Iron Metabolism Genes Haplotypes with Beta Thalassemia Major patients in Palestine" is the result of my own research and was written independently with no other sources than quoted.

Hilal Saleh Odeh
Signature:
Date: 29/3/2023

Dedication

This thesis is dedicated to my beloved father, mother, my wife, and siblings

It is also dedicated to my colleagues

Hilal Saleh Odeh

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I would like to express my deepest gratitude to my supervisor Professor Hisham Darwish for the unlimited encouragement and support during my project starting from proposal writing until my dissertation. Prof. Hisham was a great source of experiences and valuable knowledge. I acquired from him the ability to design, perform experiments, and how to evaluate and interpret results to link things together to come up with conclusions and new hypotheses. I also learned how to be a member of a team in a scientific project. In addition to non-science topics, I have learned about away from the field. Prof Hisham was not only a great supervisor but a wonderful and cheerful friend.

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Abstract

Thalassemia are a group of common inherited blood disorders caused by defect in the globin protein production. Iron overload is associated with increase morbidity in transfusion - dependent thalassemia. we hypothesized that iron dysregulation may play a significant role in thalassemia pathogenesis. The aim of this study was to investigate the association of a number of single nucleotide polymorphisms (SNPs) in selected genes involved in Fe metabolism including rs11915082 I transferrin receptor 1 (TFRC) gene, rs1048230 and rs224589 in solute carrier family 11 member 2 (SLC11A2) gene, rs1439816 in solute carrier family 40 member 1 (SLC40A1) gene, rs10421768 and rs104894696 in hepcidin antimicrobial peptide (HAMP) gene and rs1799945 in the human homeostatic iron regulatory protein (HFE) with thalassemia and the corresponding iron overload complication in these patients. The study subjects comprised of 88 β-thalassemia patients and 88 controls. Genotypes were determined by RFLP-PCR, ARMS and Sanger's sequencing. The results showed the C allele of rs1439816 in the ferroportin gene was associated with thalassemia pathogenesis ($p < 0.0001$, OR = 12.21, 95% IC =7.38- 20.22). However, genetic variants in the other indicated genes failed to show significant association with iron overload or other disease complications due to the sporadic treatment plans for most patients and absence of reliable medical records concerning regular follow up and accurate monitoring. The rs1439816 variant (specifically the C allele) in the ferroportin gene represents a fairly strong indicator in thalassemia patients which provides a reliable additional marker for the clinical complexity of the disease.

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Abbreviation

ABCG2: ATP binding cassette protein G2

DNA: Deoxyribonucleic acid

DMT1: Divalent Metal Transporter 1

FPN: Ferroportin 1

FLVCR: Feline Leukemia Virus Subgroup C Cellular receptor

HAMP: Hepcidin Anti-Microbial Peptide

HEPH: Hephaestin

HFE: Hemochromatosis

HH: Hereditary Hemochromatosis

HLA: Human Leucocyte Antigen

IRE: Iron Responsive Element

IRP: Iron Responsive Protein

Jak-2: Janus Kinase 2

MRI: Magnetic Resonance Imaging

mRNA: Messenger Ribonucleic Acid

NTBI: Non-transferrin bound iron

PCR: Polymerase chain reaction

RBC: Red Blood Cell

RFLP: Restriction Fragment Length Polymorphism

ROS: Reactive Oxygen Species

SNP: Single Nucleotide Polymorphisms

SLC11A2: Solute Carrier Family 11 Member 2

STEAP: Six Transmembrane epithelial antigen of the prostate

TF: Transferrin

TFR1: Transferrin Receptor 1

TSAT: Transferrin Saturation

UTR: Untranslated region

WHO: World Health Organization

Chapter one: Introduction

1.1 Historical Background:

In 1925, Thomas B. Cooley, a physician in Detroit, recognized the first case of thalassemia in North America. He described the disorder in detail, noting that a group of children developed various deformities during their first year of life like bone change and splenomegaly. In 1936, Whipple and Bradford, described the pathological changes of the condition for the first time, they also recognized that many of their patients came from the Mediterranean region, and hence invented the word 'thalassemia' from the Greek (θαλασσα) meaning 'the sea' (CHERNOFF, 1959; Vichinsky et al., 2005). In the last decade, it became clear that thalassemia occurs widely throughout the world in about 60 countries with the highest level in the Mediterranean region where this condition is considered a major public health issue (F. Habibzadeh, M. Yadollahie, A. Merat, n.d.; Vichinsky et al., 2005).

1.2 Thalassemia Background

Thalassemia's are a group of common inherited blood disorders, caused by defects in the globin protein production (Cao et al., 1997; Rund & Rachmilewitz, 2005). Thalassemia results from mutations in the human globin genes. These mutations can be classified into two groups including, those associated with impaired globin synthesis (Thalassemia) and those that produce structurally abnormal globin (Hb variant) (Galanello & Origa, 2010). One of the most common forms of this disorder is beta thalassemia, which affects the synthesis of the β-globin subunit. Another type of the disease, known as alpha thalassemia, results from

defects in the genes responsible for the synthesis of α-globin.(Modell & Darlison, 2008; Olivieri, 1999).. Beta thalassemia is the most widely spread form which causes severe anemia in the homozygous and compound heterozygous states while in the case of alpha thalassemia, and in order to produce a clinical phenotype, mutations in three or more of the alpha globin alleles are required (Al Haddad, 2012; Olivieri, 1999). The inheritance mode of β-thalassemia is autosomal recessive since carriers of this condition don't have clinical symptoms. When both parents are' carriers, for each pregnancy, there is a 25% chance that the child will be a patient, 50% chance that the child will be a carrier, and 25% chance that the child will be normal. Premarital diagnosis represents the only way for couples to avoid carrying a child with this condition. This disease is known as multi-organ complication and can affect various aspects of a person's life. depending on the clinical severity of the disease symptoms. Individuals inflicted with β-thalassemia are generally divided into three groups (Cao et al., 1997; Olivieri, 1999; P. Lahiry, S.A. Al-Attar, 2008) :

(1) Beta Thalassemia trait: This group include Individuals who are carriers and usually asymptomatic and do not require medical attention.

(2) Beta Thalassemia intermedia: This group include patients with two mutant alleles that lead to moderate anemia (Hb 60 - 100 g/L) and they require transfusion every 4-6 months.

(3) Beta Thalassemia major (TM): This group includes patients who carry two mutant alleles and have a severe anemia and need regular blood transfusions for survival once every 3-4 weeks

However, individuals inflicted with α-thalassemia mutations are clinicaly divided into:

(1) Individual with 1-2 gene mutations are considered carriers of the disease.

(2) Hb H disease: Alpha thalassemia with three mutant alleles and one normal allelle.

(3) Hb Bart's hydrops fetalis: the patients have four mutant alleles and this condition is incompatible with life and the fetus dies shortly after birth or in uterus.

Evidently, adult human hemoglobin is composed of four globin subunit (2 alpha and 2 beta subunits). Each of these subunits contains one heme molecule. During human development, different types of hemoglobin's are produced during the various life stages. as shown in Figure 1.1.

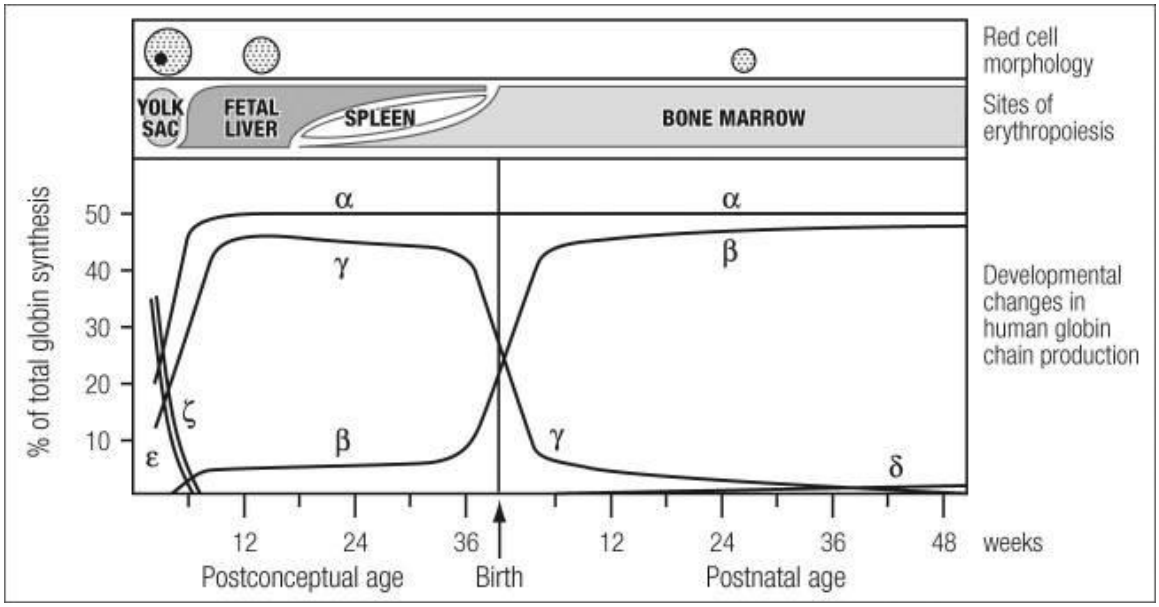


Figure 1.1 The changes in human globin chains synthesis during developmental stages of life (Schechter, 2008).

In general, the common clinical complications of thalassemia include pallor, jaundice, splenomegaly, skeletal deformities and endocrine complications (Jensen et al., 1998; Toumba et al., 2007).

1.2.1 Molecular Genetic of Human Hemoglobin's

The production of human globin chains is regulated by two clusters of genes. The β-like genes and the α-like genes cluster which are responsible for the different types of human

globin. (Elizabeth and Mary Ann, 2010). The α-like globin genes are located in the short arm of chromosome 16 and spans around 26 Kb DNA. The cluster of α genes consist of two highly homologous α genes (α1, α2), pseudogenes (ψζ, ψα2, ψα1), embryonic ζ gene, and a gene with unknown function (θ). All of these genes are spread on the chromosome with the order -′5- ζ- ψζ - ψα2 - ψα1 -α2 - α1- θ -3′, while the β-like globin genes are located in chromosome 11 near the terminus of short arm. The cluster of β-like globin genes arranged in more than 60 Kb. This cluster composed from 2γ (Gγ, Aγ) fetal genes, one pseudogene (ψβ), a single embryonic (ε) gene and the adults β and δ genes (Figure 1.2). These genes are located on chromosome 11 in the order:′5- ε- Gγ - Aγ -ψβ - δ -β -3′(Higgs et al., 1989, Ribeiro and Sonati, 2008).

1.2.2 Management of Transfusion Therapy:

Thalassemia major patients require blood transfusion especially within the first two years of life (Galanello & Origa, 2010). Individuals who undergo multiple blood transfusions due to an ineffective erythropoiesis associated with increased iron absorption develop iron overload. This condition can lead to various organ damage and death. Some of the complications that can be caused by iron overload include endocrine complications, heart problems, splenomegaly, and bone deformities.(Fung EB et al.,2007; Ladis et al., 2010; Mishra & Tiwari, 2013). For this reason, it is very important to frequently measure iron levels to provide the proper chelating therapy along with all needed clinical managements for patients. In humans, the iron storage capacity of the body is usually maintained at around 200 to 1500 mg (in female is around 5mg/kg and in male is 13 mg/kg). This level is maintained by adequate adjustment of iron absorption in the intestine.

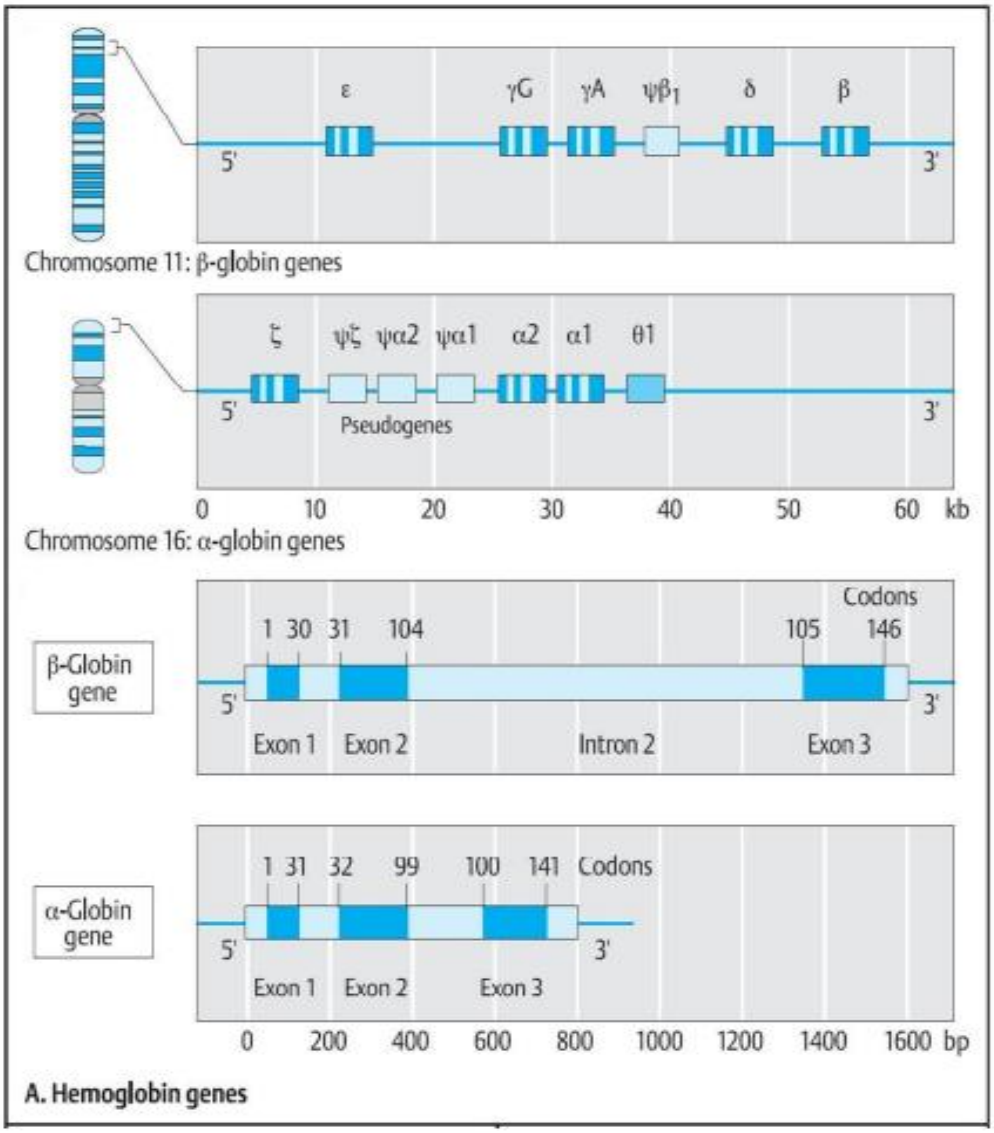


Figure 1.2 Location and Structure of the β-globin and α -globin genes.

Since no effective excretory mechanisms for iron is available in humans (Finch, 1994). One of the most common methods used to determine iron overload level is by continuously measuring serum ferritin. However, the liver biopsy method is more sensitive and can be invasive. Another technique that can be used for iron overload evaluation is the use of nuclear magnetic resonance imaging (MRI)(Galanello & Origa, 2010; Rund & Rachmilewitz, 2005). Monitoring of cardiac and endocrine function is also important (Rachmilewitz &

Giardina, 2011). Patients on regular blood transfusion acquire about 200 mg iron per unit of packed RBCs which leads to significant iron accumulation (Porter & Shah, 2010). Iron chelator (binder) is required to get rid of excess iron through urine and stool (Knovich et al., 2009a). Iron chelators administration should start after the patient have had 10-20 blood transfusion or after serum ferritin level reaches more than 1000 ng/ml (Galanello & Origa, 2010; Rachmilewitz & Giardina, 2011).

1.2.3 Prevalence of Thalassemia

According to a report released by the World Health Organization in 2008, around 40000 infants are born each year with beta thalassemia with about 25500 beta thalassemia patients need blood transfusion. The report also noted that around 20,000 newborns with this condition are expected to be born in Southeast Asia, 10,000 will be born in Eastern Mediterranean,1000 in Europe and 350 in the Americas. In most European countries, the incidence of beta thalassemia major is relatively low. In Belgium, for instance, the rate of births with this condition is estimated to be around 1 in 25000. A study conducted in France revealed that the incidence of this condition was around 1 in 112000 births from 2005 to 2008.The Greek National Registry for Hemoglobinopathies reported a significantly lower incidence of beta thalassemia than expected based on the prevalence of carriers, thereby demonstrating the efficacy of thalassemia prevention programs (Modell & Darlison, 2008). Approximately 1.5% of the global population are heterozygotes (carriers) of β-thalassemia's; there is a high incidence in populations from the Mediterranean basin, throughout the Middle East, the Indian subcontinent, Southeast Asia, and Melanesia to the Pacific Islands(Sanctis et al., 2017). In the Middle East, the prevalence of Beta thalassemia is traditionally high due in part to a sgniga high carrier

rate and a cultural preference for consanguineous marriages. However, the introduction of prevention programs in many countries in this region has led to a decrease in prevalence over the last decades (De Sanctis et al., 2017; Kadhim et al., 2017). In Palestine, the prevalence of this condition is estimated to be around 4 percent, with more than 200,000 individuals having the trait. In 2021, a report revealed that there were 492 and 309 cases of beta thalassemia major in the West Bank and Gaza, respectively (Thalassemia Patients Friends Society- Palestine,2021). Furthermore, a law implemented by the Palestinian Legislative Council which states that premarital screening for beta-thalassemia is a prerequisite for receiving a marriage license from the religious authority. Therefore, couples have to undergo a series of required blood tests, to ensure safe marriage which is strictly adopted leading to dramatic decrease in the number of thalassemia major births. The mandatory per-marital test that was adopted in 2000 was an effective tool in reducing new cases from 40 case per year before 2004 to less than 3 case per year since 2013 (Al Sabbah et al., 2017 and Thalassemia Patients Friends Society-Palestine). The average life expectancy of thalassemia patient in Palestine is merely 15 years with many thalassemia patients suffering from serious complications due to iron overload complications which significantly exceeds 1,000 mg/dL (Karmi B, 2009).

1.2.4 Molecular Genetics of β-thalassemia

Through the use of modern DNA technology, over 400 mutations have been identified in the β-globin gene that lead to the development of the disease. Only 17 mutations are common among Palestinian thalassemia patients as describe in table 1.2.

Country	Prevalence of β-thalassemia per 100 000 persons[a]	β-thalassemia carrier rate (year)	Screening program available[b] (year of initiation)		
			Premarital/carrier	Prenatal	Newborn
Bahrain	9 (2007)	2.0% (2007)	Voluntary (1991); mandatory (2004)	Yes (1994)	Yes (2007)
	–	2.9% (2013)	NR	NR	NR
Egypt	–	9.0%-10.0% (NR)	NR	No	NR
	–	5.3%-9.0% (2007)	No	No	No
	–	4.5% (2013)	NR	NR	NR
Iran	–	5.7/1000 (1997-2011)	Yes (1995)	Yes (1995)	NR
	–	4.0%-8.0% (NR)	NR	NR	NR
Iraq	–	3.7% (2006)	Pilot (2006)	Pilot (2006)	NR
	–	33.5/100 000 (2010) 37.1/100 000 (2015)	Yes (NR)	NR	NR
Jordan	–	3.0%-5.9% (2013)	NR	NR	NR
Lebanon	–	2.0%-3.0% (2007)	Mandatory (1994)	Yes (1994)	NR
Oman	40[c]	4.0% (1995)	No	No	NR
Palestine	–	3.0%-4.0% (1996-2015)	Yes (2001)	NR	NR
Qatar	–	2.0%-3.0% (NR)	NR	NR	NR
Saudi Arabia	–	1.0%-15.0% (2013)	NR	NR	NR
United Arab Emirates	–	8.5% (2013)	Mandatory (2012)	Yes (NR)	Yes (NR)

Abbreviation: NR, not reported.
[a]Prevalence values were converted into number of patients per 100 000 for consistent reporting; therefore, they are estimates.
[b]As published in the reference.
[c]Specified as β-thalassemia major.

Table: 1.1 The prevalence of β-thalassemia trait in Palestine and other Middle East populations (Karmi B., et al 2017).

1.3 Iron Background

1.3.1 Iron Metabolism:

The first description of iron metabolism at the molecular level was in 1930 and A comprehensive study about iron absorption was published in 1958 (JOSEPHS, 1958). The discovery of the transferrin receptor in 1970, IRE/IRP regulatory axis in 1980, and HFE in 1996, constituted the basic elements that provided the early description about the metabolism of iron (Feder., 1996; Trowbridge & Omary, 1981). Later, the most important finding is the discovery of iron regulatory hormone called hepcidin and its

Mutation	Jericho	Ramallah	Nablus	Qalqilia	Tulkarem	Jenin	Hebron	Total number of alleles
IVS-I-6 (T→C)	–	12 (31.6)	24 (47.0)	13 (24.0)	–	2 (4.9)	29 (51.8)	80 (28.7)
IVS-I-110 (G→A)	7 (43.8)	1 (2.6)	11 (21.6)	4 (7.4)	7 (30.4)	17 (41.5)	2 (3.6)	49 (17.6)
Codon 37 (G→A)	–	7 (18.4)	–	2 (3.7)	3 (13.0)	11 (26.8)	6 (10.7)	29 (10.4)
IVS-I-1 (G→A)	4 (25.0)	5 (13.1)	7 (13.7)	1 (1.8)	1 (4.3)	3 (4.3)	4 (7.1)	25 (9.0)
Codons 106/107 (+G)	1 (6.2)	–	–	18 (33.3)	–	–	–	19 (6.8)
Codon 39 (C→T)	–	1 (2.6)	–	6 (11.1)	4 (17.4)	2 (4.9)	–	13 (4.6)
IVS-II-1 (G→A)	2 (12.5)	–	–	–	3 (13.0)	1 (2.4)	2 (3.6)	8 (2.9)
Codon 5 (-CT)	–	–	1 (2.0)	–	–	1 (2.4)	5 (8.9)	7 (2.5)
IVS-II-848 (C→A)	2 (12.5)	1 (2.6)	4 (7.8)	–	–	–	–	7 (2.5)
–30 (T→A)	–	6 (15.8)	–	–	–	–	–	6 (2.1)
Codons 8/9 (+ G)	–	–	–	–	–	–	4 (7.1)	4 (1.4)
IVS-I-5 (G→C)	–	2 (5.3)	–	1 (1.8)	–	–	–	3 (1.1)
−28 (A→C)	–	–	–	–	2 (8.7)	–	–	2 (0.7)
IVS-II-745 (C→G)	–	1 (2.6)	–	–	–	–	–	1 (0.3)
Codon 6 (-A)	–	1 (2.6)	–	–	–	–	–	1 (0.3)
Codon 27 (G→T)	–	–	–	–	1 (4.3)	–	–	1 (0.3)
Codon 30 (AGG→ACG)	–	–	1 (2.0)	–	–	–	–	1 (0.3)
Uncharacterized	0 (0.0)	1 (2.6)	3 (5.9)	9 (16.7)	2 (8.7)	4 (9.7)	4 (7.1)	23 (8.2)
Total (100.0%)	16	38	51	54	23	41	56	279
Number of variants	5	10	6	7	7	7	7	

Table 1.2: Distribution and Frequency (%) of β-Thalassemia Mutations among Palestinian Patients (Darwish et al.,2006).

target protein, Ferroportin, in the 2000s (Abboud & Haile, 2000; Donovan et al., 2000). Iron is a very important element in various biological processes, including DNA replication, production of energy and detoxification. It is an essential component of the oxygen transport and utilization system via hemoglobin and myoglobin. Therefore, having sufficient level of iron is important for health and cells survival. Howeve, excess iron can be toxic due to the ability of iron to form a redox-active and to generate reactive oxygen species (ROS) leading to oxidative stress and initiation of signaling pathways crucial for cell survival and death, through facilitating the formation of oxygen superoxide, hydrogen peroxide and hydroxyl radicals which lead to protein, lipids and DNA damage(Ray et al.,

2012). It is therefore important that iron is recycled and absorbed properly. This is done through the buffer of storage iron. Adults utilize 20 mg of iron per day to support erythropoiesis (80%) and other cellular needs (20%) (Wang & Babitt, 2019). Plasma contains only 2 to 4 mg of iron and is replenished every hour. Most of the daily iron requirement is supplied through the breakdown of senescent red blood cells in macrophages (Hower et al., 2009).

1.3.2 Systemic Iron Homeostasis:

About 60% of iron is in hemoglobin, 10% in muscle myoglobin, and 30% in reticuloendothelial macrophages. When iron is taken out of heme, it is stored as ferritin or returned to the plasma through a special membrane iron transport system.(Hower et al., 2009). Roughly 1–2 mg of iron is lost daily through sweat, blood loss, sloughing of intestinal epithelial cells, and desquamation through menstruation in reproductive-age women)(Abbaspour et al., 2014). The body can then compensate for the loss of iron by absorbing it in the form of DMT1 takinng up to 2 mg of dietary iron daily. Hemoglobin synthesis requires 20–25 mg of iron per day and to support hemoglobin synthesis and other metabolic processes, iron must be recycled and tightly regulated within the system(Abbaspour et al., 2014). In order to maintain adequate and safe amounts of free iron, cells require the coordination of a wide variety of genes which tightly control both intracellular and systemic iron metabolism (Ganz & Nemeth, 2012; Hentze et al., 2010; MacKenzie et al., 2008). Iron exits the basolateral membrane through ferroportin and binds to transferrin in plasma (Knutson, 2017). Hepcidin and its receptor ferroportin are two of the most important factors that maintain systemic iron homeostasis in the body while the other iron-regulatory proteins play a role in regulating the intracellular iron homeostasis.

Eventually, an intracellular iron network consisting of 151 chemical species and 107 reactions and transport steps was identified (Hower et al., 2009). Although some of the key factors that play a role in iron homeostasis have been known for long time, such as transferrin and ferritin, understanding of the molecular basis of iron metabolism came in few past 5-y period from 1996 to 2001 led to the identification of many of the key proteins involved in these processes, including the iron-import protein divalent metal-ion transporter 1 (DMT1), iron export protein ferroportin 1 (FPN1), and the "master" regulator of iron homeostasis and the liver-derived peptide hepcidin (Ganz, 2013).

1.3.2.1 Iron Absorption:

After birth, the main sources of iron are through diet except iron provided from therapeutic sources. Iron come from diet as heme or nonheme form (from animal and plant sources). Nonheme iron is found in a wide variety of forms including soluble iron, iron in low–molecular-weight complexes, storage iron in (ferritin) and iron in the catalytic centers of a wide range of proteins (R. E. Fleming & Ponka, 2012). Nonheme iron is absorbed by enterocytes following reduction by Ferrireductase, Cybrd1 (DcytB), to Fe2+ before it is transported through the cellular membrane by the divalent metal transporter 1, DMT1 (SLC11A2) (Gunshin et al., 1997; Oakhill et al., 2008). Folloing heme iron absorption, it is transported into the cytosol and released by heme oxygenase 1 (HO1) (Ferris et al., 1999). Extra intracellular iron is stored in ferritin which oxidizes and sequesters excess ferrous iron into a ferrihydrite mineral core (Arosio & Levi, 2010; Theil, 2003). After a few days, iron is lost from the ferritin of enterocytes through the sloughing of the intestine's epithelium cells. Dietary cytosolic iron is exported into the plasma by the basolateral iron exporter ferroportin (FPN, *SLC40A1*) (Donovan et al., 2000; McKie et al., 2000). Export

of iron from enterocytes into circulation requires ferroxidase hephaestin (HEPH), a multicopper oxidase, that oxidizes Fe2+ to Fe3+ (Vulpe et al., 1999). Plasm Fe3+ binds to transferrin with two binding sites and maintains the iron soluble form. The discovery of transferrin as a plasma iron transporter date back to 1946 (L. & Leona, 1946). Transferrin has two important functions; it limits the formation of toxic radicals and delivers iron to cells. In healthy humans, about a third of transferrin is bound to iron. Most of iron in the body is bound to Tf, non-transferrin-bound iron accumulates (NTBI) in tissues in case of iron overload. NTBI is thought to contribute substantially to the pathology associated with iron overload (Hentze et al., 2010).

1.3.2.2 Iron Utilization, Recycling, and Storage

The erythroid bone marrow is the main consumer of iron. Almost all iron comes from internal recycling of tissue macrophages. Erythroblasts acquire iron via transferrin receptor1 (Tfr1) which is a ubiquitous protein expressed on the cell surface, transferrin receptor 1 (TfR1) via receptor-mediated endocytosis. TfR1 transfers iron-loaded Tf (Holo-Tf) into acidified endosomes where iron dissociates from transferrin with the assistance of six transmembrane epithelial antigen of the prostate (STEAP) proteins and exits the endosome via DMT1 (Figure 1.3) (Ohgami et al., 2006).

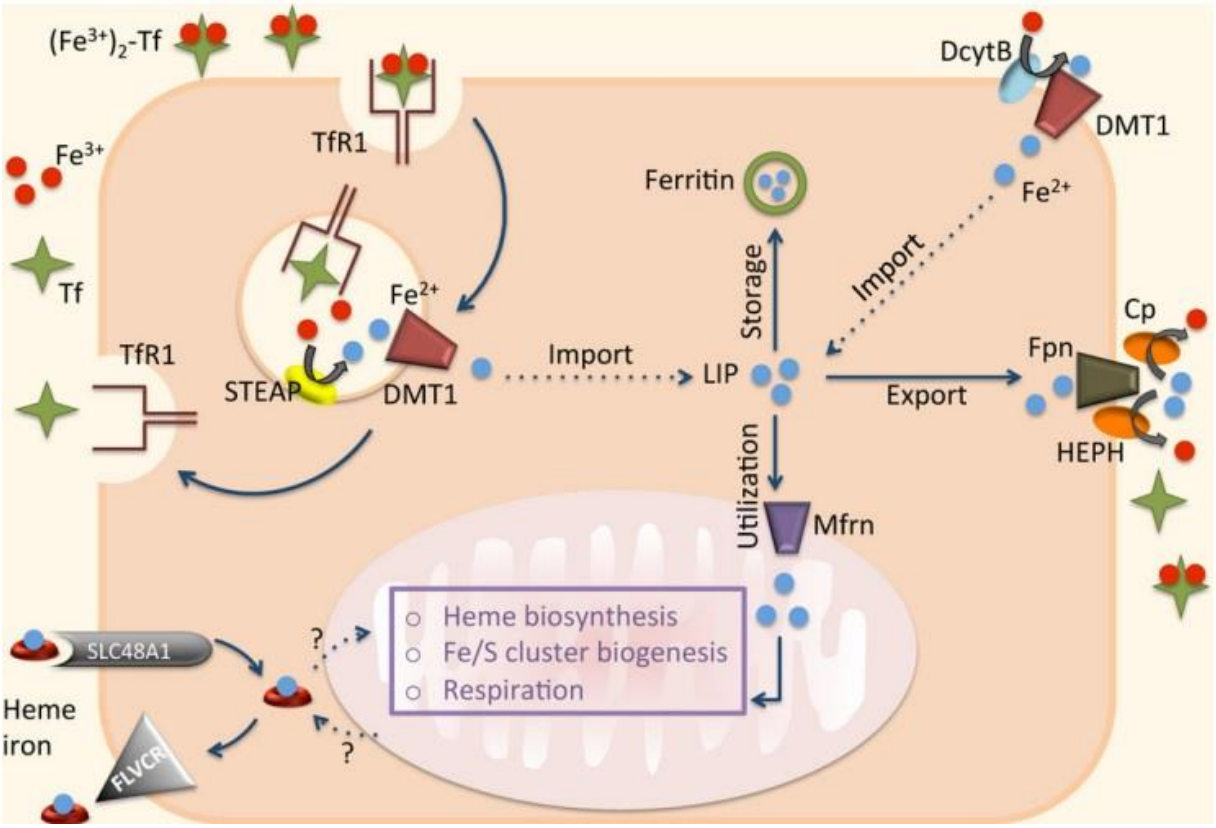


Figure 1.3 systemic iron homeostatic (Chifman et al., 2014)

Transferrin and transferrin receptor are then recycled back to the cell surface. Iron is imported into mitochondria from intracellular compartments by the inner membrane protein mitoferrin 1 to form heme, the majority of which is then used for hemoglobin production (Shaw et al., 2006). Since excess heme can lead to apoptosis and, it is important that the mechanisms that maintain heme levels are in place to prevent this problem.

It has been proposed that feline leukemia virus subgroup C cellular receptor (FLVCR) and ATP binding cassette protein G2 (ABCG2) export excess heme, although this is not completely understood (Keel et al., 2008; Krishnamurthy et al., 2007).

Macrophages retrieve iron from damaged and aged erythrocytes by phagocytosis and oxygenase catabolizing heme to release iron. Ferrous iron is exported into the plasma via

the iron exporter ferroportin (*SLC40A1*) and unused iron is stored in macrophages, mainly as ferritin (Andrews & Schmidt, 2007; Donovan, A., Lima, C. A., Pinkus, J. L., Pinkus, G. S., Zon, L. I., Robine, S., and Andrews, 2005). The main storage site for iron is the liver in form ferritin. It can be used to mobilize iron when required by the body. Hepatocytes acquire Holo-Tf through two receptors, TfR1 and TfR2, however TfR2 is believed to act mainly as a transferrin saturation "sensor" and has much lower affinity for Holo-Tf than TfR1(Johnson & Enns, 2004; Robb & Wessling-Resnick, 2004). Most important, when serum iron levels surpass transferrin binding capacity, the liver becomes the major storage site for non-transferrin-bound-iron (NTBI) (Andrews & Schmidt, 2007). It is not not fully understood how Hepatocytes acquire the (NTBI). One possible candidate for this is zinc transporter Zip14, while other tissues like heart and pancreas represent sites of iron accumulation in iron overload and are proposed to have mechanisms for NTBI uptake (Liuzzi et al., 2006).

1.3.2.3 Regulation of Systemic Iron Homeostasis

An organism must maintain balanced iron homeostasis to avoid iron overload and deficiency. This is done through the regulation of hepcidin which helps maintain iron availability in the body. It is predominantly produced by the liver although studies indicate that other tissues generate hepcidin (Park et al., 2001; Pigeon et al., 2001). The levels of hepcidin are influenced by various physiological factors that affect iron homeostasis including iron overload, iron deficiency, and inflammation. Hepcidin modulates serum iron levels and controls transferrin saturation by inhibiting iron release from duodenal enterocytes, macrophages, and hepatocyte (De Domenico et al., 2009). More precisely, hepcidin regulates iron efflux by binding to the iron exporter ferroportin, triggering its

internalization and degradation in lysosomes (Nemeth et al., 2004). The mechanism is facilitated by Janus kinase 2 (Jak2) that binds to the ferroportin-hepcidin complex, phosphorylates ferroportin and targets ferroportin for degradation through ubiquitin-mediated pathway of ferroportin (De Domenico et al., 2009; Qiao et al., 2012).

1.3.3 Intracellular Iron Homeostasis

1.3.3.1 Iron Distribution to Tissues:

Iron absorbed from duodenum or released from stores is bound to transferrin in plasma for distribution to the tissues in a regulated manner and to prevent toxicity from unbound iron (non-transferrin-bound iron or NTBI (Knutson, 2017). Under normal conditions, serum transferrin is 30% saturated with iron TSAT below 15% representing limited availability of iron to erythroblasts and other tissues. Conversely, when TSAT exceeds 70%, NTBI begins to appear in plasma which is toxic to several organs due to its reactive nature and unregulated entry into cells (Brissot et al., 2012).

1.3.3.2 Iron Uptake by Cells:

Transferrin receptor 1 (TFR1) is a cell membrane protein that binds to and activates the iron-containing transferrin complex. This complex then becomes an endosome where Ferric iron is then reduced to Fe2+ by the transmembrane family of metalloreductases (STEAP)(Ohgami et al., 2006). The acidification of transferrin results in the release of iron from transferrin by the aid of chaperon, which is then transported to the mitochondria for heme and iron cluster synthesis or stored by ferritin. Ferritin is a 24-subunit protein composed from heavy chains and light chains (Figure 1.4). It can store about 4500 atoms of iron in its center. The storage of iron in ferritin protects the cell from oxidative damage and provides a source of iron for metabolic needs (Theil, 2013).

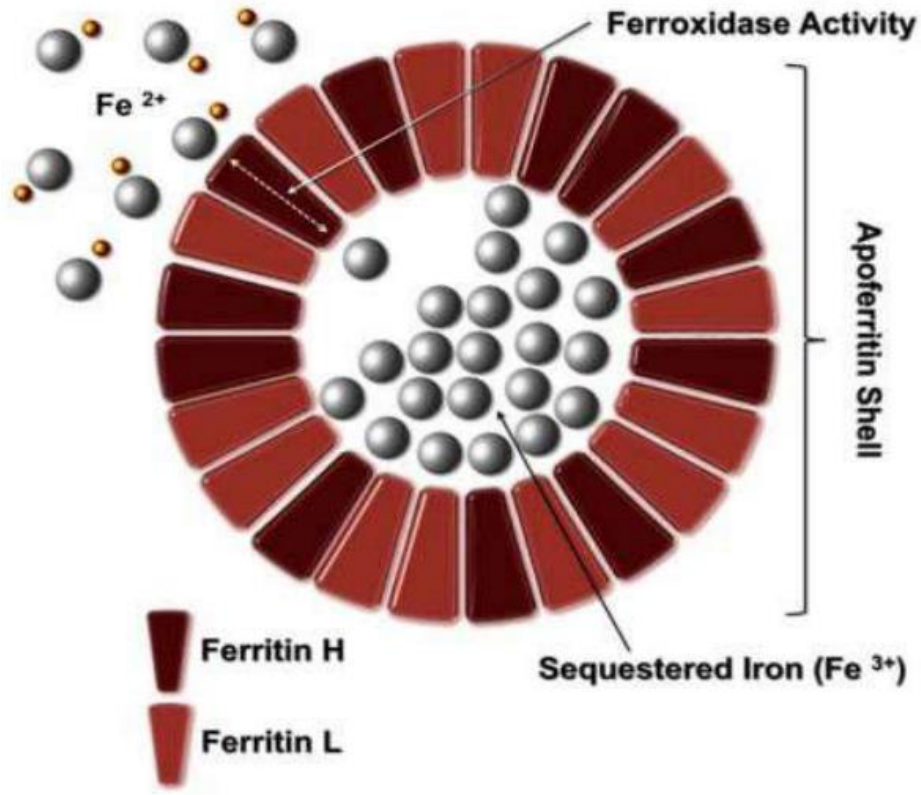


Figure 1.4. The structure of ferritin with and without iron (Knovich et al., 2009b).

1.2.3.3 Control of Iron Uptake and Utilization

The entry of iron into the cell and its fate in the cytoplasm is controlled by post-transcriptional regulation involving the interaction of iron regulatory proteins (IRP1 and IRP2) with iron-responsive elements (IRE) present in the mRNA of several iron related proteins (Anderson et al., 2012). When the concentration of iron becomes Low, this promotes IRP to bind to IRE, this binding suppresses translation of mRNA when the IRE in 5′ UTR and enhances translation when the IRE is in the 3'UTR. This process reduces the synthesis of ferritin (5' UTR IRE) but increase synthesis of TFR1 (3' UTR IRE), that allows the cell to increase iron uptake from plasma which becomes available for metabolism instead of storage in ferritin. When cellular iron is high, IRPs dissociate from IRE, the process become reverse which led to stop iron uptake and promotes storage of

cytoplasmic iron as ferritin. The mRNAs for DMT1, FPN1, 5-aminolevulinic acid, and hypoxia inducible factor 2α contain IREs, making it possible to regulate several important proteins of iron metabolism according to cellular needs (Kühn, 2015).

1.2.3.4 Iron Export

There is currently no known mechanism by which iron can be removed from the body. However, there is a well-organized regulation of iron excretion in cells. This is due to the presence of a protein known as ferroportin, which is found in various tissues and organs (Abboud & Haile, 2000; Donovan et al., 2000; Donovan, A., Lima, C. A., Pinkus, J. L., Pinkus, G. S., Zon, L. I., Robine, S., and Andrews, 2005; Lal, 2020).

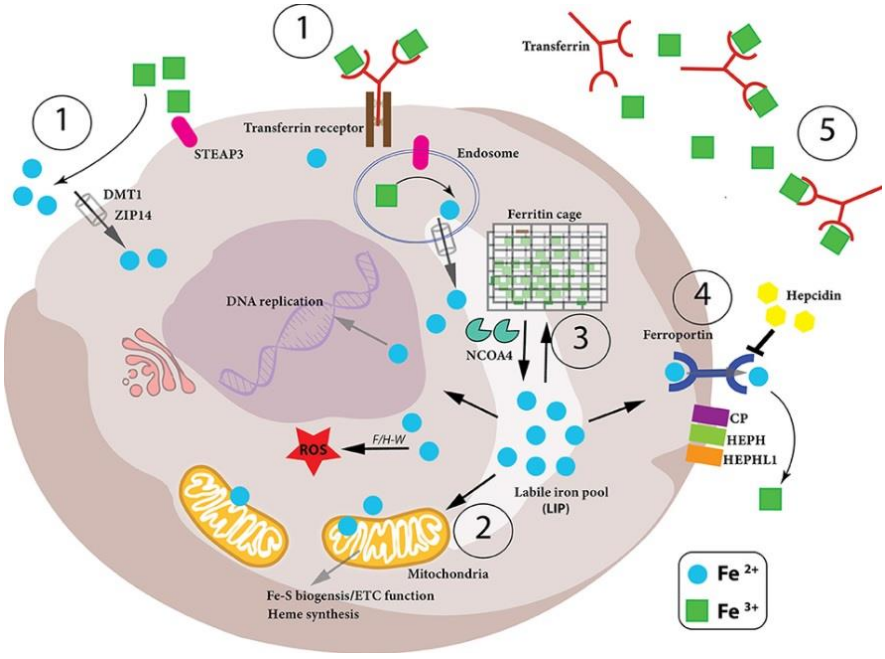


Figure 1.5 Cellular iron metabolism. Iron metabolism mainly includes (1) iron uptake, (2) iron utilization, (3) iron storage, (4) iron export, and (5) iron circulation. (Cronin et al., 2019).

1.4 Iron Regulatory Genes and Proteins

1.4.1 HAMP Gene

The hepcidin antimicrobial peptide (HAMP) gene is located on chromosome 19q13.12 (Figure 1.6), composed from 25 amino acids which plays a role in innate immunity (Ganz, 2006), and appears to play a crucial role in iron homeostasis in humans, through regulating both iron absorption from the intestine and its recycling by macrophages (Kemna et al., 2008).

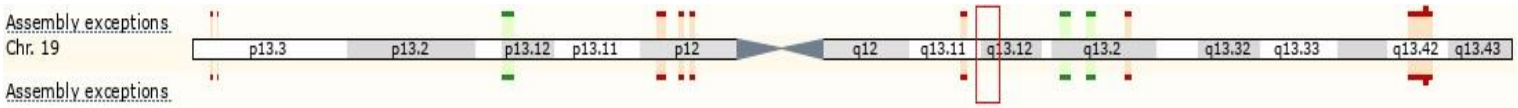


Figure 1.6 Location of HAMP gene on chromosome 19 (Howe et al., 2021).

It has been shown that inflammation and iron overload can stimulate the production of hepcidin. Erythropoietic activity can decrease this production. Hepcidin synthesis is also suppressed by anemia and hypoxia(Peyssonnaux et al., 2007). Hepcidin role in iron metabolism has been described in thalassemic patients (Camberlein et al., 2008). In most cases of hereditary hemochromatosis, hepcidin deficiency is the cause of iron overload. Several mutations of *HAMP* gene have been reported. The G71D mutation in the HAMP gene is likely to be associated with reduced hepcidin activity and was reported to be relatively common in hereditary hemochromatosis (Merryweather-Clarke et al., 2003). Hepcidin was found to be suppressed in patients with thalassemia syndromes (Papanikolaou et al., 2005). Defects in HAMP gene have been associated with iron dysregulation in many pathological conditions. Many studies have suggested this mutation to affect iron metabolism. This SNPs has been linked with iron overload in thalassemia

major patients (Andreani et al., 2009). Silva has documented that this variation predisposed beta thalassemia patients to increased serum ferritin levels and iron overload (Silva et al., 2014). It was reported that this SNP may be associated with decreased hepcidin expression (Bruno et al., 2010). In vitro, the G variant decreased HAMP gene transcriptional activity by 20% compared to the A variant (Parajes et al., 2010).

1.4.2 HFE Gene

The HFE gene is located on chromosome 6 (Figure 1.7) and it forms a protein like HLA - Class I that binds with beta 2- microglobulin and transferrin receptor (TfR) on the cell surface. The role of HFE protein is to decreases the affinity of TfR for transferrin and lowers iron absorption by the cells (Zamani et al., 2012).

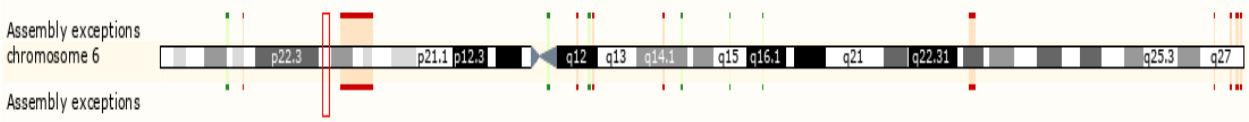


Figure 1.7 Location of HFE gene on chromosome 6 (Howe et al., 2021).

Mutations in this gene disrupt the binding of the HFE gene with beta2 microglobulin and prevent its surface expression(Kaur et al., 2003). Two most common mutations in the HFE gene involves a C > G substitution at nucleotide 187 that changes amino acid 63 from histidine to aspartic acid (H63D) and cysteine-to-tyrosine substitution at amino acid 282 in the HFE gene (C282Y mutation) (Leão et al., 2014) . Mutations in the HFE gene have been shown to be associated with haemochromatosis, an autosomal recessive disease of iron overload.

1.4.3 SLC11A2 Gene

Solute carrier family 11, member 2 (*SLC11A2*) is the only transmembrane iron transporter known to be involved in cellular iron uptake. The *SLC11A2* gene located on chromosome 12q13 (figure 1.8).

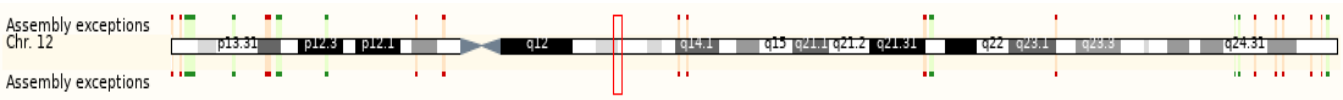


Figure 1.8 Location of SLC11A2 gene on chromosome 12q13 (Howe et al., 2021).

It is widely expressed and has been postulated to play important roles in intestinal iron absorption, erythroid iron utilization, hepatic iron accumulation, placental iron transfer, and other processes (Hiromi Gunshin et al., 2005). Although iron can be transported through cellular membranes without breaking down, it cannot pass through them unassisted. Only one protein known to transport iron responsible for this function. The SCL11A2 protein which is a member of the solute carrier family 11 and known to play a physiological role in bringing iron into cells. It can also transport other divalent metal cations including Cu2+, Mn2+, and Zn2, Co2+ (H Gunshin et al., 1997). SLC11A2 is found on the apical membrane of duodenal enterocytes, consistent with its role in transepithelial iron transport (Canonne-Hergaux et al., 1999). *SLC11A2* is also found in transferrin cycle endosomes in erythroid precursors (Canonne-Hergaux et al., 2001). Studies have shown that the presence of missense mutations in SLC11A2 could affect the transport of non-heme iron from the intestine to the erythroid iron uptake. Microcytic anemia (mk) mice and Belgrade (b) rats have systemic iron deficiency and anemia attributable to the same spontaneous missense mutation (G185R) in SlC11A2 (M. Fleming et al., 1998). Microcytic anemia mice have a mutation in Nramp2, a candidate iron transporter gene (M.

D. Fleming et al., 1997).The Nramp2/DMT1 iron transporter is induced in the duodenum of microcytic anemia mk mice but is not properly targeted to the intestinal brush border (Canonne-Hergaux et al., 2000) . The G185R mutation disrupts function of the iron transporter Nramp2 (Su et al., 1998) .A different missense mutation in SLC11A2 was described in a human patient who presented with anemia and hepatic iron overload and effect on human health through metal traffic (Mims et al., 2005). Different polymorphisms in SLC11A2 gene have been documented to be associated with many disorders including, age-related macular degeneration (Wysokinski et al., 2012a), microcytic anemia (Kloss-Brandstätter et al., 2012)and Wilson's disease (Przybyłkowski et al., 2014) .

1.4.4 *SLC40A1* Gene

This gene is responsible for regulating iron absorption and release in the intestine is known as the Ferroportin gene located on 2q32.2 chromosome (figure 1.9). Ferroportin is the sole cellular efflux channel for iron and is regulated by iron regulatory hormone hepcidin, which binds ferroportin and induces its internalization and degradation (Ganz, 2005) .

Figure 1.9 Location of SLC40A1 gene on chromosome 2 (Howe et al., 2021).

The role of ferroportin in vivo has been confounded by the finding that mutations in ferroportin occur in a subset of human patients with iron overload. Individuals heterozygous for a variety of missense mutations in ferroportin develop macrophage-predominant iron overload that may progress to iron-induced organ damage (Montosi et

al., 2001; Pietrangelo, 2004). Intestinal specific inactivation of SLC40A1 gene in mice resulted in iron insufficiency and accumulation of iron in enterocytes which indicate essential role of ferroportin in iron transport and homeostasis (Donovan, A., Lima, C. A., Pinkus, J. L., Pinkus, G. S., Zon, L. I., Robine, S., and Andrews, 2005). Several mutations in SLC40A1 have been linked with iron overload in many studies (Mayr et al., 2010; Zaahl et al., 2004). As defects in SLC40A1 gene has been linked with iron dysregulation, this project focused to investigate the correlation of rs1439816 variant, located in an intronic region as shown in figure 1.9 with beta thalassemia. This SNP has been shown to be correlated with iron dysregulation in hereditary hemochromatosis (HH) (Radio et al., 2015).

1.4.5 *TFRC* Gene

TFRC gene is located on chromosome 3q29 as shown in (figure 1.10), *TFRC* encodes for a cell surface receptor TfR1, a glycoprotein with a molecular weight of about 90kDa expressed as a homodimer ubiquitously in the majority of cells (Nadadur et al., 2008).

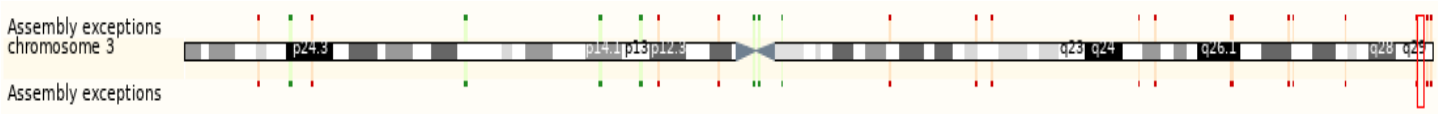


Figure 1.10 Location of TFRC gene on chromosome 3 (Howe et al., 2021) .

It is expressed at low levels in most tissues, with higher levels in the placenta, activated lymphocytes, erythroid precursors, osteoclasts, microglia, intestinal crypt cells, and many types of cancers (Wu et al., 2016).TfR1 is essential for iron uptake by erythrocytes and other cell types by mediated endocytosis. It has high binding affinity to transferrin bound iron (Nadadur et al., 2008).

1.5 Study Aims and Objectives

The aim of this research is to study the associacion between selected single nucleotide polymorohisms (SNPs) variants including (rs11915082, rs1048230, rs224589, rs1439816, rs10421768, rs1799945, rs104894696) in specific genes involved in iron metabolism (Absorption, transport, accumulation, and excretion) with beta thalassemia ans accumulative plasma ferritin level.

Chapter Two: Methodology

2.1 Subjects:

Eighty-eight Palestinian patients with Thalassemia major were enrolled in this study. Patients were diagnosed based on molecular genetic testing of the β-globin gene and hematological indicators in the CBC parameters. All patients received blood transfusion once every 3-4 weeks. The Control group comprised of 88 healthy individuals with no indicators of anemia or other related health problems. Both patients and control subjects were recruited from the same geographical area covering various districts. All patients were familiarized with the objectives of the project and gave written informed consent to participate in the project. Demographic information and serum ferritin levels of the patient's group were obtained from their medical records.

2.2 DNA Extraction and Genotyping:

2.2.1 DNA Extraction:

Venous whole blood samples (3-5 ml) were collected in EDTA-containing tubes and transferred on ice to the molecular genetics' laboratory at the Arab American University campus in Alrihan. Genomic DNA was extracted using MasterPure™ complete DNA purification Kit (Epicenter, USA) according to the manufacturer protocol as follows:

1)Blood samples were centrifuged at room temperature for 15 minutes at 1500 X g. The buffy coat (white blood cells) was withdrawn in 150 µl volume and transferred into 1.5 ml Eppindorf tube.

2) 0.6 ml of Lysis buffer (1) (10mMTris-HCl, 400 mM NaCL and 2mM Na2EDTA, pH 8.2) was added to the buffy coat, mixed gently by inverting the tube 6-8 times the bottom of the tube was flicked.

3). The tubes were incubated for 5 minutes at room temperature, inverted 6-8 time and then incubated for an additional 5 minutes at room temperature after flicking the bottom. The tubes were inverted 6-8 times one addition time and centrifuged for 25 seconds at 10,000 x g.

4) The supernatant (lysed RBCs) was discarded leaving about 25 ul with the pellet, vortexed for resuspension of the white blood cells.

5) 300 µl of Lysis buffer (2) (10%SDS, protease K solution,1mg protease K in 1 % SDS and mM Na2EDTA] were added to the tubes and the mixture was pipetted up and down 5-7 times for complete WBCs lysis.

6) 250 ul of Precipitation Solution was added to the mixture, mixed vigorously by vortex and centrifuged for 10 minutes at 10,000 x g.

7) The supernatants were transferred into new clean microfuge tubes.

8) 700 ul of ice-cold isopropanol was added to precipitate the DNA after gentle mixing

9) Tubes were centrifuged at 4° C for 10 minutes at 10,000 x g.

10) The supernatants were carefully discarded without dislodging the pellets, washed with absolute ethanol, and air dried.

11) DNA was suspended in 100 µl sterile distilled water and stored at -30° C until use for genomic analysis.

DNA was tested for quality by gel electrophoresis using 1% agarose gel in TAE buffer as shown in (figure 2.1). DNA purity and concentration were evaluated using nanodrop (Nanodrop 2000 Thermo scientific, USA). All samples had an A260/A280 ratio above 1.7.

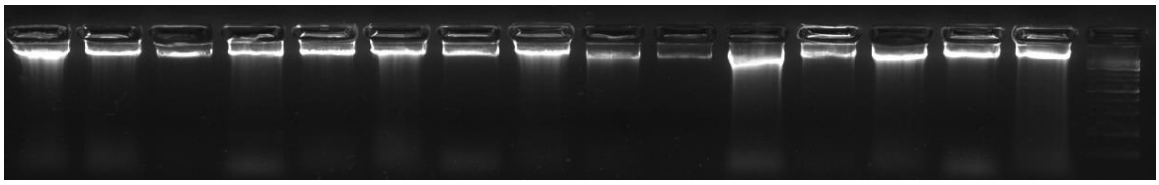


Figure 2.1 Agarose gel Illustration for some DNA samples quality on 1 % agarose gel

2.2.2 DNA Genotyping:

Several selected SNPs located in five major genes involved in iron metabolism were selected based on the association of these SNPs with iron dysregulation in several disorders (Camberlein et al. 2008 , Andreani et al, 2009, Leão et al., 2014, Hiromi Gunshin et al., 2005, and Wu et al., 2016). Both allotypes and genotypes variant of the indicated SNPs (7 SNPS) were analyzed and correlated with Beta Thalassemia. Table 2.1 describe the indicated genes SNPs reference and the forward and reverse primers used for amplification and the expected fragment size. PCR amplification programs of the indicated fragments were done for all the SNPs according the programs shown in table 2.3 and run on FlexCycler2 thermocycler (Analytik Jena, Germany). The PCR product was evaluated using 2.5% agarose gel. Electrophoresis was run at 100 V for 40 minutes in 1X Tris-Acetate-EDTA buffer (TAE) (Bio-Rad, USA) and visualized using ultraviolet transilluminator documentation system (ChemiDoc ,Bio-Rad, USA). Thermal cycling conditions for each PCR are given in (Table 2.2).

Gene	SNP reference	Position (GRCh38.p12)	Gene region	Variation	Forward primer	Reverse primer	Product size (bp)	Method used
TFRC	rs11915082	chr3:196082268	Promoter	G>A	GTCACTTCCTGAGGCACGTA	CGCAGTGCAATATCCAACAT	306 bp	DNA sequencing
SLC11A2	rs1048230	chr12:50992283	Exonic	A>G	TCCCATTCTTCTGAGGTCTCTC	AGACCACAACCATGCCTCTG	366 bp	DNA sequencing
SLC11A2	rs224589	chr12:51005267	Intronic	T>A	TGTGAGGCTGGATTTTGTTG	AGATTTTGCACATGACCTGCT	483 bp	DNA sequencing
SLC40A1	rs1439816	chr2:189579904	Intronic	C>A	TGGGGAAAGATCTTCGATG	GTACGTGGTTTGTCCTGCAA	367 bp	DNA sequencing
HAMP	rs10421768	chr19:35281996	Intron Variant/5' UTR variant	A>G	CCCAGGCTAGTCTTGAACTTCTG	AGGGAACACTAGATAGCCCTGAG	244 bp	RFLP-PCR
HAMP	rs104894696	chr19:35284999	Exonic	G>A	Fo:ATGCAGGGAGGTGTGTTAGGAGG Fi:CCATCTGCATTTTCTGCTGCGG	Ro:TGCAAGGCAGGGTCAGGACAAGC Ri:CACTTTGATCGATGACAGCAGT	521 bp 236 bp	T-AMRMS-PCR
HFE	rs1799945	Chr6:26090951	Exonic	C>G	Fo:ACATGGTTAAGGCCTGTTGC Fi:CCAGCTGTTCGTGTTCTATGATC	Ro:GCCAC ATCTGGCTTGAAATT Ri:GGCTCCACAC GGCGACTCTCATC	164 bp 89 bp	T-AMRMS-PCR

Table 2.1 SNPs details, sequence of primers and method of investigation

2.2.2.1 ARMS:

Two Tetra-primer amplification refractory mutation system polymerase chain reaction (T-ARMS-PCR) were carried out for (rs104894696 & rs1799945) in 25 ul reaction volume. Each reaction containing 12.5 µl ready mix (lyophilized mixture of Taq polymerase, Mgcl2, dNTP and buffer), 9.5 µl distilled water, 1 µl template genomic DNA, 1 µl Reverse primer, and 1 µl Forward primer. One reaction contains Forward outer and Reverse inner while the second contain Forward inner and Reverse outer. The sequences of primers are given in (table 2.1).

2.2.2.2 PCR-RFLP:

PCR-RFLP were carried out for (rs10421768) in 25ul reaction volume. Each reaction containing 12.5 µl ready mix (lyophilized mixture of Taq polymerase, Mgcl2, dNTP and buffer), 9.5 µl distilled water, 1 µl template genomic DNA, 1 µl Reverse primer, and 1 µl Forward primer. After amplification, fragments containing the rs10421768 polymorphic site were digested for 24 h with HpyCH41V allele-specific restriction enzyme. Each reaction contained, 10 µl PCR product, 1.5 µl 10 x enzyme buffer, 0.2 µl (54 units of enzyme), and 3.3 µl distilled water. After incubation at 37 °C for 24 hours, DNA fragments were separated on a 2.5% agarose gel, electrophoresis was run at 100 V for 40 minutes in Tris-Acetate-EDTA buffer. After separation, gels were analyzed by the ChemiDoc imaging system (BIO-RAD, USA). Alleles and genotypes were interpreted based on specific digestion pattern.

Table 2.2. PCR Protocols

SNP	Initial denaturation (5 min)	Cycling			Final extension (5 min)
		Denaturation 30 sec	Annealing 30 sec	Elongation 45 sec	
rs11915082	94 ◦C	94 ◦C	56 ◦C	74 ◦C	74 ◦C
rs1048230	94 ◦C	94 ◦C	56 ◦C	74 ◦C	74 ◦C
rs224589	94 ◦C	94 ◦C	56 ◦C	74 ◦C	74 ◦C
rs1439816	94 ◦C	94 ◦C	58 ◦C	74 ◦C	74 ◦C
rs10421768	94 ◦C	94 ◦C	58 ◦C	74 ◦C	74 ◦C
rs104894696	94 ◦C	94 ◦C	59 ◦C	74 ◦C	74 ◦C
rs1799945	94 ◦C	94 ◦C	59 ◦C	74 ◦C	74 ◦C

2.2.2.3 DNA Sequencing Analysis

For rs11915082, rs1048230, rs224589, and rs1439816 variants investigation, Sanger's sequencing was used. After DNA amplification, PCR product was cleaned up by mixing 5 µl of PCR product with 1 µl of clean up reagent Eppic Fast (A&A biotechnology) and incubated at 37°C for 15 minutes. Samples that required dilution were diluted using Double distilled water (DEPEK). Sanger's sequencing was performed using the BigDye™ Direct Cycle Sequencing Kit (Thermo Fisher scientific, USA) and run on the Applied Biosystems 3500 Genetic Analyzer (Applied biosystem, USA). Nucleotide variations were examined using the Sequence Scanner Software 2.

2.3 Statistical Analysis.

The Hardy–Weinberg equilibrium (HWE) was evaluated to determine the representativeness of the study population, this analysis was carried out using the SNPStats online tool (Solé et al., 2006). Overall differences in allele and genotype frequencies between Thalassemia patients and non-Thalassemia individuals were evaluated by Chi-Square ($\chi 2$) test or Fisher's exact test using SNPStats tool (Shi & He, 2005; Solé et al., 2006). Allelic associations with the disease were expressed as p value, odds ratio (OR), and 95%

confidence interval (95% CI). A p value less than 0.05 was considered statistically significant.

2.4 Bioinformatics Analysis

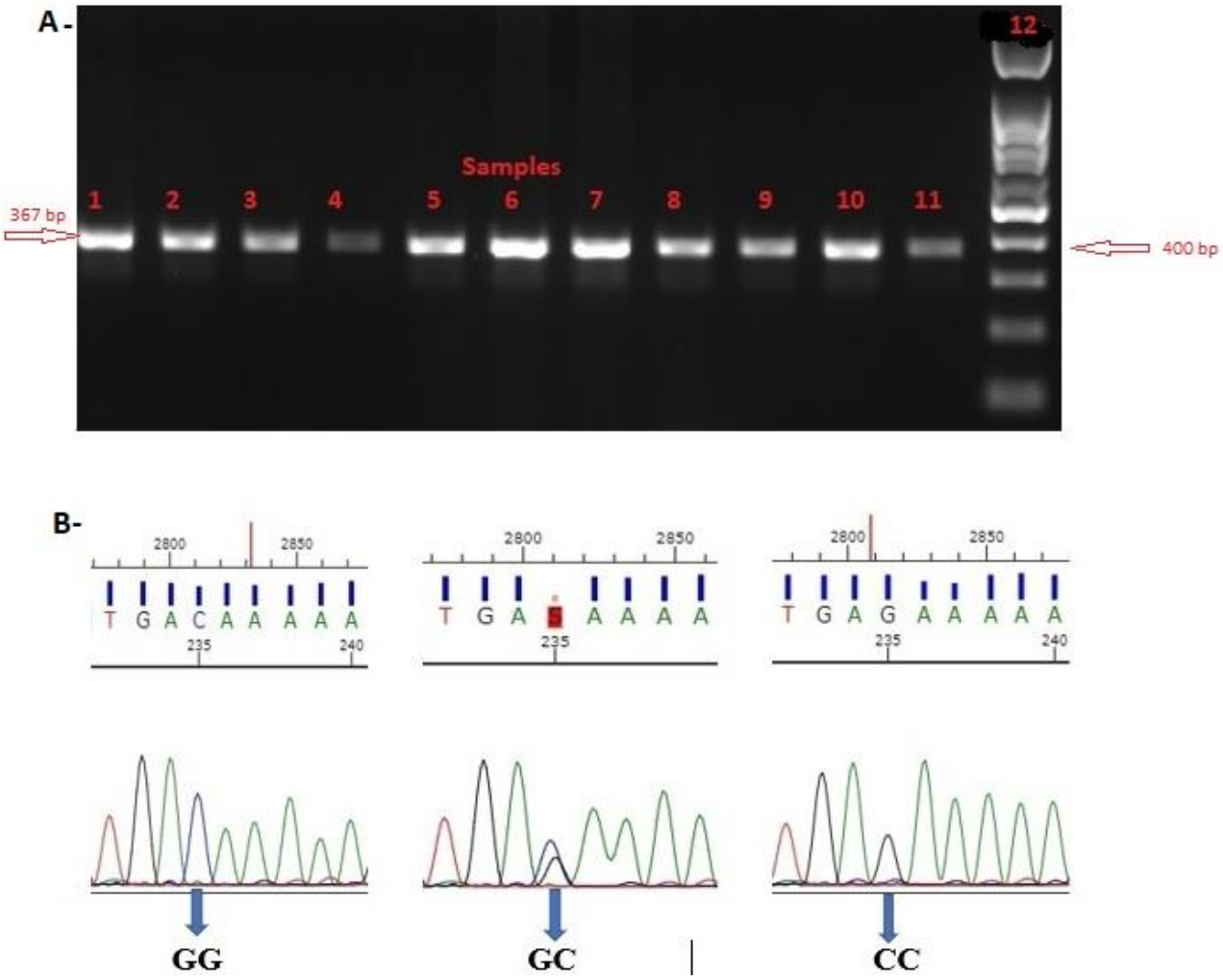
The functional consequences of SNPs that showed significant association with Iron over load was investigated by different in silico tools when appropriate, these include RegulomeDB, which is a database dedicated for noncoding SNP and annotates SNPs with known and putative regulatory elements in non-coding regions of human genome (Boyle et al., 2012). This database annotations are based on data from ENCODE project and other resources, combined together by a scoring system ranging from 1-6. A higher rank corresponds to a less functional significance (Boyle et al., 2012).In addition to RegulomeDB, the online tool HaploReg V4.1 was also used to further explore the potential role of non-coding genetic variants included in this study (Ward & Kellis, 2016 b).

Chapter Three: Results

The aim of this study was to investigate the association between selected genetic variants (single nucleotide polymorphisms SNPs) in major genes involved in iron metabolism with beta thalassemia and ferritin level in these patients. These genes include **TFRC**, which is involved in iron transport, **SLC11A2** which is involved in iron influx, **SLC40A1** which is involved in iron efflux, **HFE** which is involved in cellular iron storage and **HAMP** which is a key regulator of iron homeostasis with iron overload. Seven selected single nucleotide polymorphisms were investigated including: **rs11915082** in the **TFRC** gene, **rs224589** and **rs1048230** in the **SLC11A2** gene, **rs1439816** in the **SLC40A1** gene, **rs1799945** in the **HFE** gene and **rs104894696** and **rs10421768** in the **HAMP** gene. All investigated genotypes were in accordance with Hardy-Weinberg equilibrium (HWE) in the control group ($P< 0.05$) at the genotype levels.

3.1 Genotyping and Statistical Analysis for rs1439816 SNP Variant In The SLC40A1 Ferroprotin Gene.

PCR amplification of the rs1439816 containing sequence yielded a 367 bp product as shown in figure (3.1 A). The yielded amplicon was qualified using gel electrophoresis and shows a single clear band at the expected size (367 bp). Sanger's sequencing was used for genotyping of this amplified DNA fragment. Figure (3.1 B) shows a representative sequence of the three different genotypes (GG, GC, CC) detected in all controls and patients' samples.



GG GC CC

Figure 3.1 Genotyping of the rs1439816 variant. **A.** Representative agarose gel showing the indicated amplified 367 bp DNA fragments containing rs1439816 (1-11). Lane 12 represents 100 bp DNA ladder. **B.** Representative sequence showing the various obtained genotypes.

Table 3.1 Shows the frequency of the three indicated genotypes between controls and patients' samples. The data shows a significant difference between controls and patients (p=0.000), with the CC genotype being the most frequent in patients compared to controls with respective frequencies 90% vs.10%.

Table 3.1A Statistical analysis of genotype frequency for rs1439816.

			Subject Status		Total
			Healthy Control	Patient	
FERROPORTIN GENE	CC	Count	7	63	70
		% Within FERROPORTIN gene	10.0%	90.0%	100.0%
	GG	Count	47	5	52
		% Within FERROPORTIN gene	90.4%	9.6%	100.0%
	GC	Count	39	20	59
		% Within FERROPORTIN gene	66.1%	33.9%	100.0%
Total		Count	93	88	181
		% Within FERROPORTIN gene	51.4%	48.6%	100.0%

P= 0.000

Table 3.1B Shows statistical analysis of allele frequency (total C and total G) between controls and patients ($p < 0.0001$). The C allele of rs1439816 showed strong significant association with patients compared to control subjects (OR= 12.21, 95% CI=7.38-20.22, $p < 0.0001$).

Table 3.1 B Statistical analysis of allele frequencies for rs1439816.

SNP	Allele	Patient (%) (n=88)	Control (%) (n=88)	P Value	OR (95% CI)
rs1439816	C	146 (83 %)	53 (28.5%)	**<0.0001**	12.21 (7.38-20.22)
	G	30 (17 %)	133 (71.5%)		

3.2 Genotyping and Statistical Analysis of rs11915082 Variant In (TFRC) Gene.

PCR amplification of the rs11915082 containing sequence yielded a 306 bp product as shown in figure (3.2 A). The yielded amplicon was qualified using gel electrophoresis and showed a single clear band at the expected size (306 bp). Sanger's sequencing was used

for genotyping of the indicated amplified DNA fragment. Figure (3.2 B) shows a representative sequence of the three different genotypes (AA, GA, GG) detected in all controls and patients' samples.

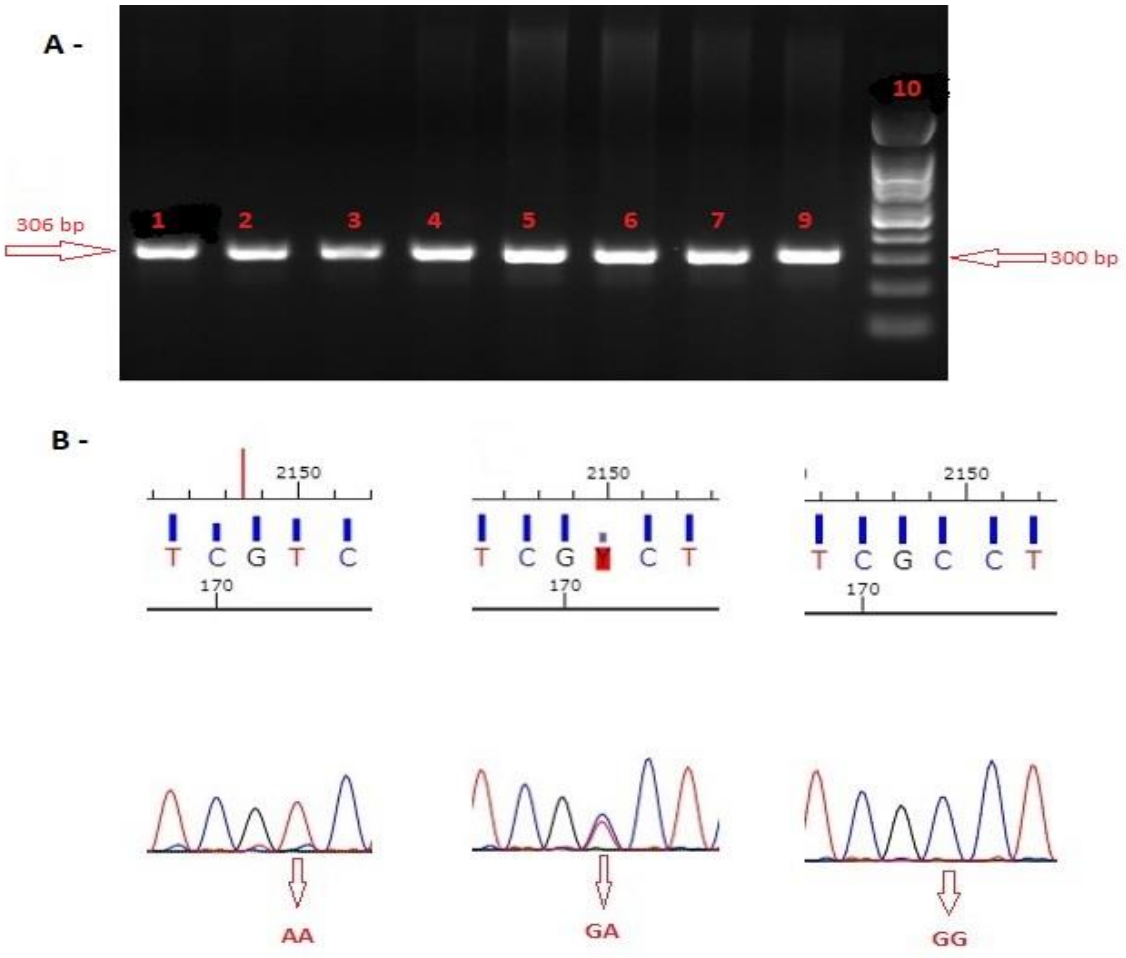


Figure 3.2 Genotyping of the rs11915082 variant. **A.** Representative agarose gel showing the indicated amplified 306 bp DNA fragments containing rs11915082 (1-9). Lane 10 represents 100 bp DNA ladder. **B.** Representative sequence showing the various obtained genotype.

Regarding statistical analysis, the genotype of the rs11915082 showed a non-significant difference between cases and controls (p=0.312) as depicted in Table 3.2A.

Table 3.2 A Statistical analysis of genotype frequency for rs11915082

			Subject Status		Total
			Healthy Control	Patient	
TFRC1 GENE	CC	Count	31	37	68
		% within TFRC1 082 GENE	45.6%	54.4%	100.0%
	TT	Count	18	11	29
		% within TFRC1 082 GENE	62.1%	37.9%	100.0%
	CT	Count	43	38	81
		% within TFRC1 082 GENE	53.1%	46.9%	100.0%
Total		Count	92	86	178
		% within TFRC1 082 GENE	51.7%	48.3%	100.0%

P= 0.312

No significant difference was seen in allele frequency between controls and cases for rs11915082 (TFRC gene) (p =0.120), as shown in table 3.2 B.

Table 3.2 B Statistical analysis of allele frequencies for rs11915082.

SNP	Allele	Patient (%) (n=88)	Control (%) (n=88)	P Value	OR (95% CI)
Rs11915082	C	112 (65.1%)	105 (57.0%)	0.120	1.4 (0.92-2.15)
	T	60 (34.9 %)	79 (43.0%)		

3.3 Genotyping and Statistical Analysis of rs224589 Variant In the SLC11A2 Gene.

PCR amplification of the rs224589 containing sequence yielded a 242 bp product as shown in figure (3.3 A). The yielded amplicon as qualified using gel electrophoresis shows a single clear band at the expected size (242 bp). Sanger's sequencing was used for genotyping of the indicated amplified DNA fragment. Figure (3.3B) shows a representative sequence of the three different genotypes (AA, AC, CC) detected in all controls and patients' samples.

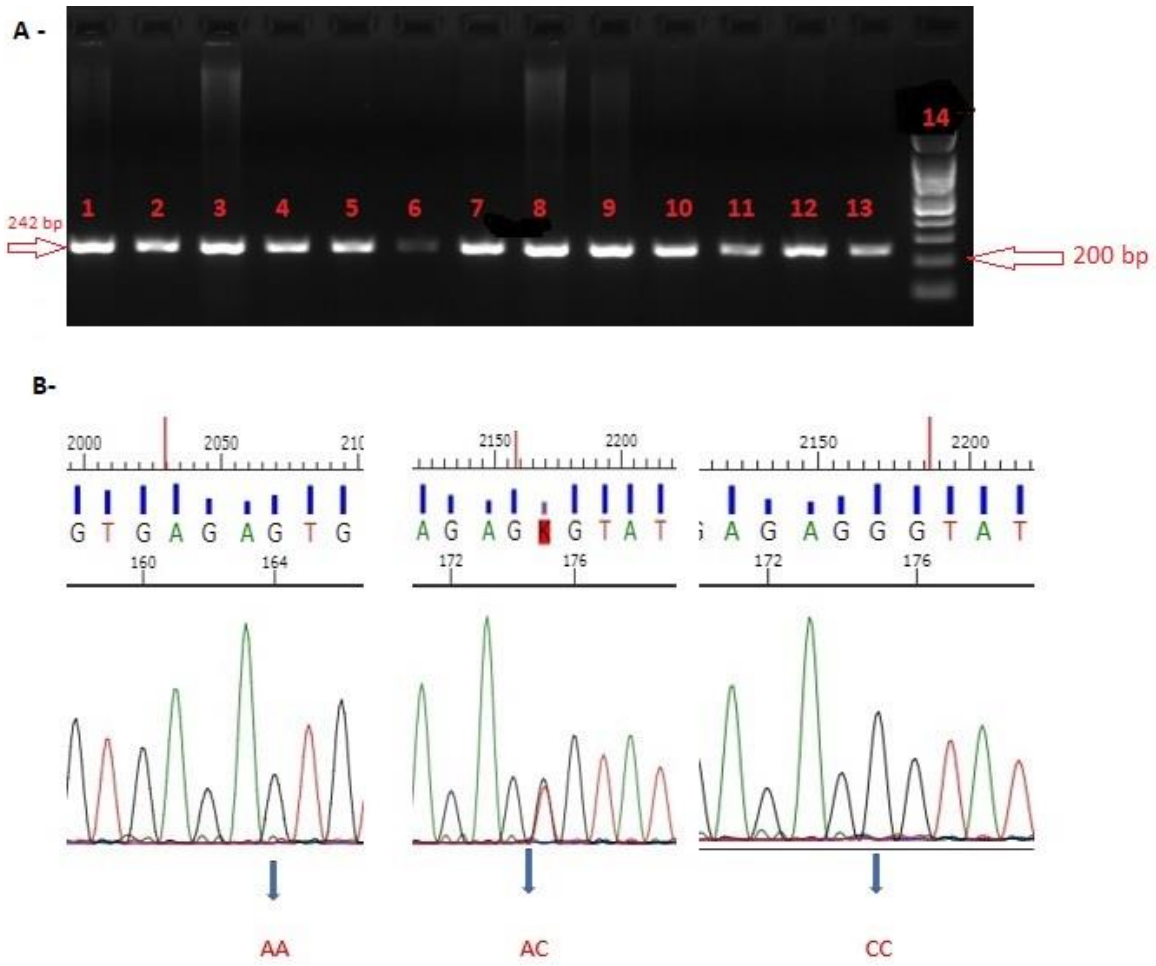


Figure3.3. Genotyping of the rs224589 variant. **A.** Representative agarose gel showing the indicated amplified 242 bp DNA fragments containing rs224589 (1-3). Lane 14 represents 100 bp DNA ladder. **B.** Representative sequence showing the various obtained genotypes.

Regarding statistical analysis, the genotype of rs224589 shows a non-significant difference between cases and controls (p=0.098), as depicted in Table 3.3A.

Table 3.3 A Statistical analysis of genotype frequency for rs224589

			Subject Status		Total
			Healthy Control	Patient	
DMT1 GENE	CC	Count	40	33	73
		% within DMT1 589 gene	54.8%	45.2%	100.0%
	AA	Count	4	9	13
		% within DMT1 589 gene	30.8%	69.2%	100.0%
	CA	Count	45	27	72
		% within DMT1 589 gene	62.5%	37.5%	100.0%
Total		Count	89	69	158
		% within DMT1 589 gene	56.3%	43.7%	100.0%

P = 0.098

No significant difference was seen in allele frequency between controls and cases for rs224589 (DMT1 gene) (p =0.589), table 3.3 B.

Table 3.3 B Statistical analysis of allele frequencies for rs224589

SNP	Allele	Patient (%) (n=88)	Control (%) (n=88)	P Value	OR (95% CI)
Rs224589	A	45 (32.6 %)	53 (29.7 %)	0.589	1.14 (0.71-1.84)
	C	93 (67.4 %)	125 (70.3 %)		

3.4 Genotyping and Statistical Analysis of rs1048230 In the SLC11A2 Gene.

PCR amplification of the rs1048230 containing sequence yielded a 366 bp product as shown in figure (3.4 A). The yielded amplicon as qualified using gel electrophoresis shows a single clear band at the expected size (366 bp). Sanger's sequencing was used for genotyping of the indicated amplified DNA fragment. Figure (3.4 B) shows a representative agarose gel of the three different genotypes (CC, CT, and TT) detected in all controls and patients' samples.

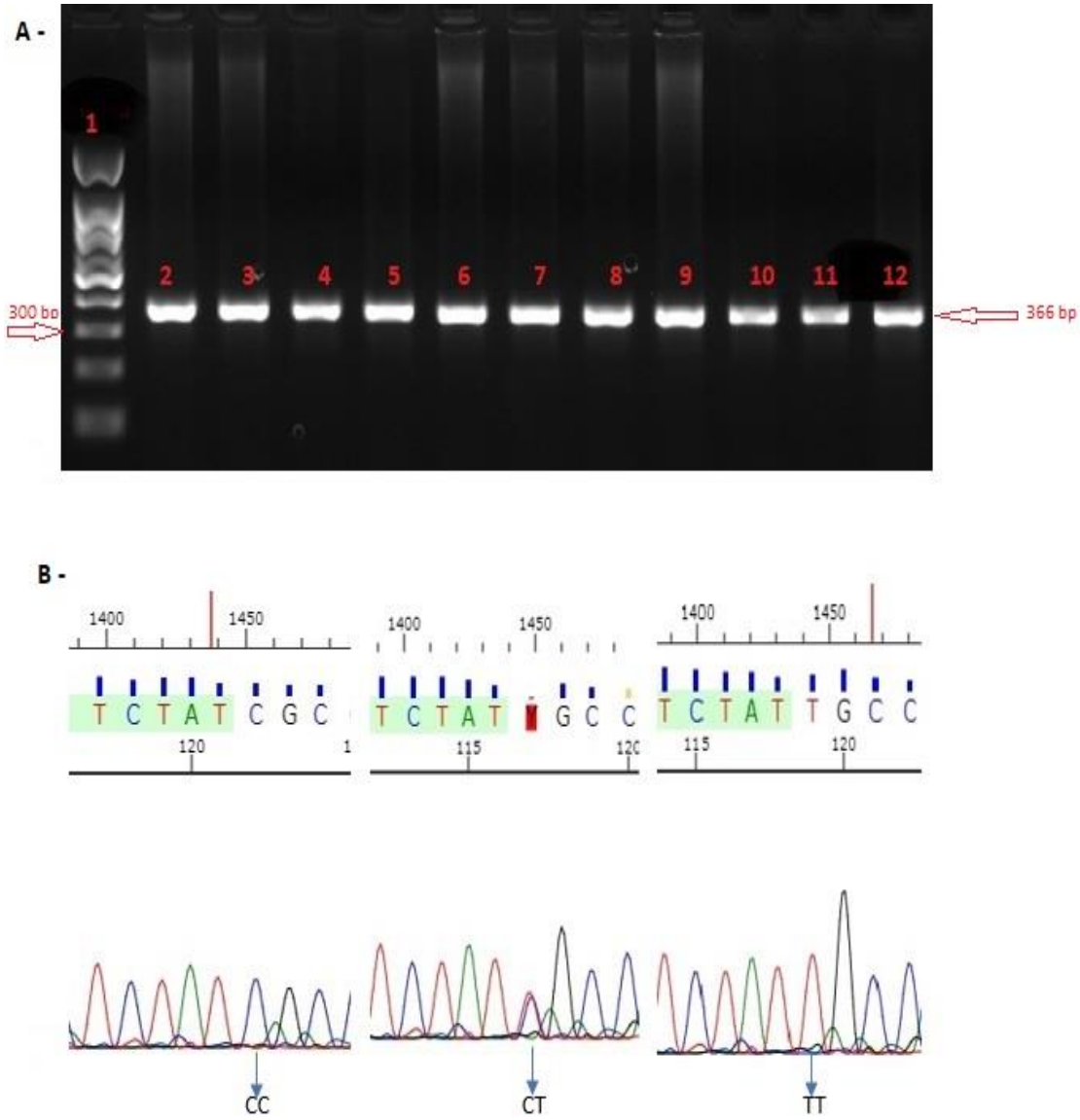


Figure 3.4 Genotyping of the rs1048230 variant. **A.** Representative agarose gel showing the indicated amplified 366 bp DNA fragments containing rs1048230 (2-12). Lane 1 represents 100 bp DNA ladder. **B.** Representative sequence showing the various obtained genotypes.

Regarding statistical analysis, the genotype of rs1048230 shows a non-significant difference between cases and controls (p=0.063), as depicted in Table 3.4A.

Table 3.4 A Statistical analysis of genotype frequency for rs1048230

			Subject Status		Total
			Healthy Control	Patient	
DMT1 230 gene	TT	Count	56	48	104
		% within DMT1 230 gene	53.8%	46.2%	100.0%
	CC	Count	1	5	6
		% within DMT1 230 gene	16.7%	83.3%	100.0%
	TC	Count	36	20	56
		% within DMT1 230 gene	64.3%	35.7%	100.0%
Total		Count	93	73	166
		% within DMT1 230 gene	56.0%	44.0%	100.0%

P= 0.063

No significant difference was seen in allele frequency between controls and cases for rs1048230 (DMT1 gene) (p =0.979), table 3.4 B.

Table 3.4 B Statistical analysis of allele frequencies for rs1048230

SNP	Allele	Patient (%) (n=88)	Control (%) (n=88)	P Value	OR (95% CI)
Rs1048230	T	116 (79.4%)	148 (79.6%)	0.979	1.01 (0.59-1.72)
	C	30 (20.6%)	38 (20.4 %)		

3.5 Genotyping and Statistical Analysis of rs10421768 In the HAMP Gene.

Amplification of the rs10421768 containing sequence using PCR yielded a 244 bp product as shown in figure (3.5 A) which shows a representative image of the yielded single clear band amplicon at the expected size.

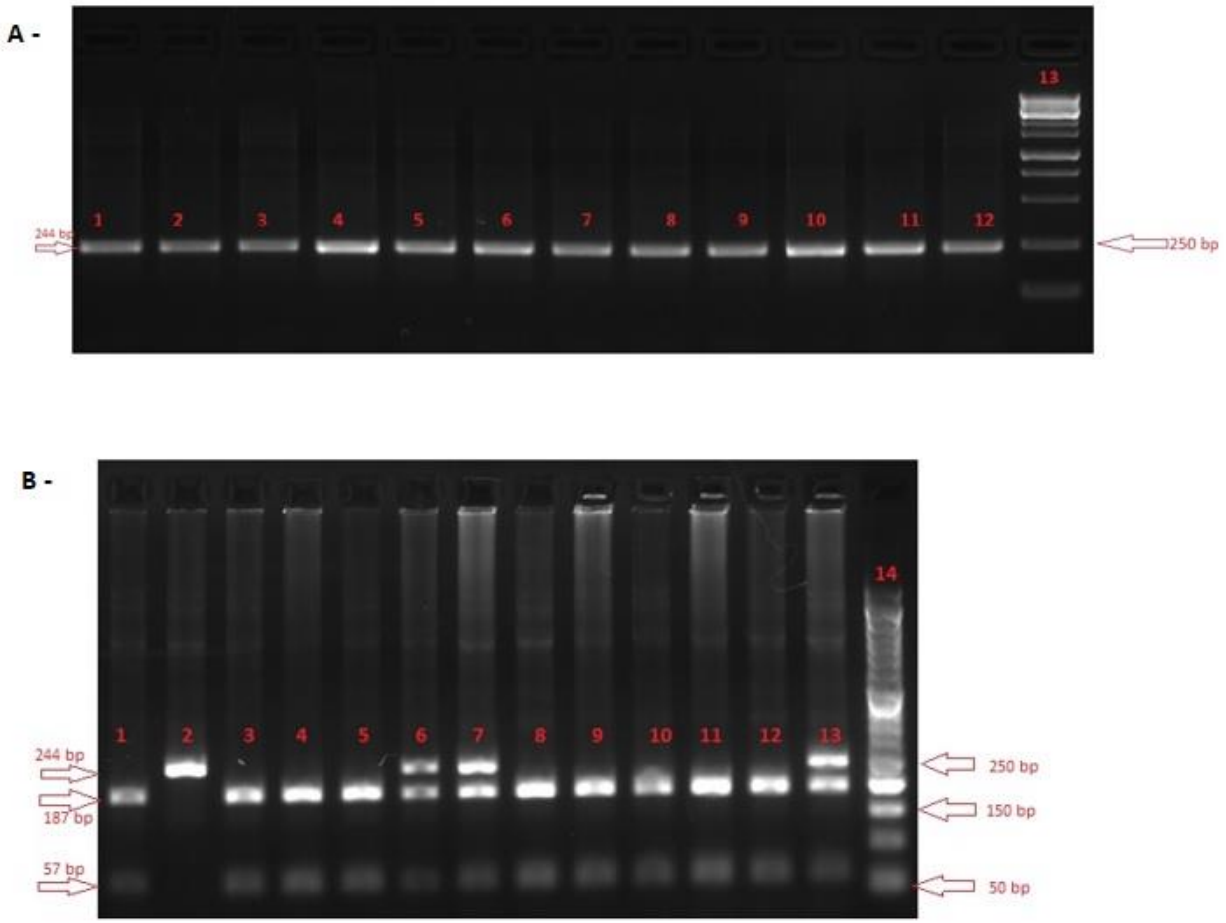


Figure 3.5 A. Agarose gel showing the indicated amplified 244 bp DNA fragment containing rs10421768 (1-12), lane 13 represent 250 bp ladder. Figure 3.5 **B.** Representative image of RFLP results with the AA genotype resulted in two bands (187 and 57 bp) (1,3,4,5,8,9,10,11,12), the AG genotype resulted in two bands (244 and 187bp) (6,7,13) and the GG genotype resulted in one band (244 bp) (2). Lane 14 represents 50 bp DNA ladder.

Genotyping of rs10421768 variant was analyzed using RFLP-PCR which yielded two DNA fragments (187 and 57 bp) for the AA genotype, and a single DNA fragment (244bp) for the GG genotype and two DNA fragments (187 and 244 bp) for the GA genotype following digestion with the HpyCH41V enzyme, (figure 3.5 B). Regarding statistical analysis, the genotype of rs10421768 shows a non-significant difference between cases and controls (p=0.20), as depicted in Table 3.5A.

Table 3.5 A Statistical analysis of genotype frequency for rs10421768.

			Subject Status		Total
			Healthy Control	Patient	
HAMP582 gene	AA	Count	60	51	111
		% within HAMP582 gene	54.1%	45.9%	100.0%
	GG	Count	1	5	6
		% within HAMP582 gene	16.7%	83.3%	100.0%
	AG	Count	32	31	63
		% within HAMP582 gene	50.8%	49.2%	100.0%
Total		Count	93	87	180
		% within HAMP582 gene	51.7%	48.3%	100.0%

P=0.20

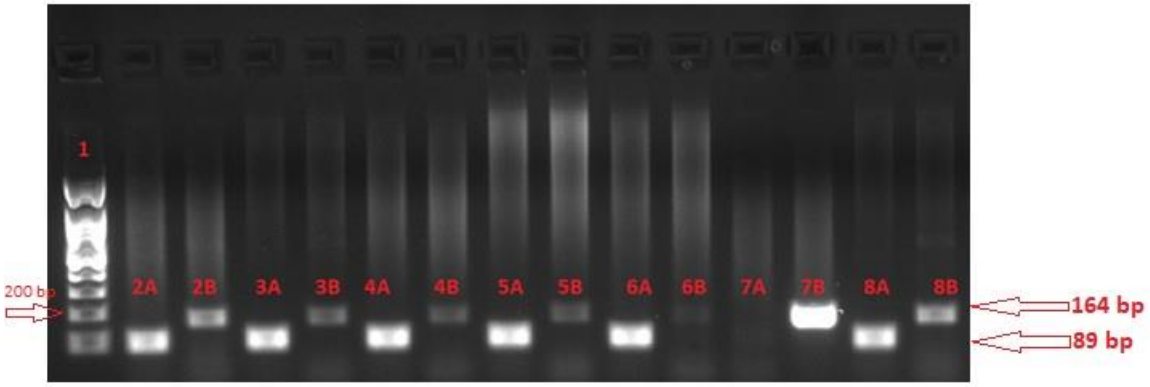
No significant difference was seen in allele frequency between controls and cases for rs10421768 in DMT1 gene (p =0.190) as described in table 3.5 B.

Table 3.5 B Statistical analysis of allele frequencies for rs10421768

SNP	Allele	Patient (%) (n=88)	Control (%) (n=88)	P Value	OR (95% CI)
rs10421768	A	133 (76.4%)	155 (82.0%)	0.190	0.71 (0.43-1.18)
	G	41 (23.6 %)	34 (18 %)		

3.6 Genotyping and Statistical Analysis of rs1799945 In the HFE Gene.

TARMS-PCR technique was performed for this variant analysis between patient and control groups. Genotyping of the rs1799945 variant was analyzed using TARMS-PCR which yielded two DNA fragments 89 bp and 164 bp for the C allele and G allele respectively, (figure 3.6).



3.6 Representative gel of T-ARMS results showing the CG genotype with two DNA A and B (89 and 164 bp) as shown in samples 2,3,4,5,8, The CC genotype resulted in one band (89 bp) as shown in sample 6, the GG genotype resulted in one band (164 bp) as shown with sample 7. Lane 1 represents 100 bp DNA ladder.

Regarding statistical analysis, the genotype of rs1799945 showed a non-significant difference between cases and controls (p=0.383) as depicted in Table 3.6.

Table 3.6 A Statistical analysis of genotype frequency for rs1799945.

			Subject Status		Total
			Healthy Control	Patient	
HFE H63D gene	CG	Count	65	82	147
		% within HFE H63D gene	44.2%	55.8%	100.0%
	GG	Count	0	1	1
		% within HFE H63D gene	0.0%	100.0%	100.0%
	CC	Count	1	4	5
		% within HFE H63D gene	20.0%	80.0%	100.0%
Total		Count	66	87	153
		% within HFE H63D gene	43.1%	56.9%	100.0%

P = 0.383

No significant difference was seen in allele frequency between controls and cases for rs1799945 (HFE gene) (p =0.777), table 3.6 B.

Table 3.6 B Statistical analysis of allele frequencies for rs1799945.

SNP	Allele	Patient (%) (n=88)	Control (%) (n=66)	P Value	OR (95% CI)
Rs1799945	C	90 (52%)	66 (50.3%)	0.777	1.07(0.68-1.68)
	G	83 (48%)	65(49.7%)		

3.7 Genotyping and Statistical Analysis of rs104894696 in the HAMP Gene.

TARM-PCR technique was performed for Genotyping of rs104894696 variant. TARMS-PCR yielded two DNA fragments 236 and 521 bp for the G allele, and A allele respectively as shown in figure3.7.

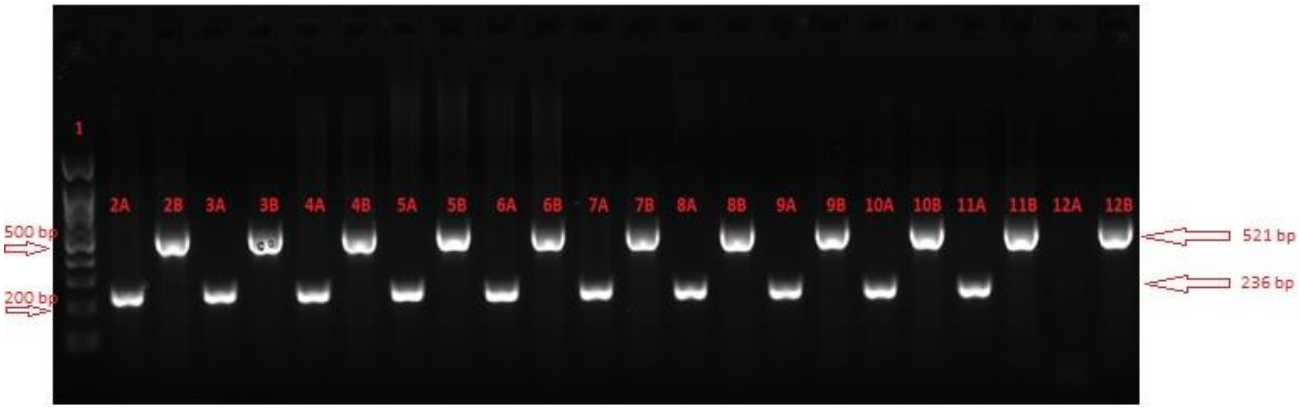


Figure 3.7. Representative agarose gel of T-ARMS results with The GA genotype resulted in two bands (236 and 521 bp) (A and B) (1-11), The AA genotype resulted in one band (521 bp) (12B). 1 represent 100 bp DNA ladder.

Regarding statistical analysis, the genotype of rs104894696 shows a non-significant difference between cases and controls (p=0.382), as depicted in Table 3.7A

Table 3.7 A Statistical analysis of genotype frequency for rs104894696

			Subject Status		Total
			Healthy Control	Patient	
HAMP G71D gene	GA	Count	66	86	152
		% Within HAMP G71D gene	43.4%	56.6%	100.0%
	AA	Count	0	1	1
		% Within HAMP G71D gene	0.0%	100.0%	100.0%
Total		Count	66	87	153
		% Within HAMP G71D gene	43.1%	56.9%	100.0%

P= 0. 382

No significant difference was seen in allele frequency between controls and cases for rs104894696 (HAMP gene) (p =0.921), table 3.7 B.

SNP	Allele	Patient (%) (n=88)	Control (%) (n=66)	P Value	OR (95% CI)
Rs104894696	G	86 (49.4%)	66 (50%)	0.921	0.98(0.62-1.53)
	A	88(50.6%)	66(50%)		

In summary, tables 3.8 demonstrate the genotype and haplotype frequencies of all investigated variants between control and patient subjects. The results clearly indicate a significant difference (P = 0.000) which was only evident for rs1439816 (in FPN1 gene) between control and patients subjects, table 3.8. Similarly, a significant different (P = 0.000) was only evident for C allele of rs1439816 (in FPN1 gene) for association with patients compared to control subjects as sown in table 3.9.

Table 3.8 Statistical analysis of genotype frequencies

SNP	Genotype	Patient (%) (n=88)	Control (%) (n=88)	P Value
rs11915082	GG	37 (43 %)	31 (33.7 %)	0.321
	AG	38 (44.2 %)	43 (46.7%)	
	AA	11 (12.8 %)	18 (19.6%)	
rs1048230	TT	48(65.7%)	56 (60.2%)	0.063
	TC	20 (27.4%)	36(38.8%)	
	CC	5 (6.9 %)	1 (1%)	
rs224589	AA	9 (13 %)	4 (4.4 %)	0.098
	AC	27 (39 %)	45 (50.5%)	
	CC	33 (48%)	40 (45.1%)	
rs1439816	CC	63(71.5%)	7 (7.5 %)	**0.000**
	CG	20 (22.7%)	39(41.9%)	
	GG	5 (5.8 %)	47 (50.6%)	
rs10421768	AA	51 (58.6%)	60 (64.5 %)	0.20
	AG	31 (35.6%)	32 (34.4 %)	
	GG	5 (5.7 %)	1 (1 %)	
rs1799945	GG	1 (1 %)	0(0%)	0.383
	GC	82 (94.2%)	65 (98.4%)	
	CC	4 (4.8 %)	1 (1.6 %)	
rs104894696	GA	86 (98.8 %)	66 (100%)	0.382
	AA	1 (1.2 %)	0 (0 %)	
	GG	0 (0%)	0 (0 %)	

Values in bold indicate significant difference.

Table 3.9 Statistical analysis of allele frequencies

SNP	Allele	Patient (%) (n=88)	Control (%) (n=88)	P Value	OR (95% CI)
Rs11915082	C	112 (65.1%)	105 (57.0%)	0.120	1.4 (0.92-2.15)
	T	60 (34.9 %)	79 (43.0%)		
Rs1048230	T	116 (79.4%)	148 (79.6%)	0.979	1.01 (0.59-1.72)
	C	30 (20.6%)	38 (19.4 %)		
Rs224589	A	45 (32.6 %)	53 (29.7 %)	0.589	1.14 (0.71-1.84)
	C	93 (67.4 %)	125 (70.3 %)		
Rs1439816	C	146 (83 %)	53 (28.5%)	**<0.0001**	12.21 (7.38-20.22)
	G	30 (17 %)	133 (71.5%)		
Rs10421768	A	133 (76.4%)	155 (82.0%)	0.190	0.71 (0.43-1.18)
	G	41 (23.6 %)	34 (18 %)		
Rs1799945	C	90 (52%)	66 (50.3%)	0.777	1.07(0.68-1.68)
	G	83 (48%)	65(49.7%)		
Rs104894696	G	86 (49.4%)	66 (50%)	0.921	0.98(0.62-1.53)
	A	88(50.6%)	66(50%)		

Values in bold indicate significant difference

3.8 The Association between various genotypes and haplotypes frequencies and ferritin levels in thalassemia patients.

The association between ferritin levels status and the variants in the indicated genes could not be achieved. A major complication emerged where eventhough patients' data showed significant variation in their serum ferritin levels (use as the standard measure of iron overload), however, no information was available indicating compliance of all patients with regular frequency of blood transfusions. In addition, patients' chelation therapy was very sporadic regarding drug (Desferroxamine or Exjade) dose, compliance in taking the drug as required and frequency of testing. All these complications could not provide the needed accurate ferritin level variation for correlation with the molecular genotyping data.

Chapter Four: Discussion

Thalassemia's are a group of common inherited blood disorders caused by defects in the globin protein production (Cao et al., 1997; Rund & Rachmilewitz, 2005). Thalassemia major patients are usually under regular blood transfusion especially beginning from the first two years of life (Galanello & Origa, 2010). Patients who are under multiple blood transfusions due to ineffective erythropoiesis and increased gastrointestinal iron absorption develop iron overload in the body. Iron overload can lead to multiple organs damage and increased mortality especially due to cardiac complications caused by iron deposition in the heart (Fung EB, Harmatz P, Milet M, Ballas SK, De Castro L, 2007; Ladis et al., 2010; Mishra & Tiwari, 2013). Thus, it is very important to frequently measure plasma iron levels in order to evaluate iron overload clinical prevalence, timing and monitoring of chelating therapy and providing the needed adjustment treatment. In the present study, the association of specific

selected single nucleotide polymorphisms (SNPs) in several genes involved in iron metabolism including rs1439816 in SLC40A1 (FPN) gene, rs11915082 in TFRC gene, rs224589 and rs1048230 in SLC11A2 (DMT1) gene, rs104894696 and rs10421768 in HAMP (Hepcidin) gene and rs1799945 in the HFE gene with thalassemia were investigated among Palestinian thalassemia patients.

Ferroportin is the sole cellular efflux channel for iron and is regulated by iron regulatory hormone hepcidin which binds ferroportin and induces its internalization and degradation. (Ganz, 2005). The present data showed strong significant difference in the ferroportin genetic variant rs1439816 frequencies between controls and patients which indicates a possible role in beta thalassemia pathogenesis among patients. The data indicate the CC genotype was significantly associated with the disease while the GG genotype was significantly with the control suggesting the C variant represents a risk genomic marker for one or more of thalassemia clinical complications (like iron overload) while the G variant seems to be protective. The rs1439816 SNP variant is located in intron 1 of the SLC40A1 gene. A study on this variant showed the C allele represents a risk of Alzheimer's disease that was significantly associated with decreased expression of the SLC40A1 gene in these patients causing increased Fe levels inside the cell(Crespo et al., 2014). Another study showed that individuals heterozygous for a variety of missense mutations in ferroportin develop macrophage - predominant iron overload that may progress to iron-induced organ damage (Montosi et al., 2001; Pietrangelo, 2004). Our results similarly showed the C allele in SLC40A1gene was significantly associated with thalassemia major patients compared to controls. In silico

analysis using RegulomeDB showed this SNP has a score of 4 indicating it has no pathogenic consequence. However, HaploReg analysis showed that this variation disrupts two regulatory motifs including the binding sites of Eomes and SREBP (Ward & Kellis, 2016a). The divalent metal transporter 1 (DMT1) is encoded by the solute carrier family 11-member 2 gene SLC11A2 and it is widely expressed and has been postulated to play important roles in intestinal iron absorption, erythroid iron utilization, hepatic iron accumulation, placental iron transfer, and other processes (Hiromi Gunshin et al., 2005). The mutation located in exon 12 was described in a human patient presented with anemia and hepatic iron overload and the lack of full-length mRNA would predict deficient iron absorption in the intestine and deficient iron utilization in erythroid precursors (Mims et al., 2005). Other polymorphisms in the SLC11A2 gene, including rs224589, have been documented to be associated with many disorders including, age-related macular degeneration. Patients with CC genotype increase risk of age-related macular degeneration (Wysokinski et al., 2012a), microcytic anemia (Kloss-Brandstätter et al., 2012) and Wilson's disease (Przybyłkowski et al., 2014) . It was speculated that the rs224589 polymorphism may affect alternative splicing or constitutive splicing of the SLC11A2 gene through the corruption of splicing regulatory cis-elements which can result in incorrect isoforms of DMT1 (Przybyłkowski et al., 2014; Tazi et al., 2009; Ward & Cooper, 2010). In addition, since this SNP is located in an intronic region, it might affect transcription, post-transcription, and ultimately mRNA translation of the SLC11A2 gene. However, our results showed both rs1048230 and rs224589, located in the SLC11A2 gene were not correlated with thalassemia patients compared to the control group as both genotypes and alleles frequencies were not significantly different between patients and the control group.

The hepcidin antimicrobial peptide (HAMP) gene is located on chromosome 19q13.12. The protein is composed of 25 amino acids involved in innate immunity (Ganz, 2006) and appears to play a crucial role in iron homeostasis in humans via regulating iron absorption from the intestine and recycling by macrophages (Kemna et al., 2008). Hepcidin was found to be down regulated in patients with thalassemia (Papanikolaou et al., 2005). The rs104894696 variant in the HAMP gene is likely to be associated with reduced hepcidin activity that is relatively common in hereditary hemochromatosis (Merryweather-Clarke et al., 2003). Another study showed this variant was predisposed in beta thalassemia patients and correlate with high serum ferritin levels and iron overload (Silva et al., 2014). Another variant (rs10421768) seems to participate in the activation of the HAMP gene expression both by upstream regulatory factor 1 and 2 (USF1/USF2) and cMyc/Max heterodimers that occur through E box within the promoter, which can alter the regulation of the HAMP gene and hepcidin function in iron homeostasis (Bayele & Srai, 2009). In comparison, our data showed both rs104894696 and rs10421768 variants in the HAMP gene were not correlated with thalassemia since both alleles and genotypes frequencies were not significantly different between the control and patient subjects. The TFRC gene encodes a cell surface receptor TfR1, a glycoprotein with a molecular weight of about 90kDa, expressed as a homodimer ubiquitously in the majority of cells with high binding affinity to transferrin bound iron (Nadadur et al., 2008). TfR1 is essential for iron uptake by reticulocytes and other cell types by endocytosis. The rs11915082 (-253G < A) polymorphism is located in the 5'- upstream region (promoter) of the TFRC gene and is expected to affect gene expression regulation level of this gene. This SNP was documented to be associated with age-related macular degeneration (Wysokinski et al., 2012b). In silico analysis of this SNP

using the RegulomeDB database showed that this SNP has a score of 4, indicating lack of evidence that it has a pathogenic effect (Boyle et al., 2012). However, HaploReg analysis showed that this variation may affect the binding of several regulatory factors including CHD2, SRF, and ZBTB33 (Ward & Kellis, 2016a). Our data showed the rs11915082 SNP was not correlated with beta thalassemia since both alleles and genotypes frequencies were not significantly different between patients and control groups. Finally, a mutation in the HFE gene (H63D) disrupts the binding of HFE with beta2 microglobulin and prevent its surface expression (Kaur et al., 2003). Mutations in the HFE gene, known to be significantly associated with hereditary hemochromatosis, was found to have a modifying effect on iron absorption in thalassemia patients (Camaschella et al., 2002). A study in India showed higher frequency of H63D mutation in beta thalassemia patients compared with controls (Melis et al 2002). However, the present data showed the rs11915082 SNP was not correlated with thalassemia were both alleles and genotypes frequencies were not significantly different between patients and control groups.

Conclusion and recommendations

In summary, the present study showed that the among the investigated gene variants in the indicated genes including (FPN1, DMT1, TFRC, HFE, HAMP), only the variant in the Ferroportin gene showed strong significant association with thalassemia. The ferroportin protein controls efflux of iron from tissues into plasma. Therefore, it is expected to influence plasma ferritin levels. However, correlation of this and the other gene variants with the steady state levels of plasma ferritin which reflects iron overload status in these patients were not conclusive. Plasma ferritin levels showed significant variation among all patients, however, these levels could not be correlated with well documented treatment

program including compliance to regular blood transfusion and the use of chelation therapy as needed in addition to the absence of closely monitored records concerning the various clinical complications. Therefore, extended efforts are needed to correlate all variants in the indicated genes with these parameters in a highly controlled group of patients. It is highly expected that one or more of the genotypes of these variants may play a significant role in the development of these complication. This could prove to be extremely variable in predicting the development of these complications among patients which will allow early clinical intervention in order to decrease the severity of these complications and allows better response to their treatment.

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الملخص

الثلاسيميا هي مجموعة من اضطرابات الدم الوراثية الشائعة التي سببها خلل في انتاج بروتين الغلوبين . يرتبط ارتفاع مستوى الحديد في الدم بزيادة حدة الحالة المرضية لمرضى الثلاسيميا اللذين يعتمدون على نقل الدم .بما ان الحديد يلعب دورا مهما بالعديد من عمليات الايض بالجسم ,فاننا افترضنا انه في حال وجود أي خلل في تنظيم مستوى الحديد بالجسم فانه قد يلعب دورا مهما في التسبب بالمضاعفات المتعلقه بمرض الثلاسيميا خاصه تراكم الحديد .لذلك فإن هدف هذه الدراسة يتعلق بفحص وجود علاقة بين بعض التغيرات المفردة في القواعد النيتروجينية في مجموعة محددة من الجينات التي تلعب دورا مهما في أيض و تنظيم الحديد مع مرض الثلاسيميا ومضاعفات زيادة مستوى الحديد .وقد شملت الدراسه المتغيرات المحدده في الجينات التاليه :

rs11915082 (TFRC) gene, rs1048230 and rs224589 (SLC11A2) gene, rs1439816 (SLC40A1) gene, rs10421768 and rs104894696 (HAMP) gene and rs1799945 (HFE). اشتملت هذه الدراسة على 88 مريض فلسطيني مصاب بالثلاسيميا و 88 شخص سليم. ولتحديد التغيرات الجينية في كل موقع جيني مذكور اعلاه لكل مشارك في هذه الدراسة , تم استخدام التقنيات التاليه :

(RFLP-PCR, Sanger Sequencing, TARMS-PCR) .

أظهرت نتائج الدراسة أن التغير الجيني (C) (rs1439816) (P=0.00) في جين Ferroportin مرتبط بشكل قوي بالثلاسيميا بينما فشلت المتغيرات الجينية في الجينات الأخرى من إيجاد ارتباط كبير بهذا المرض مع الارتفاع في نسبة الحديد بالجسم وذلك بسبب خطط العلاج المختلفة للمرضى وبسبب غياب السجلات الدقيقة لخطط العلاج لكل مريض والتزام المرضى الحثيث بالعلاج, لم يكن بالامكان ربط الجين المذكور بمستوى تراكم الحديد في حين اظهرت مخرجات الدراسه الى إمكانية استخدام المتغير rs1439816 (C) كمؤشر قوي لمرض الثلاسيميا وتعقيداتها.