Faculty of Pharmacy, Nursing and Health Professions
Master program in clinical laboratory sciences

Iron Refractory Iron Deficiency Anemia Due to Mutations in the Negative Regulator Matriptase 2/TMPRSS6 gene Down Regulating Hepcidin in Palestinian Subjects

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2018

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I dedicate this work:

To my father and mother for always believing in me unconditionally, without your support I would not be where I am today

To my beloved husband Mahmuod for always supporting and encouraging me

To my deceased uncle Dr. Yusef Arar for his encouragement and strong belief in me, I wish you were here to see it to completion

To my children Intisar, Ibtisam, Iram, and Yusef

To my mother in law for her support, especially with the kids

To my sisters and brothers

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Many individuals have contributed to the completion of this work. I wish to thank all who made this work possible with their support, advice, and effort.

Declaration

I certify that the work provided in this thesis, unless otherwise referenced, is my own work, and to the best of my knowledge and belief has not been submitted elsewhere for any other degree or qualification.

I understand the nature of plagiarism, and I am aware of the University's policy on this.

LHA
Latifa Arar

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Abbreviations

amino acid :	**aa**
Bone morphogenetic protein:	**BMP**
Center for Disease Control and prevelation:	**CDC**
Complete blood count:	**CBC**
Deoxyribonucleic acid:	**DNA**
Complement C1r/C1s, Uegf, Bmp 1 domain:	**CUB domain**
Enzyme linked immuno-sourbent Assay:	**ELISA**
Ethylene diamine tetra acetic aAcid :	**EDTA**
Iron :	**^{1}Fe**
Glycosylphosphsatidylinosito linked protein:	**GPI-linked protein**
Hematocrit concentration:	**HCT**
Hemoglobin:	**Hg**
Hepcidin Antimicrobial peptide:	**HAMP**
Human hemochromatosis protein:	**HFE**
Hemojuvelin:	**HJV**
Interleukins-6 :	**IL-6**
Iron Deficiency Anemia:	**IDA**
Iron-Refractory Iron Deficiency Anemia:	**IRIDA**
Low-density-lipoprotein receptor class A:	**LDLRA**
Liver-Expressed Antimicrobial protein 1:	**LEAP-1**
Mean Cell Hemoglobin:	**MCH**
Mean Cell Volume:	**MCV**
Polymerase Chain Reaction:	**PCR**
Ribonucleic acid:	**RNA**
Red blood cells :	**RBCs**
sperm protein, enterokinase, and agrin domain:	**SEA domain**
mothers against decapentaplegic homolog:	**SMAD**
Total Iron Binding Capacity:	**TIBC**
Transmembrane protease serine 6 gene:	**TMPRSS6**
Transferin:	**Tf**
Transferin receptor 2:	**TfR2**
World Health Organization:	**WHO**
real time polymerase chain reaction:	**Rt-PCR**
Absorbance:	**Abs**
double stranded DNA:	**dsDNA**
single stranded DNA:	**ssDNA**
Restriction fragment length polymorphism:	**RFLP**

Abstract

Maintaining iron homeostasis within specific levels is important for normal physiological function. Deviation from these levels leads to systemic iron disorders. Hepcidin is the key element that regulates systemic iron homeostasis. Positive and negative regulators of this hormone attribute greatly in maintaining hepcidin within normal ranges depending on serum iron levels and iron stores under normal physiologic conditions; mutations of which results in iron disorders. The negative regulator of hepcidin, matriptase 2 (encoded from the gene TMPRSS6) is a type 2 transmembrane serine protease. Mutations in this gene results in microcytic, hypochromic anemia that doesn't respond to oral iron and only partially respond to parenteral administration; a condition termed iron refractory iron deficiency anemia (IRIDA). Low iron absorption and decreased iron release from stores was reported due to abnormally elevated levels of hepcidin. In this research, 20 IRIDA patients and 20 age and sex matched healthy individuals were recruited for TMPRSS6 gene screening for polymorphisms using the melting analysis technique. The subjects were also tested for iron parameters and hepcidin. All iron parameters in the recruited patients were significantly lower than the control group; however, Hepcidin and TIBC were significantly higher. Parenteral iron treatment did partially treat the anemia. However, hepcidin level was not affected by the state of treatment among subjects. When screening all of the 18 exons of the gene using the melting analysis technique, the patients presented with distinct melting temperatures that were not present in the control. These distinct melting patterns may represent pathogenic polymorphisms that need to be confirmed and analyzed by sequencing the exons where the distinct melting pattern was presented.

ملخص الدراسة

نبذة مختصرة

الحفاظ على توازن الحديد داخل مستويات محددة أمر مهم للوظائف الفسيولوجية الطبيعية في الجسم. انحراف كمية الحديد عن هذه المستويات يؤدي إلى اضطرابات في هذه الوظائف . هرمون ال Hepcidin هو العامل الاساسي الذي ينظم توازن الحديد. يوجد لهذا الهرمون منظمات ايجابية وأخرى سلبية تلعب دورا هاما في الحفاظ على مستواه ضمن النطاق الطبيعي اعتمادا على مستويات الحديد في الدم ومخزونه. المنظم السلبي لهرمون ال Hepcidin يدعى matriptase 2 حيث توجد الشفرة الوراثية له على الجين TMPRSS6

(TMPRSS6 - type 2 transmembrane serine protease)

.تؤدي الطفرات في هذا الجين إلى فقر دم يتصف بصغر وشحوب كريات الدم الحمراء وعدم استجابة الجسم لتعاطي الحديد بالفم او استجابة محدودة لاعطائه بالحقن. هذه الحالة تدعى iron refractory iron deficiency anemia (IRIDA) . اثبتت التقارير وجود حالات انخفاض امتصاص الحديد مع انخفاض إطلاق الحديد المخزون بسبب الارتفاع الغير طبيعي في مستويات ال hepcidin . في هذا البحث ، تم اختيار 20 مريضاً من الـ IRIDAو 20 من الأفراد الأصحاء بنفس العمر والجنس لفحص تنوع اشكال جينات ال TMPRSS6 باستخدام تقنية البلمرة. كما تم عمل فحوصات إضافية لها علاقة في نسبة الحديد في الجسم ومستوى هرمون ال hepcidin. جميع نتائج الفحوصات التي لها علاقة في نسبة الحديد في الجسم كانت اقل بكثير في المرضى بالمقارنة مع الاصحاء. بينما كان Hepcidin و TIBC أعلى بكثير. علاج الحديد بالحقن الوريدي كان له تاثيرا ايجابيا جزئيا ومحدودا في علاج فقر الدم. ومع ذلك ، لم يتأثر مستوى hepcidin . عند فحص جميع ال exons 18 المكونة للجين باستخدام تقنية melting point by real time PCR ، كانت درجة حرارة الانصهار في عينات المرضى مختلفة تماما عن درجة انصهار عينات مجموعة الاصحاء. بالنتيجة قد تمثل هذه الأنماط المتميزة للانصهار وتعدد اشكال الجينات حالات مرضية تحتاج إلى التأكد منها وتحليلها من خلال تحديد تسلسل الإكسونات (sequencing).

Iron refractory iron deficiency anemia caused by mutations in matriptase 2 gene, a negative regulator of hepcidin, in Palestinian subjects

Chapter 1

Introduction

Chapter 1: Introduction

1.1 Overview

Iron is one of the most important elements for most living organisms. It is essential for many fundamental biological processes including DNA, RNA and protein synthesis, regulation of gene expression, electron transport as well as cellular proliferation and differentiation. It forms the active sites of many important enzymes, while it acts as a cofactor for others. And most importantly it is required for cellular respiration; as it acts as a cofactor for oxygen transport to cells through hemoglobin (Winfield, 1965), which is where most of the total body iron is present. Without sufficient iron, cells lose their ability for electron transport and energy metabolism. Nevertheless, cellular damage can be caused by the redox activity of iron due to the production of reactive oxygen radicals (Kroot et al, 2011). Therefore, iron concentrations must be closely regulated both at the cellular and systemic levels.

The balance of systemic iron level within limits is crucial for humans. Understanding the mechanisms of iron homeostasis is therefore crucial in order to prevent iron deficiency (anemia) and iron overload (hemochromatosis); which belong to the most frequent disorders in the world (Muckenthaler, 2008). Iron deficiency may occur due to physiological, nutritional, and/or genetical shortfalls, which may lead to anemia. Anemia, according to the WHO organization (2001), is the most prevalent nutritional disorder worldwide, affecting mainly women and kids.

The control of iron absorption and regulation depends on body iron needs. The liver expressed peptide hepcidin plays a central role in the regulation of iron homeostasis (Nicolas et al., 2001). Studies suggest that hepcidin is the principal molecule that directs systemic iron homeostasis. There are many positive and negative regulators that control hepcidin expression at the physiological and molecular level (Muckenthaler, 2008). Any disruption of these regulators

can cause hepcidin to play an important role as a pathogenic mediator (Kroot, et al. 2011). A defective or a depleted hepcidin or its positive regulators results in hepcidin under expression and cause hemochromatosis. Over expression of hepcidin is involved with anemia. In this study, iron refractory iron deficiency anemia (IRIDA) disorder associated with hepcidin over expression due to a defective negative regulator called matriptase 2 will be investigated.

1.2 General Objectives

1. To screen matriptase 2 gene for polymorphisms that could be involved in hepcidin over expression and IRIDA using the melting analysis technique in Palestinian subjects.
2. To investigate hepcidin status and iron parameters among iron deficient anemic patients with refractoriness to oral iron therapy (IRIDA patients) and compare them with that of controls.

1.3 Specific Objectives

1. To determine and evaluate the effect of serum hepcidin level in IRIDA patients and controls.

2. To measure serum iron, TIBC, C reactive protein & complete blood count (CBC) indices, calculate transferrin saturation, and investigate the possible relationship between hepcidin hormone and these parameters in both patients and controls.

3. To detect the effect of treatment on hepcidin level in patients.

4. To establish a variation map for each exon of the TMPRSS6 gene in controls and compare them against patients, and then eliminate these common variants to detect rare variants that could be disease causing.

1.4 Significance

Assessment of hepcidin and its related genes and assaying hepcidin levels can be an important diagnostic and monitoring application in both iron overload and anemia disorders. Studies in understanding the biology, function and role of hepcidin and its positive and negative regulators in iron metabolism is needed. The number of disease causing mutations in iron disorders is being increasingly identified but mainly in patients originating from Europe. There have been no published studies on hereditary iron disorders in the Middle East in relation to hepcidin or its related genes, despite the fact that the high degree of consanguinity makes this region more susceptible to these types of genetic mutations. Moreover, there is difficulty in recognizing hereditary iron disorders due to increased hemoglobin disorders as well as iron deficiency that is prevalent in developing countries which could mask the presentation of these iron disorders. In many cases, the diagnosis of IRIDA patients is only achieved during adulthood due to misclassification. Until now this condition is under diagnosed worldwide. Less than hundred cases have been presented in the literature since the discovery of the gene responsible for this condition in 2008 throughout the world. Because of this, no genotype- phenotype correlation has been well established (Falco et al, 2013); also no correlation between age at diagnosis and degree of anemia has been observed. The increasing number of molecular diagnosis of IRIDA will allow for better prediction of disease severity and provide better understanding in the mechanism of action of the matriptase gene. Furthermore, genotyping the disease may be useful in predicting the severity of the disease, in predicting the response to iron therapy, and in finding better iron therapies aimed to treat this condition and other iron metabolism disorders.

Chapter 2

Literature Review

Chapter 2: Literature Review

2.1 Iron Regulation and Hepcidin

Iron is a conserved element in the body, with very minimum losses each day. Iron homeostasis is maintained via iron recycling from macrophages acquired from dead red cells and release to serum transferin (20-30mg/day), and duodenal absorption of iron from diet, as shown in figure 2.1. About only 1-2mg/day of iron is absorbed daily from the diet in order to compensate the lost iron from the body (Cook et al, 1975) by skin sloughing, menstruation and excretion in feces and urine (Demaeyer, 1980), which is about 1-2mg/day. Apart from these small losses, there is no iron excretion system in humans to regulate body iron levels. Therefore, iron homeostasis is controlled through the regulation of iron uptake from duodenum, storage, recycling, and use in order to maintain extracellular iron concentration.

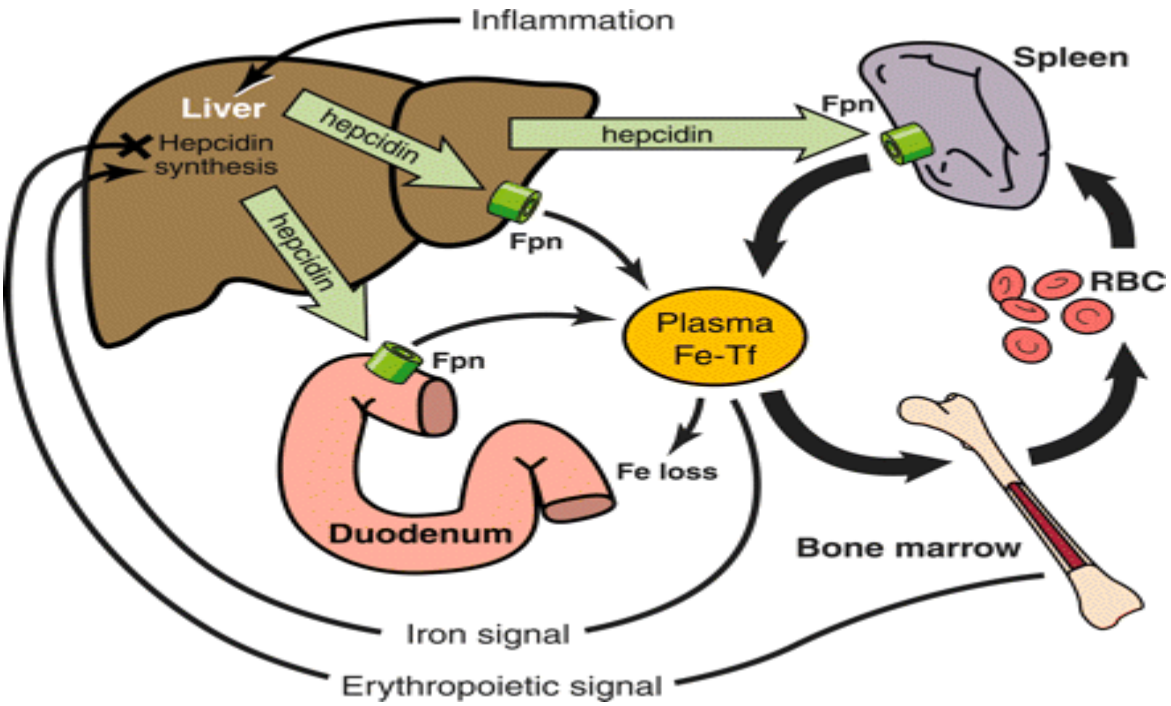


Figure 2.1: Systemic iron homeostasis (Nemeth et al, 2004)
Fpn: Ferroportin channel, Fe-Tf: Ferrous-Transferrin.

Since its discovery in the year 2000, the hepcidin hormone has evolved as the primary molecule in iron regulation and its systemic homeostasis. This role has been demonstrated in many studies (Nicolas et al., 2001; Nemeth et al., 2004 ; Nemeth and Ganz, 2009; Kanda et al, 2008; Guo et al, 2009; Bansal et al, 2009; Kwapisz et al 2009; Ganz 2008; Kennah et al, 2008; Camaschella and Silvestri, 2008; Nemeth, 2010; Sangkhae, & Nemeth, 2017). Studies have also evaluated the use of hepcidin as a biomarker for the regulation of iron metabolism and thus a great interest in quantifying circulating hepcidin levels in clinical samples, such as the serum and urine, have been employed.

Hepcidin is a small cysteine-rich peptide hormone produced mainly in the liver. In humans, HAMP (hepcidin antimicrobial peptide) is the gene that encodes for hepcidin and is located on chromosome 19q13.1 (Kemna et al., 2008). This hormone is mainly expressed in the liver, but also in heart, lung, intestine, kidneys, brain, tonsils, trachea, salivary gland and prostate gland to a much lesser degree (Krause et al., 2000; Pigeon et al. 2001). The structure of hepcidin was determined using the method of nuclear magnetic resonance spectroscopy (NMR) in solution (Hunter et al., 2002; Nemeth and Ganz 2009) and is shown in Figure 2.2. The human hepcidin gene (*HAMP*) encodes an 84 amino acid preprohepcidin that possesses a cleavage site. This precursor is modified to give 20, 22 and 25-amino acid peptides. All three forms of the peptides are traceable in urine, but only the 22aa and the 25aa peptides are present in the plasma/serum; with the 25aa peptide having the highest concentration. The structure of the major form, hepcidin-25, comprises eight cysteine residues connected by four intra-chain disulfide bonds that forms a β-hairpin-like motif containing a central core crosslinked by disulfides and a flexible six residue N-terminal region. Figure 2.2 show the solution structure and a ribbon depiction of the three-dimensional structure of hepcidin showing the beta sheet structure (broad arrows) and the four disulfide bonds (ball and sticks). The N-terminal region of hepcidin is

important for binding to ferroportin because sequential truncation of the N-terminal residues results in a progressive loss in activity of the peptide. Cleavage of the N-terminal region deactivates hepcidin, as indicated by the presence of a 20 residue inactive form of hepcidin isolated from urine (Park, et al., 2001).

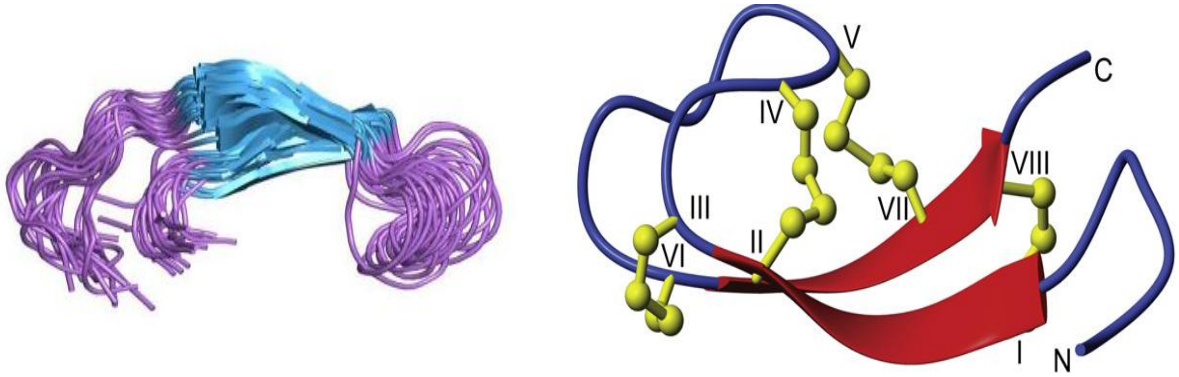


Figure 2.2 Solution structure of hepcidin-25, and a three-dimensional structure (Jordan et al, 2009; Clark et al, 2011 respectively)

Hepcidin has been shown to be a member of the cysteine-rich antimicrobial peptide family that includes defensin, with antibacterial and antifungal properties (Park et al. 2001; Klaus et al. 2000). Hepcidin has also been shown to be a type II acute-phase protein (Nemeth et al. 2003). Nonetheless, its antibacterial activity, according to Nemeth et al, currently seems to be incompatible, while scientific evidence implies its central role in regulating systemic iron homeostasis.

Mainly, hepcidin has been demonstrated to be the major iron regulatory hormone by causing a reduction in serum iron. Basically, when iron levels rise in the plasma and storage, hepcidin expression is stimulated preventing iron absorption and more storage (Ganz et al., 2008). When iron is deficient, on the other hand, hepcidin expression is restrained. This feedback loop ensures the suitable iron concentration in the plasma under normal physiological conditions. Generally, hepcidin causes a decrease in serum iron level by decreasing iron absorption from

duodenal enterocytes, iron release from macrophages and its transport across the placenta by binding to ferroportin.

2.2 Hepcidin and Ferroportin Interaction

Hepcidin adjusts the iron efflux to the blood stream by interacting with its receptor, IREG1; mainly known as ferroportin (Nemeth et al, 2004). Ferroportin is a membrane protein that forms an iron efflux channel expressed in iron-exporting cells including macrophages, eneterocytes, hepatocytes and placenta cells. Plasma iron concentration is determined by the rate of iron export from these iron-exporting cells. On the other hand, extracellular/plasma iron levels regulate hepcidin synthesis, completing the homeostatic loop, as is explained in the schematic figure (figure 2.3) (Ganz, 2007). By binding to ferroportin, hepcidin causes its phosphorylation, internalization then degradation, which in turn block the iron transport, absorption and/or release from macrophages (Nemeth et al, 2004; De Domenico et al, 2007; Ganz, 2007). Accordingly, when hepcidin concentration is high, the rate of ferroportin degradation exceeds its synthesis leading to loss of ferroportin expression from cell membranes (Rivera et al, 2005). Without ferroportin, dietary iron absorption decreases, recycled iron from senescent erythrocytes fail to be released from macrophages and hepatocytes retain their stored iron. When iron is depleted from extracellular fluids, hepcidin production is suppressed. Low concentration of hepcidin causes the rate of ferroportin synthesis to exceed its degradation and therefore increased amounts of iron enter the extracellular fluids. By modulating hepcidin expression, the body can maintain iron level and thus homeostasis.

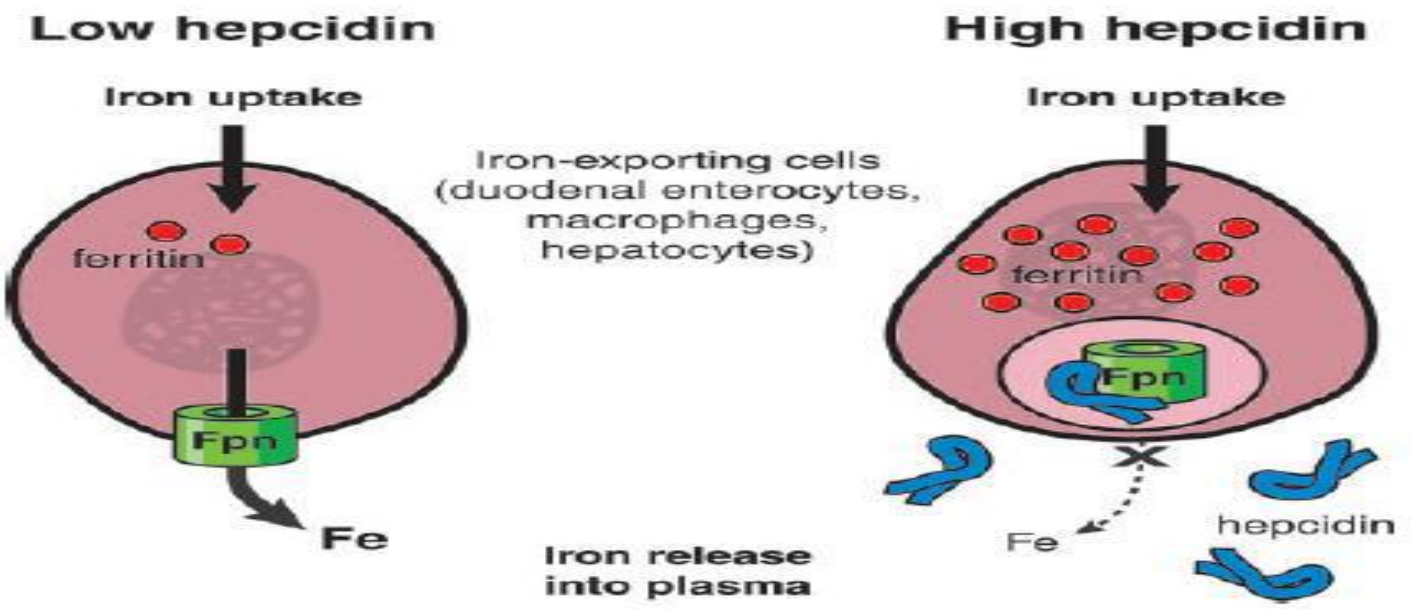


Figure 2.3 The mechanism of hepcidin-mediated cellular iron regulation (adapted from Ganz, 2007) [***Fpn***: Ferroportin channel]

2.3 Factors Regulating Hepcidin Levels

There are many regulatory influences that control hepcidin expression at the physiological and molecular level. These regulatory influences are represented in the schematic figure (figure 2.4) shown below. These can be divided into positive and negative regulators (Muckenthaler, 2008). The positive physiologic regulators of hepcidin production include increased iron stores and inflammation. The negative regulators include decreased iron stores, hypoxia, anemia, and increased erythropoisis.

All studies suggest that these factors regulate hepcidin concentration primarily through molecular mechanisms. There are many iron related molecules involved in the iron homeostasis system that can sense iron concentration and affect the expression of hepcidin (Kennah et al, 2008; Ganz, 2008; Kwapisz et al 2009, Darshan and Anderson, 2009; Femming 2008; Camaschella and Silvestri, 2008). Under normal conditions, hepcidin expression is regulated by the BMP/SMAD pathway (Babitt et al, 2006, 2007). Due to elevated iron levels, BMPs get

activated and bind to their receptor in hepatocytes that results in the phosphorylation of SMAD receptors and forms a complex with SMAD 4. This complex translocates into the nucleus and facilitates the expression of hepcidin. A membrane isoform hemojuvelin (a GPI-linked protein expressed by the liver) acts as a co-receptor for BMP 2 and 4 and its receptor to facilitate BMP signaling. Therefore, hemojuvelin is a positive regulator of hepcidin expression. Other positive sensors include STA2T3, HFE, and transferin receptor 2. Recent studies done by Babitt et al, (2006, 2007) suggest that both HFE and TfR2 exert their effect on hepcidin by interacting with hemojuvelin. A soluble form of hemojuvelin present in plasma acts as an antagonist to BMP signaling pathway and suppress hepcidin synthesis in response to low serum iron levels by binding to the BMPs and inhibiting the BMP/SMAD pathway. Another component of hepcidin suppressors is the serine protease matriptase-2 (TMPRSS6) (Muckenthaler, 2008; Traglia et al, 2009; Nai et al, 2011; Finberg 2009; Benyamin et al 2009; Chambers et al 2009; and Valenti et al, 2012). It exerts its effect by cleaving the membrane bound hemojuvelin and stopping hepcidin expression when systemic iron level is low under normal physiologic condition (Finberg et al., 2010; Lenoir et al., 2011).

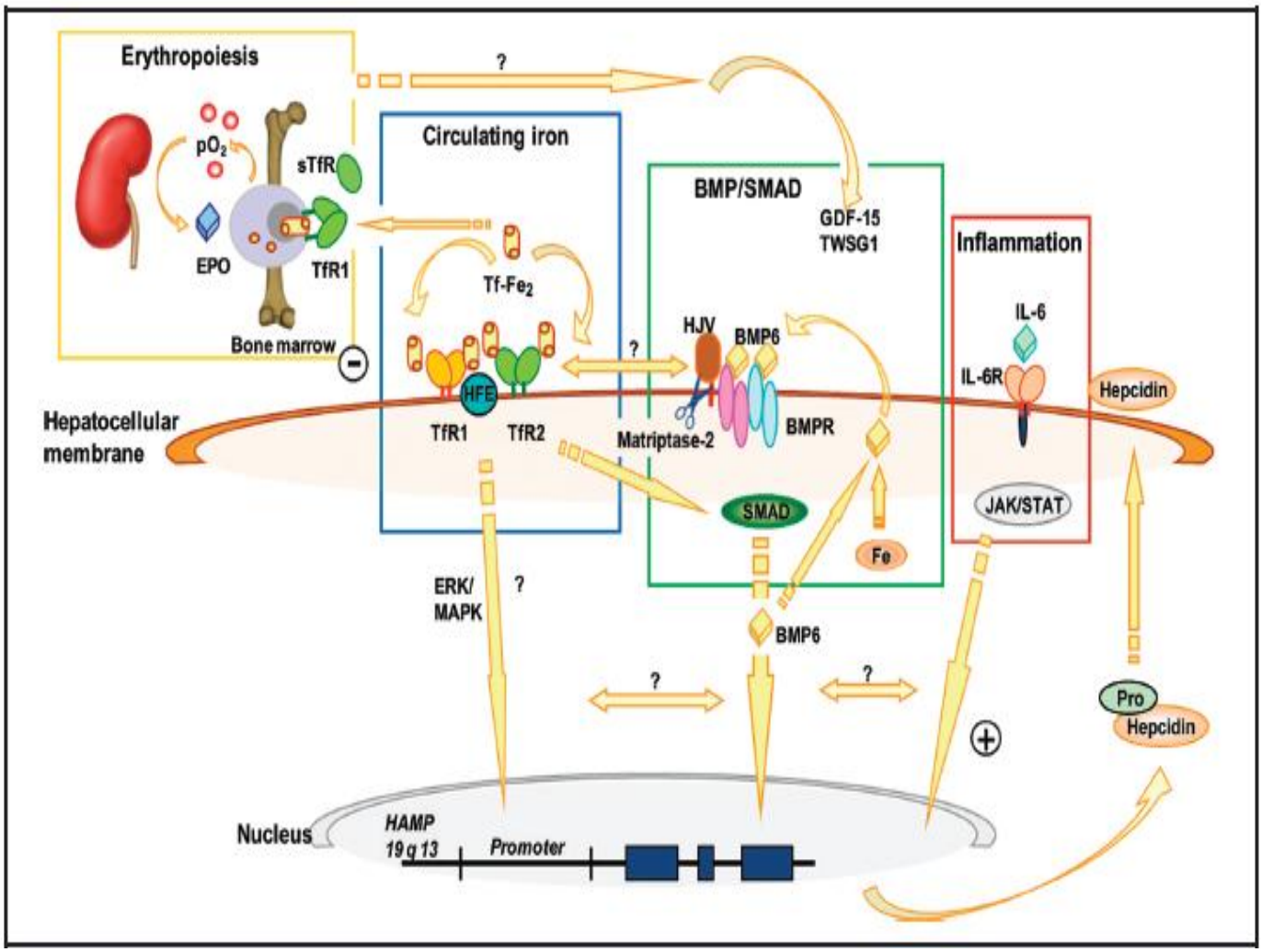


Figure 2.4: a schematic of molecular and physiological regulation of hepcidin synthesis (adapted from Kemna et al, 2008)

2.3.1 Decreased Hepcidin Synthesis

Decreased hepcidin expression is observed in increased erythropoisis activity in two clinically important conditions: hypoxia and anemia (Kroot, et al. 2011). Increased production of red blood cells has been speculated to decrease production of hepcidin, functionally increasing the absorption and movement of iron in the body. Under expression of hepcidin due to defective

or depleted hepcidin gene or its positive regulators cause hemochromatosis. In these circumstances as shown in figure 2.5 ((Weiss and Goodnough), hepcidin deficiency causes increased ferroportin mediated iron export, resulting in increased absorption and iron recycling onto plasma.

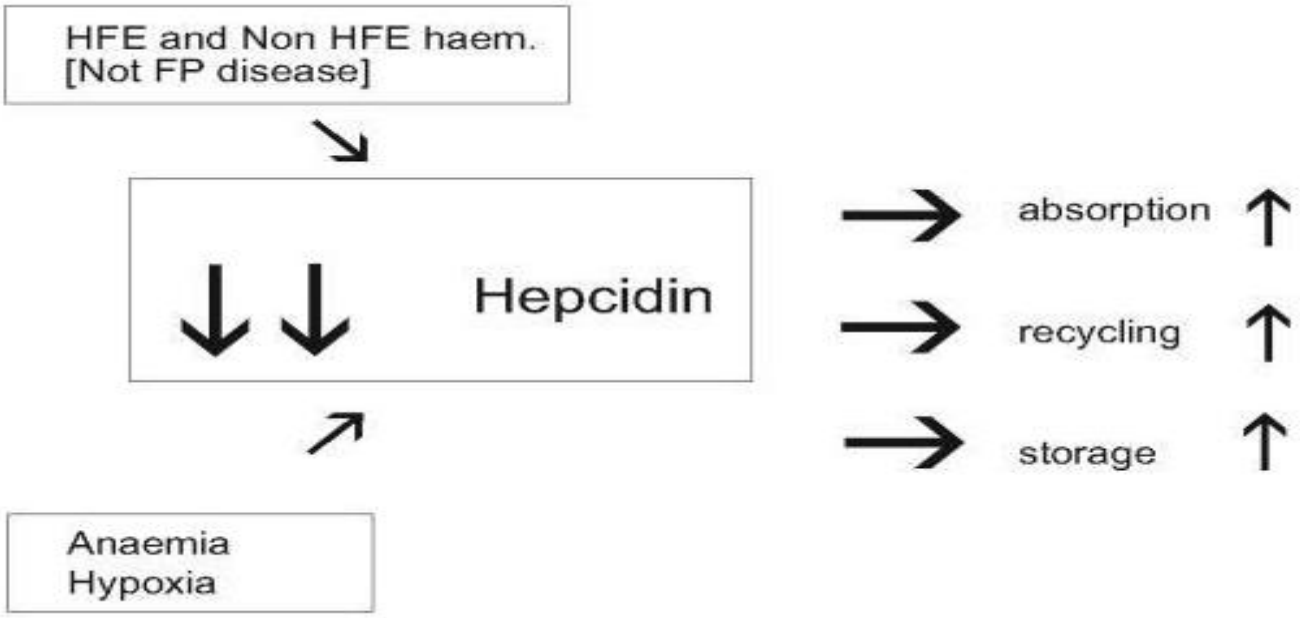


Figure 2.5 Down-regulation of liver hepcidin synthesis increases iron export from absorptive cells (enterocytes), recycling cells (macrophages) and storage cells (hepatocytes)

2.3.2 Increased Hepcidin Synthesis

Hepcidin expression is increased as a physiological response in two clinically important circumstances: iron overload and inflammation, shown schematically in figure 2.6 (Weiss and Goodnough, 2005; Papanikolaou et al., 2005). Under normal circumstances, the hepatocytes sense increased transferin saturation or increased iron stores in hepatocytes and mediates the hepcidin shut down of iron absorption, recycling and storage by increasing

hepcidin production. In addition, hepcidin expression increases as a response to cytokine release, especially interleukin-6. During inflammation or infection, hepcidin acts as an acute-phase responsive gene to retain iron from the cells that provide it. Anemia of chronic disease is thought to be mediated via this pathway.

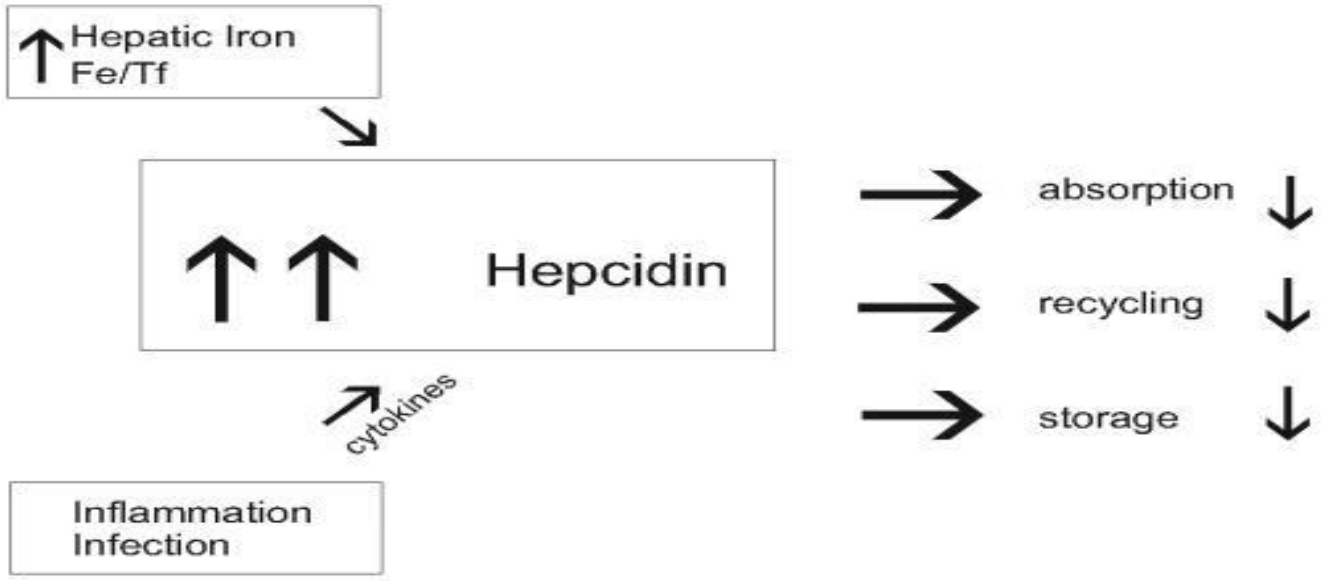


Figure 2.6 Induction of liver hepcidin synthesis decreases iron export from absorptive cells (enterocytes) recycling cells (macrophages) and storage cells (hepatocytes)

Hepcidin over expression plays an important role as a pathogenic mediator in many diseases involved with anemia, including anemia of inflammation, chronic kidney disease, hemoglobinopathies, coronary artery disease, myelodysplasia, dysmetabolic iron overload syndrome, hepatitis C, non-hereditary mild iron overloading hepatic disease, and IRIDA and other congenital microcytic anemia's (Kroot, et al. 2011). In these diseases, the overly expressed hepcidin causes a decrease in iron absorption and impair mobilization of iron stores.

2.4 Anemia

Anemia literally means lack of blood in Latin. Clinically, it refers to a decrease in red blood cells (RBCs) number, decreased hemoglobin in the red blood cell, and/or the decreased ability of oxygen binding to hemoglobin (MedicineNet.com, 2000). Anemia leads to hypoxia due to decreased hemoglobin, which carries oxygen to the capillaries. Clinical consequences of anemia vary depending on the degree of anemia and the underlying causes of anemia. Anemia can be classified based on morphology. These include microcytic, macrocytic, and/or normocytic anemias. It can also be classified using the kinetic approach, where production (as in ineffective hematopoiesis), destruction (as in hemolysis) and/or loss (as in bleeding) of RBCs are evaluated.

Iron-deficiency anemia (IDA) is the most common form of anemia, causing approximately half of all anemia cases worldwide (Baker & DeMaeyer, 1979). It probably affects more than one billion people. It can be caused by a number of reasons including insufficient dietary intake, insufficient iron absorption, and/or iron loss due to bleeding. In patients with IDA, hepcidin levels are low because of the suppressive effect of deficient iron stores and decreased erythropoiesis on hepcidin expression (Ganz and Nemeth, 2006). Recently, the *TMPRSS6* gene has been identified as the hepcidin negative regulator required to sense low iron stores (Mariani et al., 2008). Mutations in this gene cause iron-refractory iron deficiency anemia (IRIDA) (Finberg et al, 2008).

2.5 Iron Refractory Iron Deficiency Anemia

2.5.1Clinical presentation

Various clinical and laboratory findings have been presented in the literature. Also, discrepancy in the response to treatment among patients was observed. However, individuals with IRIDA are usually diagnosed during childhood. Most patients with IRIDA are opposed to dietary iron supplementation due to a defect in the TMPRSS6 gene that encodes the serine protease matriptase 2 enzyme. Loss of the matriptase 2 enzyme activity, results in continuous hepcidin synthesis, even when iron is deficient (Silvestri et al, 2008). Accordingly, innate hepcidin concentration in these patients is increased relative to the degree of anemia resulting in extremely low transferin saturation and lower range of reference value of ferritin concentration (Cherian et al, 2008; Altamura et al, 2010; Tchou et al, 2009; Finberg et al, 2008; Melis et al, 2008; Hartman and Barker, 1996; Beutler et al, 2010). Intravenous iron repeated administrations have been reported to partially increase hemoglobin and MCV values. Serum ferritin levels increase to normal or even slightly above normal after intravenous iron administration. The degree of anemia varies from mild to severe and is more prominent during childhood and under conditions of iron restriction or an increased iron requirement, such as pregnancy (Nai et al, 2010; Finberg et al; 2010)

2.5.2 History

The IRIDA disorder was reported in literature long before the genetic basis of the disease has been described. Buchanan and Sheehan (1981) described an iron deficiency anemia case of three siblings who didn't respond to oral iron therapy and only partially responded to intravenous iron therapy with no history of poor dietary iron absorption or gastrointestinal blood loss. The

authors recommended that a specific iron absorption disorder with a hereditary nature due to occurrence in 3 siblings may explain the phenotype. Similar findings have been subsequently reported in literature (Brown et al, 1988; Hartman and Barker, 1996; Andrews, 1997; Gallanello et al, 1998; Mayo and Samuel, 2001) until the discovery of the genetic basis of this disease. The genetic basis of this disease was suggested through genetic characterization of a large and consanguineous Sardinian family; in which the disease in affected individuals were attributed to a homozygous mutation arising from a common ancestor (Melis et al., 2007). The IRIDA phenotype was mapped to the long arm of chromosome 22 (22q12.3–13.1) in a recessive inheritance mode. Subsequently, IRIDA was shown to be caused by mutations in the gene TMPRSS6, which resides within this critical region of chromosome 22q and for which a key role in iron balance had recently been revealed through study of the orthologous gene in mice.

The negative regulator, matriptase 2, and its role in iron homeostasis were discovered by Du et al (2008) while studying mask mice with truncated TMPRSS6 lacking the protease domain and by Folgueras et al. (2008) while separately studying TMPRSS6 knockout mice. The mask and knockout mice showed similar phenotype that was characterized by progressive body hair loss, microcytic anemia, low plasma and iron stores levels. The TMPRSS6 mutated gene in those mice showed impaired dietary iron uptake and failure to suppress hepcidin expression in response to dietary anemia. Similar to the findings in TMPRSS6-mutant mice, mutations of this enzyme in humans mirrors the phenotype present in mice and cause abnormally increased hepcidin levels, congenital microcytic and hypochromic anemia, low mean corpuscular erythrocyte volume, decreased iron stores, low transferrin saturation, and defects in iron absorption and utilization represented as iron refractory iron deficiency anemia (IRIDA); where patients do not respond to oral iron administration and only partially respond to intravenous administration (Finberg et al., 2008; Guillem et al., 2008; Melis et al., 2008).

2.5.3 Biochemistry of matriptase 2 and its role in iron homeostasis

Matriptase 2 is the protein expressed highly in the liver from TMPRSS6 gene located at chromosome 22q12.3 and belongs to the type 2 transmembrane serine protease (TTSP) family (Velasco et al, 2002; Finberg et al, 2008; Melis et al, 2008; Ramsay et al, 2009). The gene structure consists of 18 exons with 17 intervening introns. The matriptase 2 protein is formed into 6 domains depending on exon encoding boundaries. These domains, as shown in figure 2.7, are: a transmembrane domain and SEA domain (encoded by the first 6 exons), 2 CUB domains (1^{st} one encoded by exons 7 and 8 and 2^{nd} one encoded by exons 9-11), 3 LDLR domains (each encoded by a separate exon 12-14 respectively), and the activation/prodomain and a C-terminal trypsin-like serine protease domain (encoded by exons 15-18). Matriptase-2 is synthesized as a single chain inactive proenzyme that auto-activates itself by cleaving itself between the prodomain and the catalytic domain (Ramsay et al., 2009; Altamura et al., 2010). The single disulfide bond links the prodomain and catalytic domain and keeps the enzyme membrane-bound (Ramsay et al., 2009).

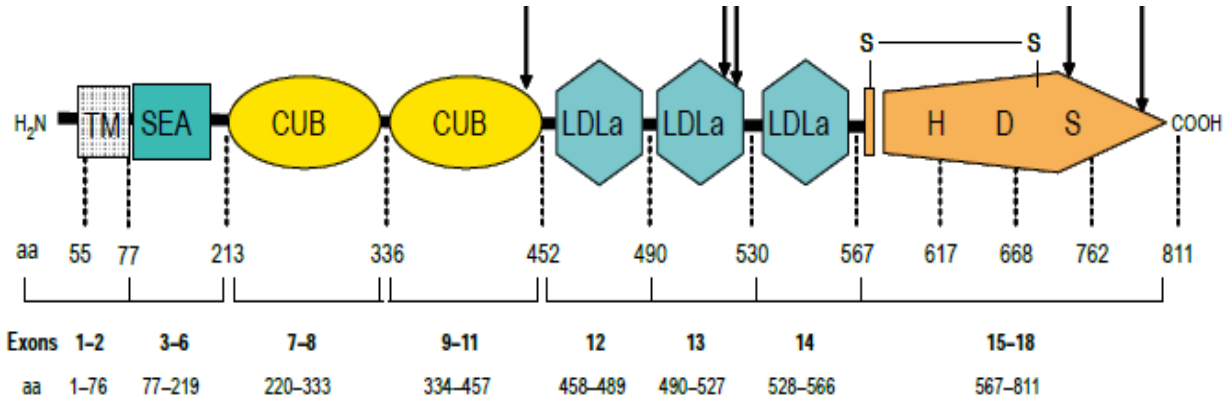


Figure 2.7: the matriptase 2 protein domains (adapted from Lee PL, 2009).

The TMPRSS6 gene structure is highly conserved across species, including chimpanzee, Rhesus monkey, dog, cow, mouse, rat, chicken, and frog (Ramsay et al., 2008, HomoloGene database).

Proteins used in sequence comparisons show conserved domain architectures with human protein sharing >80% with the other species. The protein is highly expressed in the liver (Velasco et al, 2002; Finberg et al, 2008) but also to a much lower extent in the kidneys, lung, brain, spleen, mammary gland, testis, and uterus (Ramsay et al., 2008).

Matriptase 2 role in iron homeostasis is to decrease hepcidin transcription when iron is low by cleaving the membrane bound hemojuvelin hindering its activity in stimulating hepcidin expression via the BMP/SMAD pathway (Selvestri et al, 2008; Du et al., 2008; Finberg et al., 2008; Finberg et al., 2010; Lenoir et al., 2011) as explained in figure 4. In a more recent study done by Wahedi et al (2017), matriptase 2 suppresses hepcidin independently of hemojuvelin using mouse homologues was demonstrated. It was reported that apart from HJV, matriptase 2 cleaves other components of the hepcidin induction pathway including BMP receptors, Hfe and Tfr. The relatively inappropriate high levels of hepcidin expression when the enzyme is mutated results in the observed phenotype (Du etal., 2008; Folgueras et al.,2008; Finberg et al.,2010).

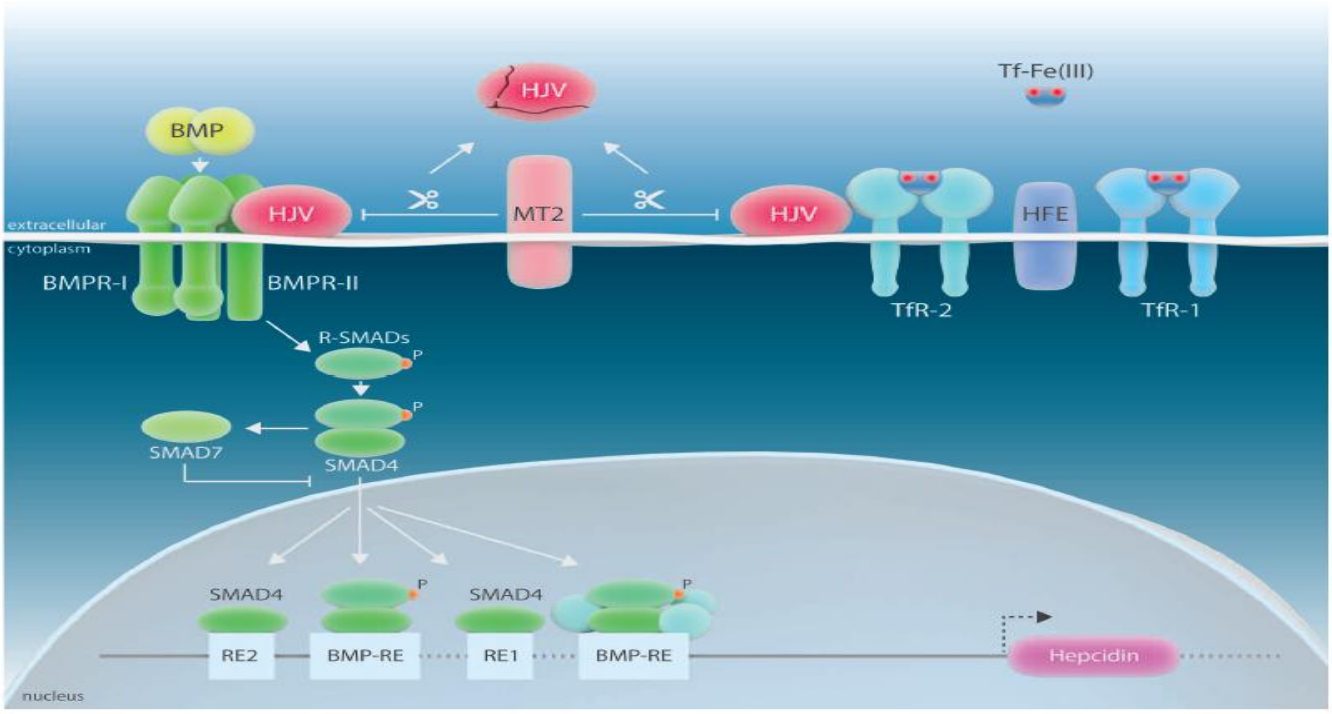


Figure 2.8a: role of matriptase in systemic iron homeostasis (De Falco et al, 2013)

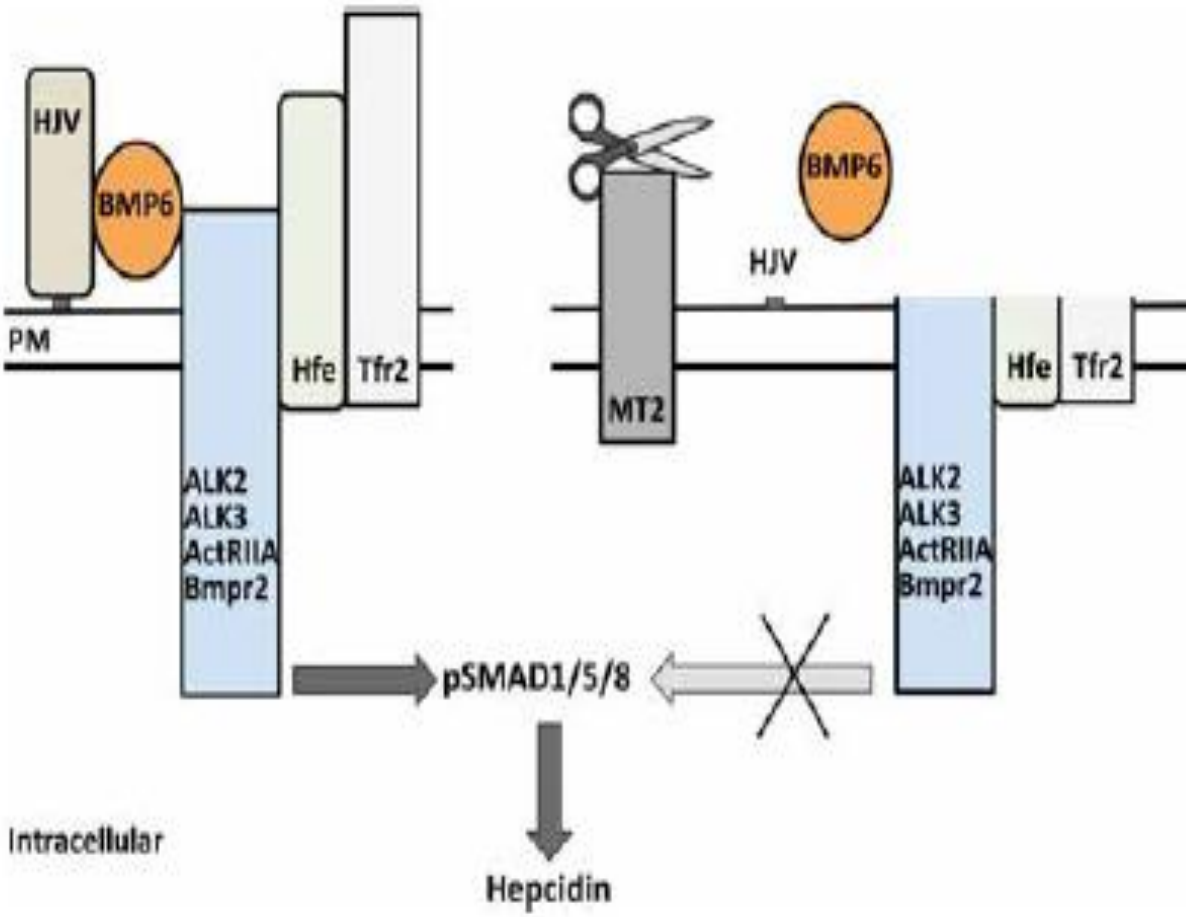


Figure 2.8b: MT2 suppresses hepcidin expression by cleaving the extracellular portion of multiple substrates.

2.5.4 Mutations and Polymorphisms

Finberg et al (2008) was the first to relate IRIDA with mutations in matriptase 2. His findings were complemented by many other subsequent studies. Now, over 50 TMPRSS6 mutations in individuals with the IRIDA phenotype have been reported since its discovery (Finberg et al, 2008; Melis et al, 2008; Guillem et al, 2008; Chambers et al, 2009; Ramsay et al, 2009; Silvestri et al, 2009; Edison et al, 2009; Tchou et al, 2009; Kannengiesser et al, 2009; Benyamin et al, 2009; De Falco et al, 2010; Altamura et al, 2010; Beutler et al, 2010; Cuijpers et al, 2010; Palare et al, 2010; Choi et al, 2012; Guillem et al, 2012; Pellegrino et al, 2012; Jaspers et al, 2013; Lehmberg et al, 2013; Khuong-Quang et al, 2013; Yılmaz-Keskin et al, 2013; De Falco et al, 2014; De Falco et al, 2015; Xiong, *et al*. 2015; Bhatia et al, 2017).

In these studies, biallelic homozygous and heterozygous mutations have been identified that include frameshift, nonsense, missense, large in-frame deletion, and splice junction mutations (Falco et al, 2013; Wang et al., 2014; Yılmaz and Yenicesu, 2015). These mutations are present in patients of different ethnic and geographic backgrounds. They account for more than 50 different mutations that are spread throughout the extracellular domains of the gene; where they fully or partially prevent hepcidin inhibition depending on the severity of mutation. These mutations have no founder effect and there is no frequent mutation with greater contribution to the phenotype. As of 2014 (Wang et al), these mutations and their locations are represented in the following schematic figure. Furthermore, a review by Keskin et al. (2015) highlights 20 odd case reports on IRIDA describing TMPRSS6 gene mutations in a total of 65, where the mutations are either homozygous or compound heterozygous and the ethnicity is varied with case reports from all over the world.

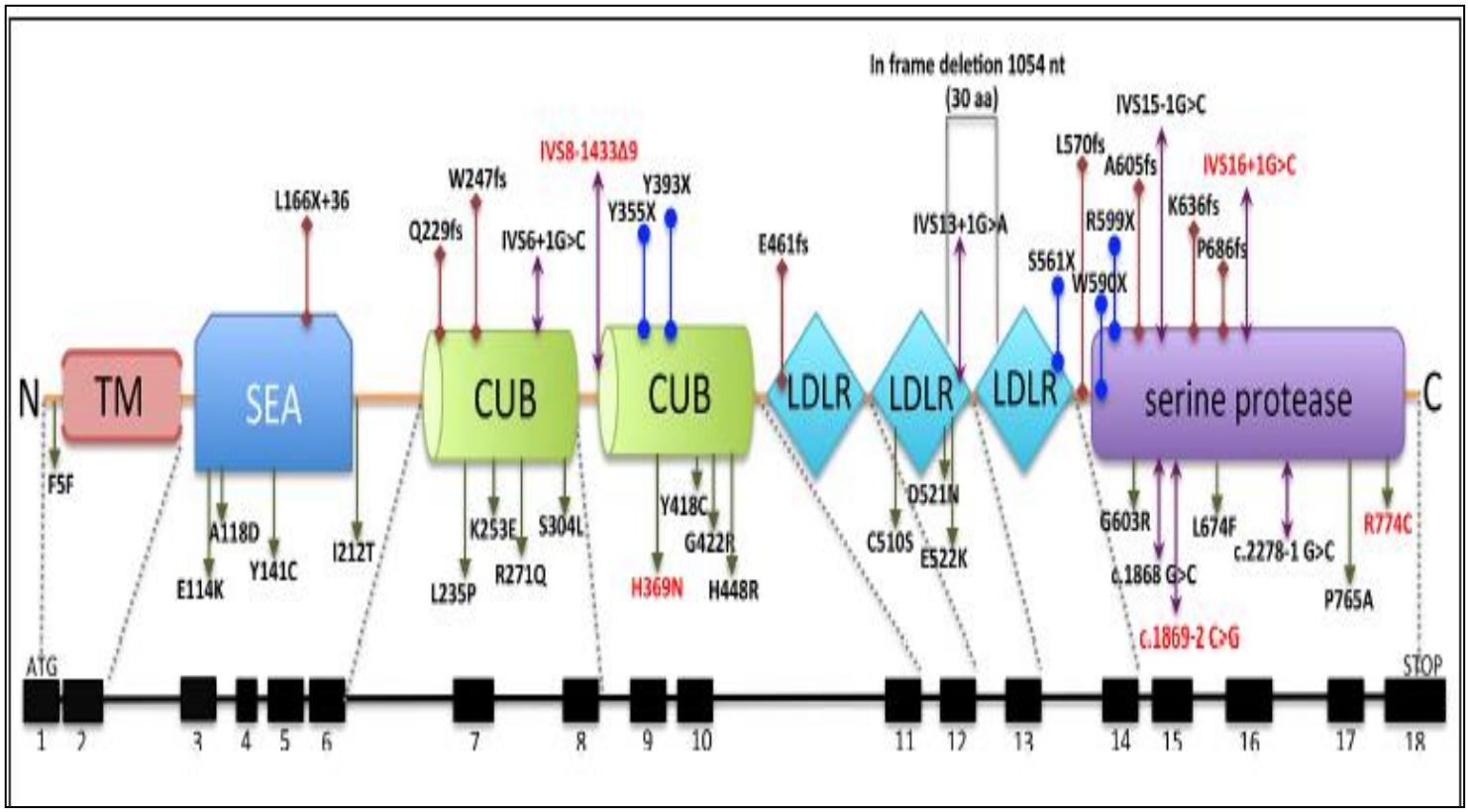


Figure 2.9: TMPRSS6 gene structure and schematic representation of corresponding mutations reported in IRIDA anemia (Wang et al, 2014).

Genome wide association studies (GWAS) have also shown that TMPRSS6 gene is highly polymorphic (Beutler et al, 2010; Wainaina et al, 2015). Polymorphisms can either be common, uncommon or rare. The common polymorphisms are many and unlikely considered risk factors for iron deficiency anemia. Uncommon and rare polymorphisms (R446W and G228D and V795R respectively) are slightly observed in the anemic population. Therefore, polymorphisms of *TMPRSS6* might be risk factors for common forms of iron deficiency anemia and mutations results in familial forms of iron deficiency related to IRIDA.

2.5.5 Phenotype-genotype correlation studies

Genotype-phenotype studies were done by De Falco (2015) to predict the clinical severity in IRIDA patients in relation to the type of mutation they have and their response to iron treatment. In their study, 17 additional novel mutations that are spread throughout the gene have been identified, including a frameshift mutation located in the cytoplasmic and transmembrane domain. These new mutations are represented in figure 2.10 along with the type of mutations they are. Their findings signify the fact that any domain of matriptase 2 can be affected and that the *TMPRSS*6 gene should be fully screened in order to detect IRIDA causal mutations. They also performed genotype-phenotype correlation analysis (70 patients). They found that patients with severe mutations including homozygous nonsense, frameshift, splicing mutations or heterozygous one nonsense and one frameshift mutation and one frameshift and one splicing mutation have statistically significant lower hemoglobin and MCV values and higher serum hepcidin levels than patients with two (homozygous) missense mutations or one missense mutation in combination with other type of mutations (heterozygous). They also found that patients carrying homozygous nonsense mutations have the most severe microcytic anemia and higher hepcidin levels than the other patients.

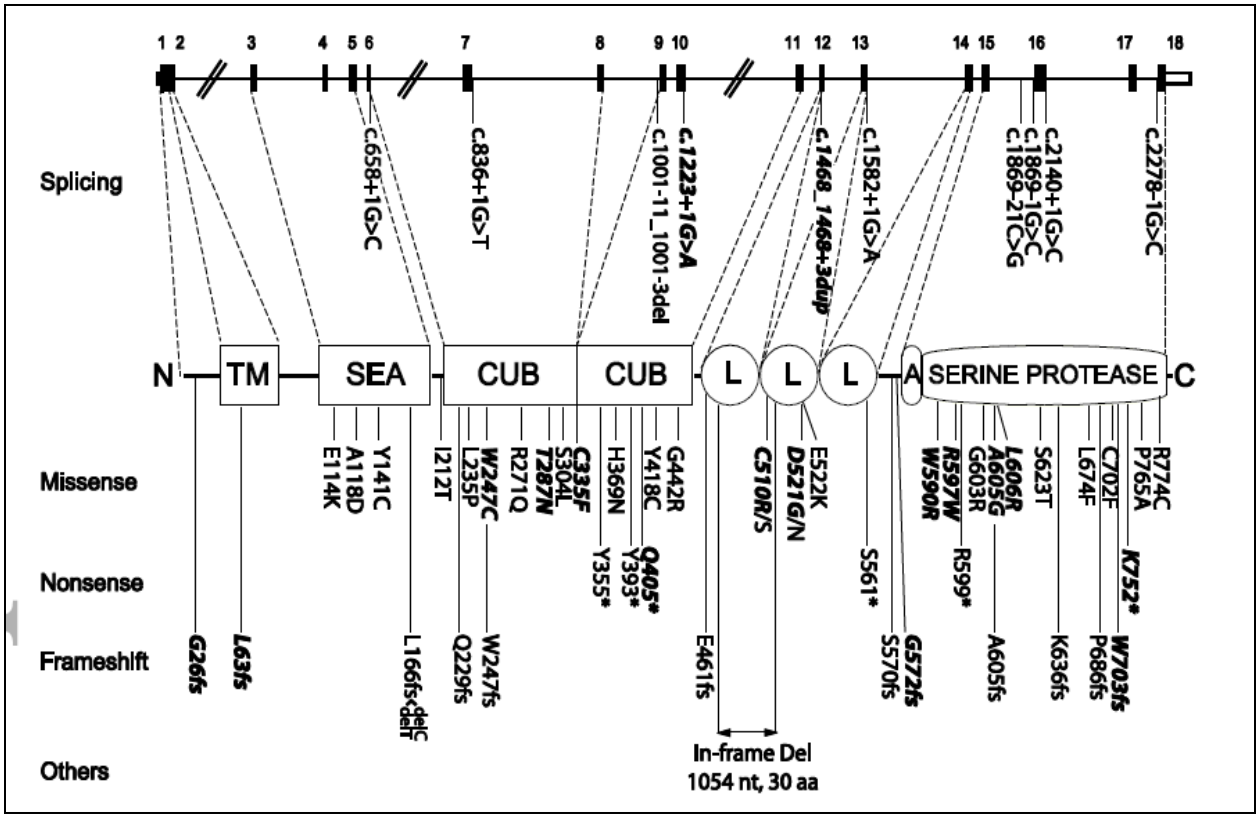


Figure 2.10: IRIDA mutations. Novel mutations in this study are in bold and italics (De Falco, 2015)

Mcdonald and his colleagues (2015) have performed a systematic functional analysis of IRIDA causing mutations in matriptase 2 and the role of the protein domains in terms of the mutational effect. They found that the disease associated mutations in matriptase 2 affect the proteolytic activity in three ways: by the loss of protease domain due to mutations in either the protease domain, CUB domain or the SEA domain; by reduction in autoactivation (Altamuara et al, 2010) due to mutations in the SEA domain; and by reduction in cell surface localization due to mutations in the SEA and LDLRA domains. These mutations result in impaired ability to decrease the expression of hemojuvelin at the cell surface or in intracellular retention of the protein. In the later, mutated proteins are retained in the ER and are not transported to the cell surface. In their study, Mcdonald et al. showed that TMPRSS6 gene harboring missense

mutations can escape the ER and traffick to the cell surface when ER retention threshold is reached. According to them, when the threshold is reached even at low levels of surface localization, hemojuvelin can still be cleaved by the mutated enzyme. Maybe this accounts for the mild phenotype observed in IRIDA patients carrying this type of mutation. On the other hand, Silvestri et al (2013) argues that an ideal simple method to assess the functions of matriptase 2 polymorphisms/mutations is not yet available. According to Selvestri et al (2013) and Camaschella (2013), measuring serum hepcidin levels should substitute the costly and time consuming in-vitro assessment of matriptase 2 mutations.

Chapter 3

Materials and Methods

Chapter 3: Materials and Methods

3.1 Study Design

This study is a genetic, case-control study.

3.2 Study Population

Subjects with iron deficiency anemia that are not responding to oral iron therapy and only partially responsive to parenteral iron administration from both genders were recruited. Subjects were referred to us by blood clinics at Al-Mutalaa Hospital, National Governmental Hospital of Nablus, and Beit Jala Governmental Hospital (Al-Hussein).

Controls were healthy individuals with no anemia present at time of test and matched cases with age and gender.

The sample size in ratio of case-control was 1:1.

3.3 Patient Recruitment Criteria

Patients that have all or most of the following features, presented at diagnosis, were recruited:

- Lifelong anemia
- Micrcytosis (MCV of 45-65fl)
- Low iron levels in blood
- Low transferrin saturation (less than 15%)
- No or partial response to oral iron therapy (demonstrating abnormal iron absorption)
- Partial paranteral response (demonstrating abnormal iron utilization)
- Other affected family members with an autosomal inheritance pattern if present
- Negative electrophoresis
- No chronic disease present (by assessing CRP)

3.4 Ethical Considerations

The necessary approval to conduct the study was obtained from the scientific research ethical committee of the Faculty of Pharmacy, Nursing and Health Professions at Birzeit University and conformed to the declaration of Helsinki principles.

The subjects or their guardians were informed of the research and their written consent was obtained (Annex 1). Measures to protect the privacy of these subjects and their personal information were taken. The purpose of the research was explained to the subjects or their guardians and their understanding of the information and expectation of the research outcome was ensured. They were informed of their right to refuse to participate in this research.

In addition, an official letter of request was sent to the Ministry of Health to obtain permission to facilitate the conduction of the study at the above mentioned hospitals.

3.5 Data Collection

- **Questionnaire Interview**

Q A meeting interview was used for filling in a questionnaire which was designed to match the study need (Annex 4). All interviews were conducted face to face by the researcher herself. During the survey the interviewer explained any of the questions that were not clear.

- **Patients' files**

Data for hematological, chemical and any other tests done at diagnosis was also obtained when possible from the patients records at the hospital or from the patients/patients' guardians themselves.

3.6 Specimen Collection and Processing

Blood samples were collected from 20 IRIDA patients and 20 healthy controls. Blood samples were drawn by the lab technicians present at the above mentioned hospitals or at the Birzeit University lab into two tubes from each control and case. The first is serum tube for chemistry and the second is EDTA tube for CBC and genetic analysis. Blood samples, when obtained outside the university were transferred in ice. CBC was conducted on fresh blood for all cases and control. To obtain serum from blood samples, the blood was left for a while without anticoagulant to allow blood to clot. Then, serum samples were obtained by centrifugation at 2000 rpm/10 minutes for biochemical analysis. EDTA tubes for genetic analysis and serum tubes were kept at -20°C until analysis.

3.7 Hepcidin Hormone, Biochemical Parameters and Complete Blood Count (CBC) Analysis

For all the study population, hepcidin hormone and biochemical parameters such as CRP, ferritin, serum iron and TIBC were measured. Transferrin saturation was calculated, and CBC was analyzed. Serum specimens were thawed before analysis and they were inverted several times prior to testing. All biochemical parameters and hepcidin hormone tests were conducted within 2 days of thawing. Samples were kept at 4°C for the next working day.

3.7.1 Determination of Complete Blood Count (CBC)

The test was performed by using Cell-Dyn-1800 hematology autoanalyzer. A complete blood count (CBC) is a series of tests used to evaluate the composition and concentration of the cellular components of blood. It consists of the following tests: red blood cell count (RBC), hemoglobin (Hg), hematocrit (HCT), mean red cell volume (MCV), Mean cell hemoglobin concentration (MCHC), Red cell distribution width (RDW), platelet count, white blood cell count, neutrophils, lymphocytes, monocytes, eosinophils and basophils. Red blood cell indices are calculations derived from the red blood cell count, hemoglobin, and hematocrit that aid in the diagnosis and classification of anemia. RBC, Hg, MCV, and MCH values were assessed for the purpose of this study.

Reference values:
RBC: 4.2-6.3* 10^6/u
Hg: 11-16 g/dl
MCV: 80-97 fl
MCH: 26-32 pg

3.7.2 Determination of Serum Ferritin

Quantitative serum ferritin was determined by the Immulite Automated Immunoassay System using Siemens DPC IMMULITE 1000 Reagents Ferritin – LKFE1.

Ferritin serves as the body's principal storehouse for excess iron. It is mainly found in liver cells and in erythrocyte recycling centers (RE cells) of the liver, spleen and bone marrow. Ferritin is also present in human plasma, where its concentration is normally a satisfactory index of body iron stores.

IMMULITE/IMMULITE 1000 Ferritin is a solid-phase, two-site chemiluminescent immunometric assay. The solid phase (bead) is coated with monoclonal murine anti-ferritin and the liquid phase consists of alkaline phosphatase (bovine calf intestine) conjugated to polyclonal

goat anti-ferritin antibody. The serum sample, the enzyme reagent and the coated beads are incubated together. During this incubation, ferritin in the serum forms the antibody sandwich complex with the monoclonal anti-ferritin antibody on the bead and the enzyme conjugated anti-ferritin antibody in the reagent. Unbound ferritin in the serum sample and enzyme conjugate are then removed by centrifugal washes. Finally, the chemiluminescent substrate is added to the test unit containing the bead and the signal is generated in proportion to the bound enzyme.

Table (3.1): Reagents used for ferritin test.

MATERIALS PROVIDED	
Ferritin Test Units (LFE1) LKFE1:100 units	Each barcode-labeled unit contains one bead coated with monoclonal murine anti-ferritin. Stable at 2–8° Allow the Test Unit bags to come to room temperature before opening
Ferritin Reagent Wedge (LFE2) With barcode LKFE1:1 wedge.	7.5 mL alkaline phosphatase (bovine calf intestine) conjugated to polyclonal goat anti-ferritin in buffer. Store capped and refrigerated at 2–8℃
Ferritin Adjustors (LFEL, LFEH) LKFE1: 1 set	Two vials (Low and High), 2.5 mL each, of ferritin in a human protein-based matrix. Stable at 2–8°C Do not freeze

Expected Values : Males 28 – 365 ng/mL
Females 5 – 148 ng/mL

3.7.3 Determination of Serum C Reactive protein

Quantitative serum **CRP** level was determined by the vitalab Selectra E analyzer using the Elitech Diagnostic Systems kit.

C-reactive protein (CRP) is a special type of protein produced by the liver in response to inflammatory cytokines such as Interleukin-6 (IL-6). The most important role of CRP is its interaction with the complement system, which is one of the body's immunologic defense mechanisms. CRP is classified as an **acute phase reactant**, which means that its levels will rise within 24-48hrs after tissue injury, the start of an infection, or other causes of inflammation.

The CRP test is based on an immunological reaction between anti-human CRP bound to biologically inert latex particles and CRP in the test specimen. The agglutination of the latex particles is proportional to the CRP concentration and can be measured by turbidimetry.

Table (3.2): Reagents used for CRP test.

MATERIALS USED	
Reagent A	Glycine buffer 0.1 mol/L, sodium azide 0.95 g/L, pH 8.6. Store at 2-8ºC.
Reagent B	Suspension of latex particles coated with anti-human CRP antibodies, sodium azide 0.95 g/L Store at 2-8ºC.
CRP Standard	Human serum. C-reactive protein concentration is stated on the vial label Store at 2-8ºC.
Rheumatoid Control Serum level I and II	to verify the accuracy of the measurement procedure Store at 2-8ºC.

REAGENT PREPARATION:

Working reagent B vial was poured into reagent A bottle and mixed thoroughly.

A serial dilution was conducted for the standard in order to generate the standard curve:

Standard 1: 58.6ul water

Standard 2: 29.3ul water with 29.3ul standard

Standard 3: 58.6ul standard

REFERENCE VALUE: Up to 5 mg/L.

3.7.4 Determination of Serum Iron

Quantitative serum Iron level was determined by the vitalab Selectra E analyzer using the Elitech Diagnostic Systems kit.

65 to 70% of total iron enters in hemoglobin composition: 20 to 25% is stored in cells thanks to ferritin. Plasma contains about 3 mg of iron bound to transferrin. Serum iron concentration increases in case of hemochromatosis, liver damage and iron intoxication. Decreased iron levels can be consequence of increased needs, dietary deficiency, bleeding, or an impaired absorption (gastro-intestinal disorders, mal absorption), serum iron level will always be interpreted with transferrin saturation data.

It uses a Colorimetric – Chromazurol B. [End point] method, where Serum iron reacts with chromazurol B and catyltrimethyl ammonium bromide to form a colored complex. The intensity of the color is proportional to the iron concentration.

Table (3.3): Reagents used for Iron test

MATERIALS USED	
Reagent R	Composed of: Acetate buffer, pH 5.0 45 mmol/L Chromazurol B 0.2 mmol/L Catyltrimethyl ammonium bromide 2 mmol/L Guanidine hydroxide 3 mol/L
Standard STD	Iron : 100 μg/dL

Reference Value : Infant 40-100 μg/dl
Child 50-120 μg/dl
Woman 50-170 μg/dl
Man 65-175 /dl

3.7.5 Determination of TIBC

Quantitative serum TIBC level was determined by the vitalab Selectra E analyzer using the Elitech Diagnostic Systems kit.

Total iron-binding capacity (TIBC) corresponds to the maximum amount of iron that Plasma proteins can bind. It is therefore an indirect way of measuring transferrin levels, protein which transports iron in plasma. Measuring TIBC, together with iron and ferritin, is indicated in nutritional assessments and differential diagnosis of anemia's, as well as for the evaluation and the control of patients presenting a risk of iron overload.

It uses a Saturation / precipitation method. First, transferrin iron binding sites are saturated by reagent R1. Then the iron in excess reacts with magnesium carbonate (reagent R2) to give an insoluble complex which is eliminated by centrifugation. Last, total iron binding capacity (TIBC) is obtained by measuring the iron concentration in the supernatant using a reagent for determination of iron in serum.

Table (3.4): Reagents used for TIBC test

MATERIALS USED	
Reagent R1	Composed of Iron saturating solution 520 µg/dLmol/L
Reagent R2	Magnesium carbonate (one measuring spoon supplied)

Procedure:

First, Saturation of Iron Binding Sites

Reagent R1 and sample was introduced by mixing 1ml of reagent 1 with 0.5ml serum sample. Mixture was incubated for 5 minutes. then:

1 level measuring spoonful of Reagent R2 was added to the mixture and incubated for 20 minutes, shacked several times during this period.

Second , The sample was precipitated by centrifuging at 3000 r.p.m. during 10 minutes. The supernatant was collected for TIBC determination.

Then, TIBC Determination:

Iron content of the supernatant is measured colorimetrically with the Iron Chromazurol method. With the dilution 1:3 by the reagent 1 and multiply by 3 each measurement value.

Reference Value : [250 – 450 µg/dL]

3.7.6 Calculation of Transferrin Saturation

Formula used: [TS = (Serum Iron / Total Iron Binding Capacity) x 100%].

Reference Range of transferrin saturation: Adults: 20% - 50%
Children: More than 16%

3.7.7 Determination of Serum Hepcidin Hormone

Quantitative serum hepcidin hormone was determined by ELISA (enzyme linked immunosourbent assay) using Intrinsic Lifesciences kit.

The Intrinsic Lifesciences Hepcidin ELISA Kit is a solid phase ELISA, based on the principle of competitive binding. The microtiter wells are coated with a monoclonal antibody directed towards the antigenic site of the bioactive Hepcidin 25 molecule. Endogenous Hepcidin of a sample competes with the added Hepcidin-biotin conjugate for binding to the coated antibody. After incubation the unbound conjugate is washed off. Incubation with a streptavidin-peroxidase enzyme complex follows and a second wash step is done. The addition of substrate solution results in a colour development which is stopped after a short incubation. The intensity of colour developed is inversely proportional to the concentration of Hepcidin in the specimen sample.

Table (3.5): Reagents used for Hepcidin test

MATERIALS USED	
Microtiterwells, 12x8 (break apart) strips, 96 wells	Wells coated with anti-Hepcidin antibody (monoclonal).
Standard (Standard 1-8), 8 vials Concentrations:0, 2.5, 10, 25, 50, 100, 250, 1000ng/mL	0.5 mL synthetic Hepcidin
Control low and high, 2 vials	0.5 mL
Hepcidin-25 Biotin tracer, 1 bottle (ready to use)	13ml
Streptavidin-HRP Conjugate, 1 bottle (ready to use)	12
TMB Substrate, 1 bottle (ready to use)	12 ml
Stop Solution, 1 bottle (ready to use)	12 ml
Wash Solution, 1 bottle (conc., 20X) dilute before use	25ml
Sample Diluent, 1 bottle (ready to use)	3ml
Microplate sealing film	2 covers
Polypropylene (PP) 96-well SampleSetupPlate	1 plate

General Remarks of Manufacturer:

- All reagents and specimens must be allowed to come to room temperature before use. All reagents must be mixed without foaming.
- Once the test has been started, all steps should be completed without interruption.
- A new disposable plastic pipette tip was used for each standard, control or sample in order to avoid cross contamination.
- Absorbance is a function of the incubation time and temperature. Before starting the assay, it is recommended that all reagents are ready, caps removed, all needed wells secured in holder, etc. This will ensure equal elapsed time for each pipetting step without interruption.
- As general rule, the enzymatic reaction is linearly proportional to time and temperature.

Procedure:

Before running the test, deionized water was added to the 20-fold concentrated wash solution. 25ml of concentrated wash solution was diluted with 475 ml deionized water to final volume of 500ml. Wash buffer was stored at room temp. 110 µl of Hepcidin-25 Biotin tracer was added to each well of the PP plate using a multichannel pipette. 22 µl of each standard, control and samples with new disposable tips were disposed into appropriate wells and mixed. All 40 samples and kit controls were run in duplicates. 120ul of the mixture was transferred into the corresponding Microtiterwells using a multichannel pipette. Reaction mixture was incubated for 60 minutes at room temperature on a plate shaker at ≈ 350 rpm. The wells were rinsed 3 times with the diluted Wash Solution (300 µl per well). The wells were stroked sharply on absorbent paper to remove residual droplets. A volume of 100 µl Streptavidin-HRP Conjugate was added to each of these wells. Reaction mixture was incubated for 30 minutes at room temperature on a plate shaker at ≈ 350 rpm. The wells were rinsed 3 times with the diluted Wash Solution (300 µl

per well) and stroked sharply on absorbent paper to remove residual droplets. A volume 100 µL of TMB Substrate Solution were added to each well. Reaction mixture was incubated for 15 minutes at room temperature. The enzymatic reaction was stopped by adding 100 µL of Stop Solution to each well. The absorbance (OD) of each well was determined at 450 ± 10 nm with a microtiter plate reader. The wells were read within 15 minutes after adding the Stop Solution.

Calculation of Results for hepcidin

1. The average absorbance values were calculated for each set of standards, controls and specimen samples. The mean of the duplicate absorbance readings for each sample was calculated.

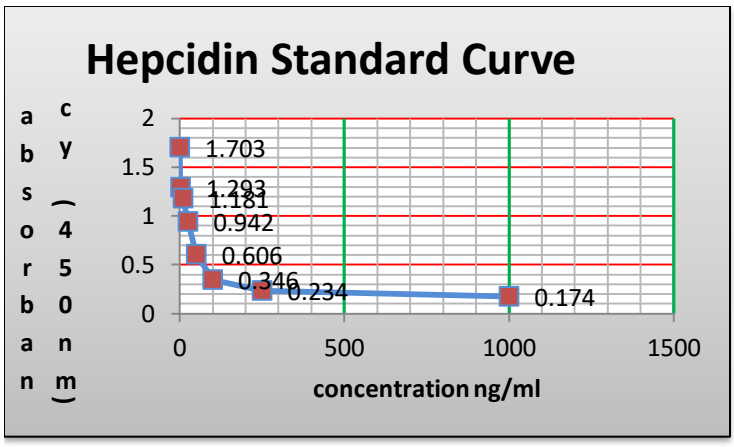
2. A standard curve was constructed by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis, as shown in figure.

3. The mean absorbance value was used for each sample to determine the corresponding concentration from the standard curve.

Table 3.6 absorbance and concentration Values for the standard

standard	Concentration	abs
1	0	1.703
2	2.5	1.293
3	10	1.181
4	25	0.942
5	50	0.606
6	100	0.346
7	250	0.234
8	1000	0.174

Figure 3.1 constructed standard curve



Reference values of Hepcidin : Males and Females 17-254 ng/ml

3.8 Molecular testing

3.8.1 DNA extraction

DNA was extracted from peripheral blood using standard lab protocols. The QIAamp DNA mini kit 250 was used. QIAamp DNA Mini Kits are designed for rapid purification of an average of 6 µg of total DNA from 200 µl of whole human blood. The QIAamp DNA purification procedure comprises 4 steps and is carried out using QIAamp Mini spin columns in a standard microcentrifuge. The first step is lysis of blood cells using proteinase K and lysis buffer provided by the kit manufacturer. The lysate buffering conditions are adjusted to allow optimal binding of the DNA to the QIAamp membrane before the sample is loaded onto the QIAamp Mini spin column. DNA is adsorbed onto the QIAamp silica membrane during a brief centrifugation step. Salt and pH conditions in the lysate ensure that protein and other contaminants, which can inhibit PCR and other downstream enzymatic reactions, are not retained on the QIAamp membrane. Then, the DNA bound to the QIAamp membrane is washed in 2 centrifugation steps using 2 different wash buffers, Buffer AW1 and Buffer AW2 provided by the manufacturer. This is done to significantly improve the purity of the eluted DNA. Wash conditions ensure complete removal of any residual contaminants without affecting DNA binding to the membrane. Purified DNA was then eluted from the QIAamp Mini spin column in a concentrated form in either Buffer AE also provided by the manufacturer. The purified DNA was then stored at –20°C until further analysis. The extracted DNA was then used for PCR amplification of the TMPRSS6 gene.

The protocol used is called the spin protocol and the procedure is as follows:

Samples were equilibrated to room temperature and a water bath was heated to 56°C.

200 µl blood sample was added to a labeled microcentrifuge tube accordingly. 25 µl QIAGEN proteinase K was pipeted into the microcentrifuge tube. Proper mixing after adding the enzyme was ensured. 200 µl Buffer AL was added to the sample. The mixture was mixed by pulse-

vortexing for 15 s. To ensure efficient lysis, the sample and AL were mixed thoroughly to yield a homogeneous solution. The sample was then incubated at 56°C for 30 min. the microcentrifuge tube was then briefly spinned to remove drops from the inside of the lid. 200 µl absolute alcohol (96–100%) was added to the sample, and mixed again by inverting the tube and then by briefly spinning for 15 s. The mixture was then carefully transferred to the accordingly labeled QIAamp Mini spin column that has the silica membrane (in a 2 ml waste tube) without wetting the rim. The tube was centrifuged at 8000 rpm for 1 min. If the lysate was not completely passed through the column after centrifugation, centrifuge again at higher speed until the QIAamp Mini spin column was dry. QIAamp Mini spin column was placed in a clean 2 ml waste tube and the tube containing the filtrate was discarded. The column was then washed by adding 500 µl Buffer AW1 without wetting the rim. And then centrifuged at 8000 rpm for 1 min. the QIAamp Mini spin column was then placed in a clean 2 ml waste tube and the tube containing the filtrate was discarded. The column was then washed again with the second salt buffer by adding 500 µl Buffer AW2; and then centrifuged at 14000 rpm for 3 min. To eliminate the chance of buffer AW2 carryover, the column was centrifuged again for 1 min after placing it in a new waste tube. The waste tube was discarded and the QIAamp Mini spin column was then placed in a clean and labeled microcentrifuge tube. 100 µl Buffer AE was added to the column and was incubated at room temperature for 10 min, then centrifuged at 8000 rpm for 1 min. A second elution step with a further 50 µl Buffer AE can be applied to the columns (placed in a new and labeled microcentrifuge tube) to increase the DNA yields. The microcentrifuge contain the extracted DNA, this was stored at –20°C until further analysis.

3.8.2 Determination of DNA concentration and purity

To determine the concentration and purity of DNA, a spectrophotometer was used. The concentration of DNA in the eluate was measured by absorbance at 260 nm. Purity of DNA was determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. Pure DNA has an *A*260/*A*280 ratio of 1.2–1.9. The spectrophotometer readings of sample and control concentrations and absorbencies are recorded below in table 3.7.

CONTROL CASE #	CONTROL DNA PROFILE		**PATIENTS CASE #**	PATIENTS DNA PROFILE	
	Concentration (ng/ul)	Ratio *A*260/*A*280		Concentration (ng/ul)	Ratio *A*260/*A*280
1	75.2	1.79	**1a**	30.2	1.56
2	38.8	1.60	**1b**	31.1	1.95
3	37.8	1.85	**1c**	49.8	1.83
4	48.1	1.78	**2**	65.8	1.86
5	27.3	1.60	**3a**	81.1	1.79
6	39.4	1.50	**3b**	19.7	1.60
7	34.4	1.48	**4a**	20.8	1.87
8	29.2	1.56	**4b**	15.4	1.50
9	30.9	1.5	**5**	18.0	1.65
10	20.6	1.51	**6**	20.8	1.61
11	33.2	1.67	**7**	21.5	1.56
12	34.5	1.74	**8**	28.7	1.67
13	27.4	1.59	**9**	30.7	1.88
14	21.9	1.53	**10**	20.6	1.64
15	36.4	1.64	**11**	28.6	1.48
16	27.3	1.57	**12**	33.0	1.67
17	18.3	1.50	**13**	15.1	1.53
18	22.0	1.54	**14**	31.7	1.62
19	28.5	1.58	**15**	21.9	1.69
20	18.6	1.48	**16**	25.3	1.80

Table 3.7: readings of concentration and absorbance ratios of patients' and control extracted DNA

3.8.3 PCR amplification of TMPRSS6 gene

Exons 1-18 and exon/intron boundaries of the TMPRSS6 gene were amplified by PCR using primer pairs published previously (Finberg et al., 2008). The PCR primers were designed to cover every coding exon as well as at least 40 nucleotide flanking sequences on both sides. The forward and reverse primers for each exon and their amplicons size are provided in table 2. The UCSC In-silico PCR website (https//genome.ucsc.edu) was used for primer alignment to ensure specificity of the primers (Annex 2). The stock primers were dissolved in sterile distilled water to 100Mm. 10ul of each stock primer was then dissolved in 90ul sterile distilled water to obtain a final concentration of 10uM. Reactions were performed using the extracted DNA with the above mentioned concentrations. The conventional PCR reactions were performed in 25ul total volume for the 20contrlos and 20patients samples. The reaction tube contained a final concentration of 1X (12.5ul) of the 2X ReddyMix PCR Master Mix with 1.5mM (Thermo Scientific), 0.2uM (0.5ul of the 10uM) of forward and reverse primers, 2.5ul of DNA template, and 9ul distilled water. Amplification by conventional PCR was performed with an initial hold at 96°C for 5minutes, 35 cycles at 96 °C for 30seconds, 59°C for 30 seconds, 72 °C for 40seconds, and a final extension at 72 °C for 5minutes. These temperatures provided the best enrichment with efficient amplification. The PCR product was visualized with 2% agarose gel prepared with 1X TEA buffer for 30minutes.

EXON #	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')	Amplicon size (bp)	GC content %
1	CTGAGACCTCCGTCTGTCCTC	TGGAAACAGCCTCGCATTTG	271	58
2	TGCCGCCTGATGTTGTTACTC	GCCTGCTACAGTCACCCCAAG	395	62
3	GCAGGAGAAGGCATGGAAGAG	TCCCTGTGAATGCTCCAGATG	323	55
4	AGTAGGAGCAAAGGGCACCTC	GACATGCAGGAAGCCAAGTTC	301	53
5	CTTCTGCGTGAAGACGGACAG	GGCCACACCACAGCTTGTTTC	378	61
6	AGACAAGGCTGGCTCCAAGG	CCCTGCACACACAACAGAAGC	255	38
7	AGGCGTGAAGCTCAGTGTGTG	CTAGCCGTCCTGTCTCCCAGA	584	60
8	GATGTCCAGACTCCCGTCCAC	GAATCTTCCCTCTCCCCATCC	364	64
9	ATTTGCTGGCAGAGGTGGTAG	GGAAACACAGAATCCCAGGTG	458	62
10	TGTTGTTAGGGAGGTGGGTTCAC	GAGATTGGGGACTTGGGCTTC	287	58
11	AGGGAGAAATCAGGGCAGAGG	CCTTGGTGGTTCCAGGGATG	356	64
12	GCCACAAGGGTTTGCAGGAAT	GAGGCTGCATTGCTGGTCTGT	523	56
13	GTGATTGGTAACGTGCAATACAGC	TGAAGCATGTAGCAGGCCTAGA	285	56
14	CTCTTCTGGCTCCATCGTTCC	TGAGATTTCCCTCCAGCTTCC	295	62
15	TCTCCCCCTCCATCATTCTCC	CCACCACCCTTCCCTCTATCTG	399	61
16	ACCACCAGCTAGGCGACCTTC	GCCCAATTTGAATCCCAGCAC	571	67
17	GTGGGCAGAGCAGGAGAGAAG	GATGTGAGCAAAGGGCCAGAC	337	56
18	CCCAGTCAATTCCCAACAGTC	GAATACTTGTCCCCCTGCTTG	344	58

Table 3.8: primer pairs for PCR amplification of exons 1-18 of the TMPRSS6 gene, amplicon size, and the GC content in each amplicon

3.8.4 Realtime PCR and Melting Curve Analysis

Sequencing is the most extensively used method for screening IRIDA patients for mutations in the TMPRSS6 gene (Falcon et al., 2013); since there is neither founder-effect mutation nor frequent mutations with greater contribution to the disease have been found. Also, the gene is highly polymorphic with variations that may result in benign, increased risk of iron deficiency anemia, microcytic anemia responsive to oral iron, or IRIDA phenotypes according to genecards.org database. However, sequencing is an expensive technique for labs with limited financial and material resources. Therefore, another sensitive and efficient method had to be used in order to reduce lab cost. In 1997, fluorescent melting analysis of real time-PCR (rt-PCR) was introduced on the LightCycler in the presense of DNA binding dyes (Wittwir et al., 1997). The melting curve analysis is a post-PCR technique used to investigate the variance in nucleic acid sequences and scanning and genotyping the genetic mutations in population based on different melting points of DNA fragments. This technique enables researchers to discover new genetic variants (without sequencing), rapid detection and categorization of genetic mutations or genetic variation in population. Furthermore, in comparison with other real-time PCR methods, low-cost fluorescent dyes are used in this technique (Herman et.al. 2006). Different types of double stranded DNA (dsDNA) intercalating dyes with particular specifications are used for real-time PCR and melting analysis. These dyes should provide the detailed information of melting behavior of an amplicon and the T_m of the amplicon should not be changed under the same conditions. In addition, dyes should not be bound preferentially to purines or pyrimidines bases and inhibit DNA amplification ideally (Vali et al., 2014). dsDNA intercalating dyes are divided into three main classes: non-saturating, saturating and release-on-demand dyes. Second (SYTO9® and LCGreen®) and third (EvaGreen®) classes of dsDNA intercalating dyes are suitable for high resolution melt

(HRM) analysis. However, the cost of second and third classes of intercalating dyes is more than the first class (SYBR® Green I).

The main principle of melt curves is the integration of intercalating dye to double stranded DNA (dsDNA) and disintegration of the fluorescence dye when the dsDNA are separated to single stranded DNA (ssDNA). Mainly, the real-time thermal cycler is programmed to produce the melt curve after the amplification cycles end; starting at a preset temperature and measuring the amount of fluorescence continuously as the temperature increases (Downey, 2014). As the temperature increases, the dsDNA denatures becoming ssDNA and the intercalating dye dissociates resulting in decreased fluorescence. By using a real time measurement of the fluorescence level, a melting curve of level against time is formed. The melting profile is mainly influenced by the length of DNA fragment, the GC content and heterozygosity. Sequence factors can cause the amplicons to melt in multiple phases where an intermediate state of the DNA is in both double-stranded and single-stranded configurations. These factors include GC rich, amplicon misalignment in A/T rich regions, amplicon length, and secondary structure designs in the amplicon regions. This intermediate phase results as the dsDNA starts to melt, the more stable regions of the amplicon do not melt immediately and maintain their double-stranded configuration until the temperature is high enough to cause it to melt, resulting in different melting phases with different melting domains.

This study aimed to obtain an optimal rt-PCR-melt curve analysis condition for screening of *TMPRSS6 gene* variance in order to reduce lab costs. So far, it is the first study which screens variance in *TMPRSS6* using melt curve analysis technique; therefore, establishment of sequence variance in control samples and optimization of the technique is very important in order to get reliable results.

The amplification reaction was performed on a LightCycler 2.0 (Roche) using a 20 µL total volume containing 1.5 µL DNA template, 1X (2 µL of the 10X) PCR Buffer II containing 100 mM Tris-HCl, pH 8.5, 500 mM KCl (applied biosystem), 4mM (3.2µL of the 25 mM) $MgCl_2$ (applied biosystem), 0.2 mM (0.4 µL of the 10mM) dntp mix (applied biosystem), 0.75 µL of the AmpliTaq DNA polymerase (applied biosystem), 0.15uM (0.3 µL of the 10uM) previously used primers, 1 µL of a 1000-fold dilution of SybrGreenI dye from Roche, and adjusted with water (nuclease free) to a final volume of 20µL in glass capillaries also provided by Roche. The cycling parameters of the rt-PCR were as follows: 10 min initial denaturation at 95°C and 50 cycles of denaturation for 5 s at 95°C, annealing for 10s at 58-60°C, and elongation for 15, 20, or 30 s at 72°C (for the 255-356, 364-458, or 500s bp fragments, respectively). Subsequently, PCR products were heated at 95°*C* for 2 *min* and then cooled at 50°*C* for 1 *min* in preparation of a melting curve analysis. The melting curve analysis was performed afterward with an increment of 0.2$_o$ *C/s* from 50°*C* to 95°*C*. Then, the melt curve data were subjected to gene scanning analysis by LightCycler software, version 3.5.28 (Roche) to identify the melting temperature peak in melting curves, which indicate the presence of variation in target sequence. The specificity of PCR products were also checked by 2% agarose gel electrophoresis for 30min.

Optimization of a Homemade SYBR Green I Reaction Mixture:

SYBR Green I homemade Real-time PCR master mix was prepared according to Karsai et al descriptions and was used in this study to evaluate the effect of melting behavior of TMPRSS6 amplicons. The amplification efficiency and reproducibility of the real-time PCR was compared with the SYBR Green I master mixture from Roche Molecular Biochemicals. The BSA was excluded according to Karsai et al due to better amplification. In this study, also excluding Triton X-100 gave better amplification sensitivity. The SYBR Green I was diluted in TE 1:1000 (pH 8.0)

and stored at −20°C. After thawing, the SYBR Green I aliquot was kept at 4°C and protected from light. Freshly suspended aliquots were made when the already prepared aliquot exceeded 3 weeks; since the dye remain stable for as long as 18 days according to Karsai et al, afterward the SYBR Green I degradation products become potent inhibitors of PCR. The influence of SYBR Green I concentration was assessed by using different volumes of the 1000-fold dilution for the 20 µL reaction volume: 0.4 µL, 1 µL, or 1.4 µL. Good results for all primer pairs were obtained with the 1 µL volume per reaction. A concentration of 4 mM $MgCl_2$ was first used and good results were obtained with this concentration.

The amplification parameters had to be optimized as well based on target length and GC content. Some of the amplicons have crossing points of less than 30 with an amplification curve increasing before the 30th cycle; while other amplicons required amplification after the 30th cycle. 50 cycles were required for some amplicons in order for the reaction to reach a plateau pattern. When double bands on agarose gel with a specific band and a band below the 100bp ladder band was observed, increasing the annealing temperature to get rid of unspecific PCR products (raw material amplification) was done (figure 3.2a). In larger amplicons, nonspecific banding was observed on the agarose gel (figures 3.2b and c). When the extension time was increased, the reaction efficiency increased and a single band on agarose gel was observed (figure3.2d).

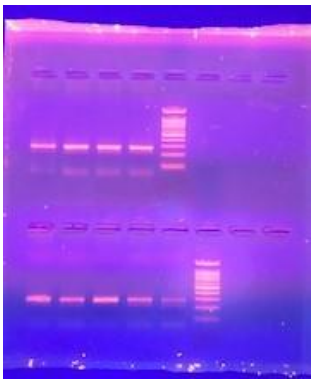


Figure 3.2a

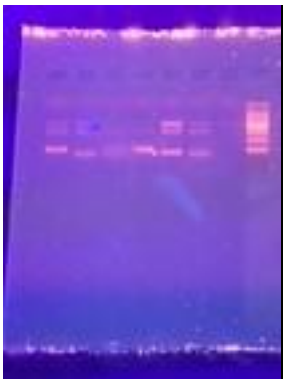


Figure 3.2b

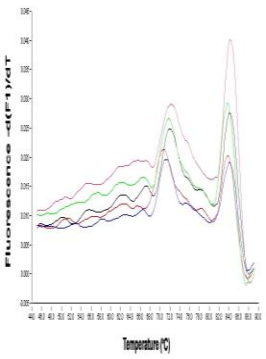


Figure 3.2c

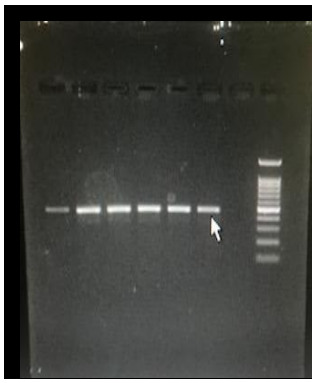


figure 3.2d

In the next step, the efficacy of our homemade master mix for discrimination of different genotypes was checked using the unpublished data of a colleague student at Birzeit University working on insulin resistance due to polymorphisms present in the insr (insulin receptor) gene. 12 samples, in which their genotypes of exon 8 had been determined by PCR-RFLP previously, were selected for melting analysis using the same PCR and melt analysis conditions. After PCR amplification, melting analysis was performed by the mentioned program. Then, the genotypes of samples were compared by the two different methods (melting analysis and PCR-RFLP). Our results showed that these two methods confirm each other and accuracy of the developed SYBR Green I based melting analysis method is 100%.

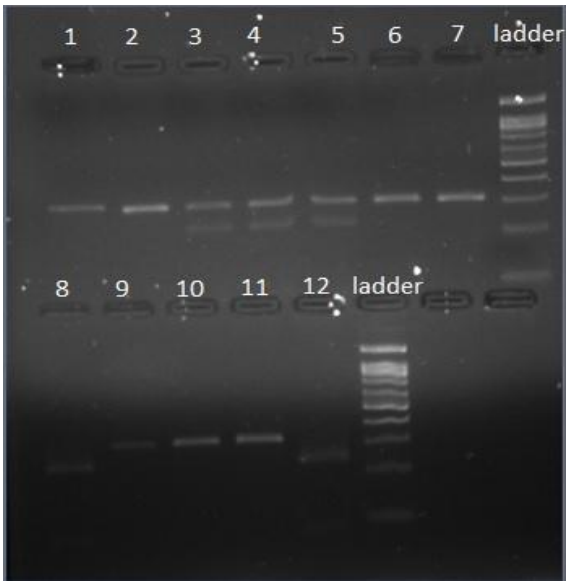


Figure 3.3a: RFLP gel electrophoresis of the insr gene exon 8 treated with the nsil restriction enzyme

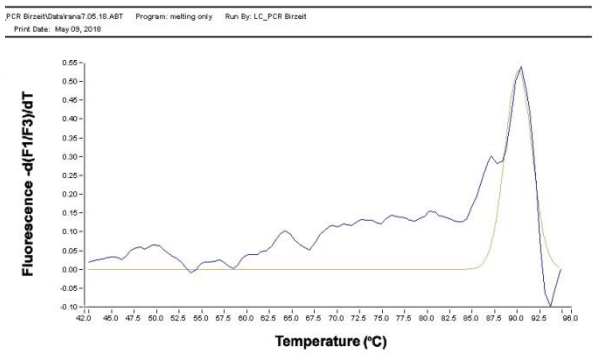


Figure 3.3b : melting point curve of sample 1 after incubation with nsil restriction enzyme , comparing with RFlP gel electrophoresis results (lane 1) **(sample 1 show G allele)**

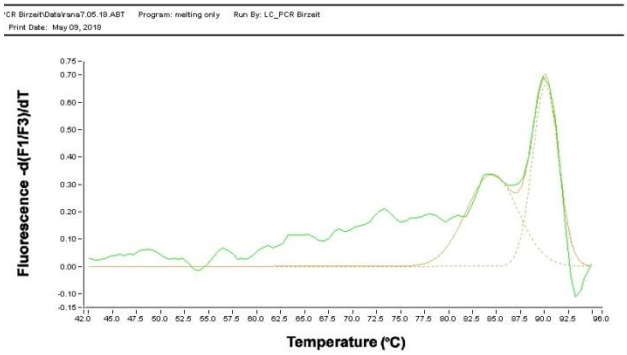


Figure 3.3c : melting point curve of sample 2 after incubation with nsil restriction enzyme , comparing with RFlP gel electrophoresis results (lane 3) **(sample3 show A allele**

Furthermore, uMelt [SM] Melting Curve Predictions Software was used to predict the melting curves of the PCR products by predicting the melting curve of the reference sequence of the primers used (annex 2). uMelt [SM] is a web-based application created to predict DNA melting curves of PCR products (the Wittwer lab for DNA analysis). In this software, the user defines an amplicon sequence and chooses a set of thermodynamic and experimental parameters and a temperature range. Using an accelerated partition function algorithm along with chosen parameter values, uMelt calculates the melting curve and the data were downloaded, where the melting curve and peak are provided. Results from fluorescent high-resolution melting experiments match the number of predicted melting domains and their relative temperatures. However, the absolute melting temperatures vary with the selected thermodynamic and experimental parameters. The software was applied for all of the 18 exons sequences and the results obtained matched the data that resulted from our samples. Figure 3.4a-d show examples of predicted melting profiles.

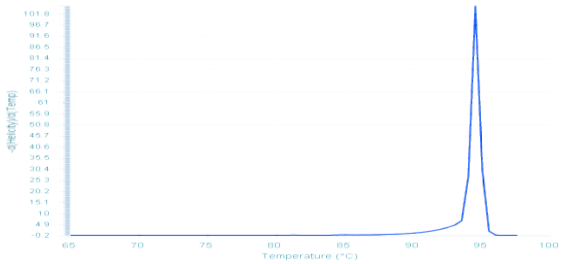


Figure 3.4a predicted melting peak of exon 1

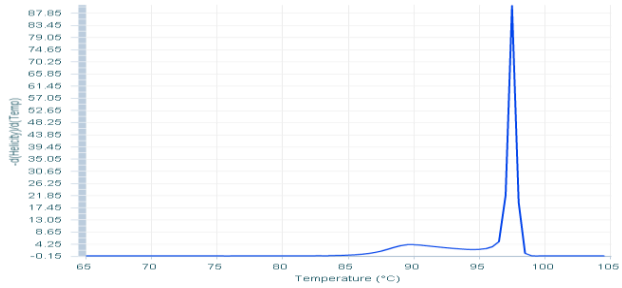


figure 3.4b predicted melting peak of exon 15

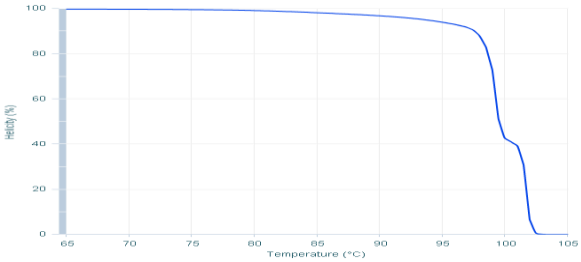


Figure 3.4c predicted melting curve of exon 16

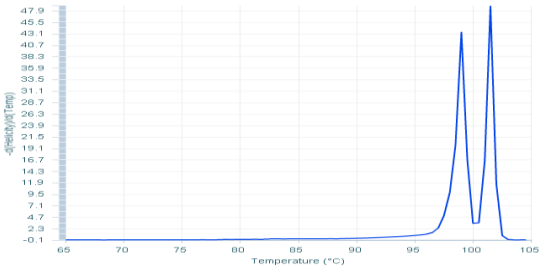


Figure 3.4d predicted melting peak of exon 16

3.9 data analysis

Data were computer analyzed using Microsoft office Excel 2007 real stats feature.

The statistical tests of significance were used depending on the nature as follows:

- Simple distribution of the study variables and cross tabulation was applied.
- The two-sample t-test for means and assuming unequal variances t-test was used to compare means of quantitative variables into two qualitative groups such as the relationship between cases and controls hepcidin and biochemical parameters.
- The paired two sample t-test for means was used to compare means of one variable at different time frames. As in the relationship between iron, ferritin and Hg at time of diagnosis and at time of phlebotomy for this study.
- The one-way ANOVA test was a comparing mean test used for analysis of variance for biochemical tests level as quantitative dependant variable by qualitative variables such as the relationship between hepcidin hormone by state of treatment
- Range as minimum and maximum values was used.
- The results in all statistical procedures were accepted as statistically significant when the p-value is less than 5% ($p<0.05$)

Chapter 4

Results

Results

The present study is a case control design. The study population comprised of 20 patients from 14 different families diagnosed with oral iron refractory and iron deficiency anemia, and sex and age matched healthy controls with normal hemoglobin levels. These 20 patients did not have any obvious cause for anemia based on their medical history and examination and had normal CRP and blood electrophoresis. Patients with a current or past microcytic hypochromic anemia, not or partially responsive to oral iron with a low TSAT (<15%) was considered as a suspected case, after which serum hepcidin analysis was performed. Very low to elevated ferritin levels, either before or after treatment with iron supplementation were included in the study.

4.1 General characteristics of study population

Table 4.1 summarizes the general characteristics of the cases according to age at diagnosis, age at present study, sex, consanguinity, treatment and state of treatment. The study population consisted of 70% females (n= 14) and 30% males (n= 6). 45% out of the females (n= 9) were adult females and 25% (n= 5) were females under the age of 18 years. All males were under 18 years. 85% (n= 17) had no parental consanguinity. 85% of the cases have had or are having intravenous iron treatment, and 15% have had or are having oral iron treatment. 40% (n= 8) have had recently ended their treatment, 45% (n= 9) are having ongoing treatment, and 15% (n=3) have not had treatment for at least 2years. The controls were age and sex matched healthy individuals. They were selected based on their normal Hg levels. None of the controls are having or have recently had iron treatment.

Table (4.1): General Characteristics of the cases

Case# (n=20)	Sex	Consanguinity	Age at diagnosis	Age at present study	Treatment	State of treatment
1a	F	NO	9months	7years	intravenous iron	recently ended 8 sessions
1b	F	NO	9 months	6years	intravenous iron	recently ended 8 sessions
1c	F	NO	9 months	1.5years	intravenous iron	recently ended 8 sessions
2	M	NO	9 months	8years	intravenous iron	not on treatment since age 4
3a	F	NO	1year	8years	intravenous iron	received 8 sessions a month ago
3b	M	NO	1year	10years	intravenous iron	received 8 sessions a month ago
4a	M	NO	I year	16years	intravenous iron	received 7 sessions 2 weeks ago
4b	M	NO	1 year	11years	intravenous iron	received 7 sessions 2 weeks ago
5	M	NO	9 months	6years	intravenous iron	not on treatment since age 4
6	M	YES	9 months	4years	intravenous iron	received 8 sessions 2 months ago
7	F	NO	26 years	39	intravenous iron	ongoing treatment
8	F	NO	24 years	32	intravenous iron	ongoing treatment
9	F	NO	22years	37	intravenous iron	ongoing treatment
10	F	NO	18years	32	intravenous iron	ongoing treatment
11	F	NO	20years	42	intravenous iron	ongoing treatment
12	F	NO	1year	1.7	intravenous iron	ongoing treatment
13	F	NO	25	35	intravenous iron	ongoing treatment
14	F	YES	16	27	oral iron	ongoing treatment
15	F	YES	18	37	oral iron + vit C intravenous iron twice in life time at age 16&31	ongoing treatment
16	F	YES	33	33	oral iron	not on treatment

4.2 Hepcidin Hormone, Biochemical Parameters and Complete Blood Count (CBC) Indices among the Study Population

Tables 4.2 and 4.3 show CBC indices, biochemical parameters, and hepcidin hormone concentrations for controls and cases respectively. **For the controls**, RBC indices had a range of 4.21-4.95; Hg had a range of 11.2-13.7; MCV had a range of 75.9-92.5, MCH had a range of 24-30.8; Serum Iron had a range of 31.9-67.7; TIBC had a range of 189.6-264; transferrin saturation had a range of 15.4-31.4; ferritin had a range of 9.5-118; and hepcidin concentration range was 19-99. **For the cases**, RBC indices had a range of 4.0-5.51; Hg had a range of 6.7-13.8; MCV had a range of 62.1-80.9; MCH had a range of 16.3-28.6; Serum Iron had a range of 15.7-67.5; TIBC had a range of 184.8 - 311.1; transferrin saturation had a range of 5.9-30.3; ferritin had a range of 2.2-22.4; and hepcidin concentration range was 33-98. CRP test was conducted to eliminate anemia of chronic disease as a cause of iron refractoriness. All cases had a CRP value below 5 mg/L. Meanwhile, 20% of the control (n=4) had higher than 5 mg/L values.

Table 4.4 shows the comparison between cases and control group according to CBC, biochemical parameters and hepcidin hormone indices. There is a statistically significant difference between the values of cases and those of control group for hepcidin hormone, transferrin saturation, TIBC, serum ferritin and all blood indices values ($P < 0.05$). However, there was no statistical significance difference in serum iron level ($P \geq 0.05$). The mean difference of RBCs, TIBC, and hepcidin hormone were higher in cases compared to controls. Meanwhile, the mean difference of serum iron, transferrin saturation, ferritin, Hg, MCV, and MCH were higher in controls compared to cases. Figure 4.1 shows the distribution of the mean hepcidin between cases and control.

Table 4.2: Hepcidin Hormone, Biochemical Parameters and Complete Blood Count (CBC) Indices among **controls**

Control # N = 20	**RBC 10^6/ul**	**Hg g/dl**	**MCV fl**	**MCH pg**	**S. iron ug/dl**	**TIBC**	**TS%**	**ferritin ng/ml**	**CRP mg/l**	**hepcidin ng/ml**
C 1	4.41	11.2	77.8	25.4	42.4	200.1	21.1	17.8	1.6	38
C 2	4.25	11.6	83.8	27.3	36.8	238.5	15.4	15.1	2.3	19
C 3	4.66	13	82.4	27.9	36.7	201.4	18.2	29.6	1.5	46
C 4	4.95	13.7	85.6	27.7	35.2	210	16.7	90.2	9.3	38
C 5	4.21	11.5	88	29	41.9	225.3	18.6	25.1	2.1	20
C 6	4.81	13	86	27	59.1	230.4	25.6	15	1.7	39
C 7	4.92	13.5	85.7	27.4	45.4	248.4	18.3	17.5	1.7	55
C 8	4.69	12.6	82.7	26.9	48.8	264	18.4	16.9	1.5	54
C 9	4.62	12.7	86	27.5	35.9	192.8	18.6	28.3	4.2	30
C 10	4.49	13	86.8	29	36.8	223.8	16.4	35.2	2.3	53
C 11	4.8	13.1	82.1	27.3	59.7	189.6	31.4	27.3	6.5	66
C 12	4.71	11.3	75.9	24	34.4	203.7	16.8	15.7	10.2	48
C 13	4.93	12.7	77.1	25.8	39.4	205.5	19.2	61	11.5	38
C 14	4.31	11.4	80.8	26.5	36.2	190.8	19	118	2.4	65
C 15	4.39	11.4	79.5	26	31.9	196.2	16.3	70	4.4	59
C 16	4.9	13.6	86.3	27.5	42.8	209.1	20.4	13.7	2.6	39
C 17	4.78	12.5	78.9	26.2	67.7	261.9	25.8	9.5	3.5	35
C 18	4.29	13.2	92.5	30.8	44.2	203.7	21.7	10.4	2.9	39
C 19	4.42	12.9	85.8	29.2	49.5	236.1	20.9	22.8	0.6	32
C 20	4.66	13.7	85.2	29.4	63.8	218.7	29.1	43.9	2.6	99

Table 4.3: Hepcidin Hormone, Biochemical Parameters and Complete Blood Count (CBC) Indices among **cases**

Case # N= 20	**RBC 10^6/ul**	**Hg g/dl**	**MCV fl**	**MCH pg**	**S. iron ug/dl**	**TIBC**	**TS %**	**ferritin ng/ml**	**CRP mg/l**	**hepcidin ng/ml**
1a	4.94	12.1	74.1	24.5	28.7	221.4	12.9	18.4	4.1	82
1b	5.41	13.8	75.3	25.5	28.3	220.2	12.8	22	4.4	98
1c	5.11	11.1	66.3	21.7	32.6	234.6	13.9	8.4	3.6	65
2	5.04	12.9	76.2	25.6	43.4	269.7	16.1	10.6	4.9	50
3a	4.61	11.7	79	25.5	57.8	250.5	23	14.7	1.9	37
3b	4.87	12.5	78	25.7	45.6	302.7	15	10.9	2.9	45
4a	5.19	13.5	80.3	26	62.5	216	28.9	10.8	3.4	78
4b	4.43	10.6	76.7	23.9	67.5	245.1	27.3	10.8	2.6	77
5	5.3	11.6	68.3	21.9	56.1	184.8	30.3	17.4	2.4	40
6	5.51	9.8	58.8	17.8	28.8	311.1	9.3	6.68	1.6	35
7	4.57	12.3	80.9	28.6	40.7	225.3	18.1	34	2.2	53
8	3.99	6.7	62.6	18.3	32.5	238.5	13.6	22.4	3.4	70
9	4.34	9.4	69.4	21.7	64.4	269.4	23.9	3.27	3.5	48
10	4.56	10.5	71	23.7	17.5	295.2	5.9	16.5	3.3	50
11	3.67	8.3	68.1	22.6	26.6	233.4	11.4	5.27	3.9	82
12	4.12	6.71	58.7	16.3	20.1	231.9	8.7	2.2	2.5	95
13	4.68	9.6	65.1	20.5	21	252.9	8.3	4.2	1.2	48
14	4	10.3	70.4	25.7	21.4	260.4	8.2	5.62	1.2	48
15	4.91	9.5	62.1	19.3	18.6	277.8	6.7	4.43	3.7	82
16	4.95	11	69.3	28.2	15.7	264.9	5.9	3.91	2.7	33

Table4.4: Comparison between cases and controls according to hepcidin hormone, biochemical parameters and complete blood count (CBC) indices

ITEMS	CONTROLS (N=20) MEAN ±SD	CASES (N=20) MEAN ±SD	T-TEST	P-VALUE
RBC (M/UL)	4.6±0.2	4.7±0.5	-8.7	0.000*
Hg (g/dl)	12.6 ±0.9	10.7±1.99	1.8	0.04*
MCV (fl)	83.4±4.2	70.5±6.9	6.6	0.000*
MCH (pg)	27.4±1.6	23.1±3.4	3.8	0.001*
TIBC (µg/dL)	217.5±22.8	250.8±32.1	-3.9	0.000*
Serum iron (µg/dL)	44.4±10.5	36.5±17.1	1.5	0.13
Transferrin saturation (%)	20.4±4.4	14.8±7.8	2.2	0.03*
Serum ferritin (ng/ml)	34.1±29	11.6±8.2	3.2	0.004*
Hepcidin hormone (ng/ml)	45.6±18.1	60.8±20.6	-2.6	0.01*

* the relation is significant at the p-value <0.05 level.

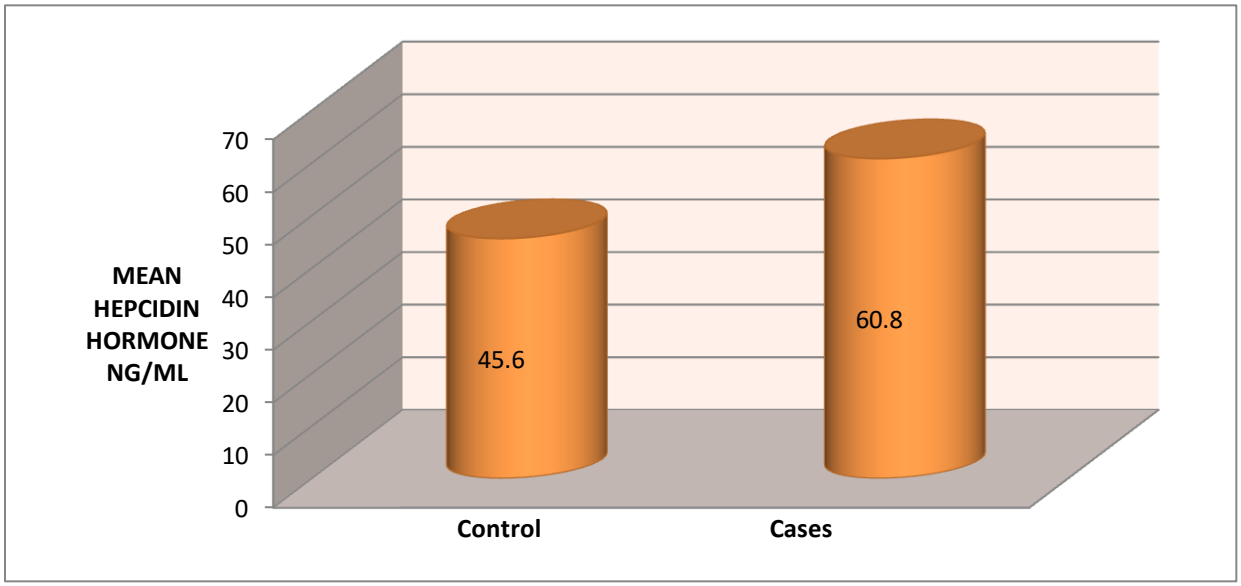


Figure (4.1): Distribution of the mean of hepcidin hormone (ng/ml) among controls and cases.

4.3 Comparing cases at diagnosis and cases at time of phlebotomy for this study according to Hg, serum Iron, and ferritin.

When comparing Hg, serum Fe, and ferritin levels between cases at diagnosis provided in the table below, and cases at time of phlebotomy for this study, the difference was significant for hemoglobin and serum iron levels. The mean difference of hemoglobin and serum iron levels was higher at time of this study than at diagnosis; meanwhile ferritin was higher at time of diagnosis than at time of this study. The means, t-test values and P values are listed below in table 4.5.

Table 4.5: Hg, serum iron and ferritin data at diagnosis and at time of phlepotomy for this study

	DATA AT DIAGNOSIS			DATA FOR THIS STUDY		
CASE#	Hg g/dL	Serum Iron ug/dL	Ferritin ng/ml	Hg g/dL	Serum Iron ug/dL	Ferritin ng/ml
1a	7.5	15	3.4	12.1	28.7	18.4
1b	8.1	12.9	2.6	13.8	28.3	22
1c	7.6			11.1	32.6	8.4
2	8.1	23.1	25	12.9	43.4	10.6
3a	8.6		4.16	11.7	57.8	14.7
3b	8.5	12.9	2.6	12.5	45.6	10.9
4a	10.3	26	4.29	13.5	62.5	10.8
4b	8.78	20.4	3.78	10.6	67.5	10.8
5	9.74		9	11.6	56.1	17.4
6	7.6	21	118	9.8	28.8	6.68
7	9.3		1	12.3	40.7	34
8	6.21L		2	6.7	32.5	22.4
9	7.1		1.2	9.4	64.4	3.27
10	7.97	18.4	2.14	10.5	17.5	16.5
11	7.4		1.1	8.3	26.6	5.27
12	6.5	12.4	1.5	6.71	20.1	2.2
13	7.6	13.5	8.9	9.6	21	4.2
14	9.7		35.7	10.3	21.4	5.62
15	8.1		16.1	9.5	18.6	4.43
16				11	15.7	3.91

Table 4.6: comparison between cases at diagnosis and cases at time of this study according to Hg, serum iron, and ferritin

TEST	**CASES AT DIAGNOSIS MEAN ±SD**	**CASES FOR THIS STUDY MEAN ±SD**	**T-TEST**	**P-VALUE**
Hg (g/dl)	8.1 ±1.1	10.7 ±2.04	-10.07	0.000*
Serum iron (µg/dL)	17.56 ± 18.7	36.34 ±17.7	-4.09	0.002*
Serum ferritin (ng/ml)	13.5±27.7	12.2 ±8.4	0.03	0.97

* the relation is significant at the p-value <0.05 level.

4.4 Association of biochemical parameters and hepcidin according to state of treatment among cases

Tables 4.7-4.11 show the association of Hg, S. iron, transferin saturation, ferritin and hepcidin according to state of treatment among cases. Table 4.7 shows that there is significance between Hg level and state of treatment among cases. The mean difference of hemoglobin was highest in the recently ended treatment group and lowest in the ongoing treatment group. Table 4.8 shows that there is no significance between S. iron level and state of treatment among cases. The mean difference of S. iron of the cases was highest in patients with the recently ended treatment group, and lowest in patients with oral iron treatment. Table 4.9 shows that there is no significance between transferrin saturation and state of treatment among cases. The mean difference of transferrin saturation of the cases was highest in the recently ended treatment group, and lowest in patients with oral iron treatment. Table 4.10 shows that there is no significance between ferritin and state of treatment among cases. The mean difference of ferritin of the cases was also highest in

the recently ended treatment group, and lowest in patients with oral iron treatment. Table 4.11 shows that there is no significance between hepcidin level and state of treatment among cases. The mean difference of hepcidin hormone of the cases was highest in patients with oral iron treatment, and lowest in patients that are not on treatment.

Table 4.7 Hemoglobin level of cases by state of treatment

State of treatment	N (20)	Hg (g/dl) Mean ±SD	F	P-value
Recently ended	8	11.9± (1.38)	4.64	0.0162*
Ongoing treatment	7	9.1± (2.0)		
Not on treatment	3	11.8 ± (0.97)		
Oral iron ± Vit C	2	9.9± (0.57)		

* the relation is significant at the p-value <0.05 level.

Table 4.8 S. iron level of cases by state of treatment

State of treatment	N (20)	SERUM IRON (ug/dl) Mean ±SD	F	P-value
Recently ended	8	43.9± (16.6)	1.39	0.280
Ongoing treatment	7	31.8 ± (16.5)		
Not on treatment	3	38.4± (20.7)		
Oral iron ± Vit C	2	20± (1.9)		

Table 4.9 transferin saturation level of the cases by state of treatment

State of treatment	N (20)	Transferrin saturation % Mean ±SD	F	P-value
Recently ended	8	17.9 ± (7.4)	1.31	0.304
Ongoing treatment	7	12.8± (6.3)		
Not on treatment	3	17.2 ± (12.3)		
Oral iron ± Vit C	2	7.45 ± (1.06)		

Table 4.10 ferritin level of the cases by state of treatment

State of treatment	N (20)	Ferritin (ng/ml) Mean ±SD	F	P-value
Recently ended	8	12.8± (5.2)	0.492	0.693
Ongoing treatment	7	12.5 ± (12.2)		
Not on treatment	3	10.6 ± (6.7)		
Oral iron ± Vit C	2	5.03 ± (0.84)		

Table 4.11 Hepcidin level of the cases by state of treatment

State of treatment	N (20)	Hepcidin Mean ±SD (ng/ml)	F	P-value
Recently ended	8	64.625± (23.2)	1.11	0.373
Ongoing treatment	7	63.7 ± (18.9)		
Not on treatment	3	41 ± (8.5)		
Oral iron ± Vit C	2	65 ± (24)		

4.5 Melting analysis results

All 18 exons of the TMPRSS6 gene were amplified and subjected to melting analysis for all study population. For each exon, primers were chosen to generate single gene sequences. However, the amplicon size for some of the exons had higher than 400bp, and the GC content of all the amplicons was also high (higher than 50 %) except exon 6 (38%), because these primers were designed for sequencing purposes, not for melting analysis and thus required further optimization than the smaller amplicons. The melting analysis was performed in the presence of the ds-DNA binding fluorescent dye Sybr Green I. When melting analysis is performed using the intercalating dye, the melting temperature is measured continuously until a temperature where the double strands of DNA dissociate (the melting temperature) is reached. The LightCycler software, version 3.5.28 (Roche) produces raw melt curve analysis and melt peak analysis. The raw melt curve data reveals individual variation between reaction samples because of differences in starting template concentrations and raw material concentration. The software allows users to normalize the fluorescence intensity between samples. This allows melting curves to be better compared between the samples. Sequence variants that do not cause disease occur at a frequency much greater than that of disease-causing variants. To eliminate these variants from consideration, we screened DNA from normal age and sex matched individuals to detect variant melting curves and establish a variation map for each exon. Samples from our patients could then be compared against such variation maps to eliminate common variants and focus on rare variants that are most likely disease causing. Samples with unique melting profiles could be rare variants not present in the control DNA samples; thus limiting the need for sequencing to only these rare variants not

presented in the control. An example of such melting curves and peaks is presented in figure 4.2

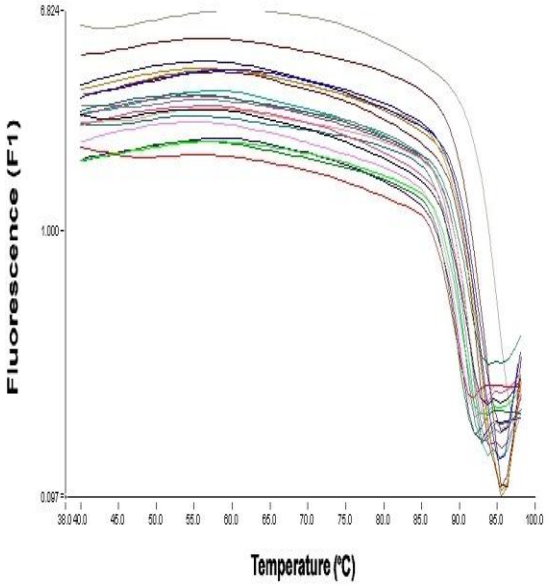


Figure 4.2a: melting curve of exon 1 control

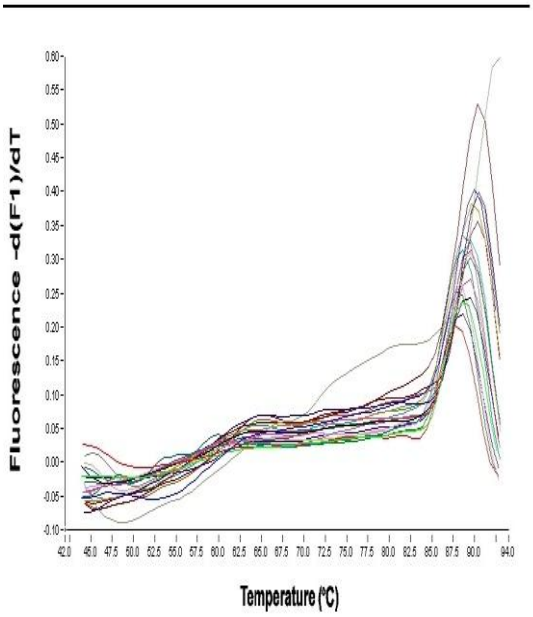


figure 4.2b: melting peak of exon 1 control.

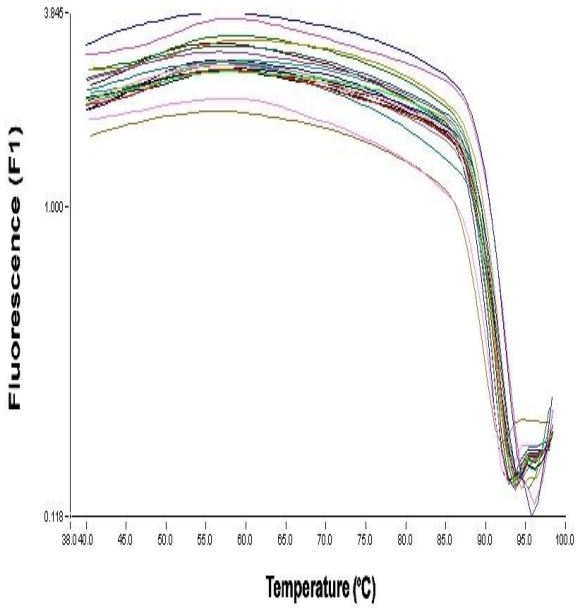


Figure 4.2c: melting curve of exon 1 patients

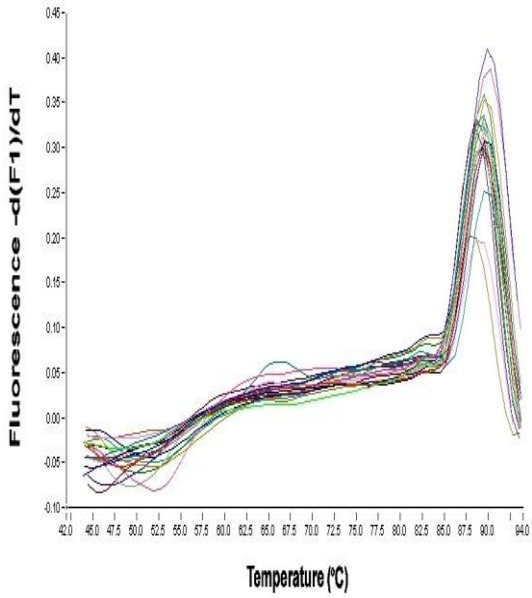


Figure 4.2d: melting curve of exon 1 patients

The melting temperature for each exon in all study population was measured manually and recoded. Table 4.4 shows range of melting temperatures, number of distinct melting patterns, and the frequency of each distinct melting temperature for each exon of all study population. Distinct melting profiles was observed in exon 1, 6, 10, and 18 that are present only in the control group and was not observed in the patients. Probands 1a-c and 2 had distinct melting pattern in exon 17. Probands 1a-c were sibling girls and proband 2 was their first degree cousin. Probands 3a and 3b were siblings and showed distinct melting profile at exon 12. Probands 4a-b and 8 had distinct melting temps in exon 13. Cases numbered 5, 13, and 14 presented with a distinct melting profile in exon 16. Probands 6 and 10 showed distinct melting pattern in exon 15. Probands 7,9,11 and 15 presented with distinct melting profile in exon 10. Patient 16 also had a distinct melting pattern in exon 15. Patient 12 had a distinct variation in exon 8.

Table 4.12: melting temperatures for all exons of study population and the frequency of each Tm value

EXON #	CONTROL N= 20		CASES N= 20	
	Tm values	frequency	Tm values	frequency
EX 1 **Range (min-max)**	87.7 88.7 89.5 90 90.37 93+ Range (87.7-93)	2 (10%) 5 (25%) 7 (35%) 1 (5%) 4 (20%) 1 (5%)	87.7 88.7 89.5 90 90.37 Range (87.7-9.37)	1 (5%) 3 (15%) 13 (65%) 2 (10%) 1 (5%)
EX 2 **Range (min-max)**	89.5 90 90.4 90.9 Range (89.5-90.9)	1(5%) 5 (25%) 3 (15%) 11 (55%)	89.5 90 90.4 90.9 Range (89.5-90.9)	3 (15%) 6 (30%) 5 (25%) 6 (30%)
EX 3 **Range (min-max)**	85.7 86.5 87.4 88.3 Range (85.7-88.3)	4 (20%) 7 (35%) 6 (30%) 2 (10%)	85.7 86.5 87.4 88.3 Range (85.7-88.3)	10 (50%) 6 (30%) 3 (15%) 1 (5%)
EX 4 **Range (min-max)**	89.6 90.4 91.2 92 Range (89.6-92)	5 (25%) 6 (30%) 4 (20%) 5 (25%)	89.6 90.4 91.2 92 Range (89.6-92)	2 (10%) 10 (50%) 3 (15%) 5 (25%)
EX 5 **Range (min-max)**	91.2 91.8 92.3 92.7 Range (91.2-92.7)	2 (10%) 7 (35%) 3 (15%) 8 (40%)	91.2 91.8 92.3 92.7 Range (91.2-92.7)	3 (15%) 3 (15%) 7 (35%) 7 (35%)
EX 6 **Range (min-max)**	84+ 84.7 85.4 86 86.5+ 87+ Range (84-87)	1 (5%) 5 (25%) 1 (5%) 5 (25%) 4 (20%) 4 (20%)	84.7 85.4 86	7 (35%) 8 (40%) 5 (25%)
EX 7 **Range (min-max)**	92.5 93.3 Range (92.5-93.3)	4 (20%) 16 (80%)	92.5 93.3 Range (92.5-93.3)	7 (35%) 13 (65%)
EX 8 **Range (min-max)**	91 91.6 92.4 Range (91-92.4)	2 (10%) 8 (40%) 10 (50%)	91 91.6 92.4 93 Range (91-93)	2 (10%) 8 (40%) 9 (45%) 1 (5%)*

EX 9 **Range (min-max)**	90.4 91.2 91.8 93.6 Range (90.4-93.6)	8 (40%) 9 (45%) 2 (10%) 1 (5%)	90.4 91.2 91.8 93.6 Range (90.4-93.6)	2 (10%) 11 (55%) 6 (30%) 1 (5%)
EX 10 **Range (min-max)**	87.4+ 88 88.4 89 Range (87.4-89)	2 (10%) 8 (40%) 4 (20%) 6 (30%)	88 88.4 89 90.1* 91.1* 92* Range (88-92)	12 (60%) 2(10%) 2(10%) 1 (5%) 1(5%) 2(10%)
EX 11 **Range (min-max)**	89.8 90.8 91.6 Range (89.8-91.6)	5 (25%) 10 (50%) 4 (20%)	89.8 90.8 91.6 Range (89.8-91.6)	8 (40%) 10 (50%) 2 (10%)
EX 12 **(min-max)**	90 90.7 91.3 Rang (90-91.3)	4 (20%) 8 (40%) 5 (20%)	90 90.7 91.3 92.8* Range (90-92.8)	3(15%) 7(35%) 8(40%) 2
EX 13 (min-max)	87.8 88.7 89.2 90.7+ Rang (87.8-90.7)	5(25%) 6(30%) 6(30%) 2(10%)	87.8 88.7 89.2 89.7* Rang (87.8-89.7)	5(25%) 8(40%) 4(20%) 3(15%)
EX 14 (min-max)	91.8 92.7 Rang (91.8-92.7)	5(25%) 15(75%)	91.8 92.7 Rang (91.8-92.7)	4 (20%) 16 (80%)
EX 15 (min-max)	91.7	20 (100%)	91.7 93.4* (91.7-93.4)	17 (85%) 3 (15%)
EX 16 (min-max)	92.1 93.1 Range (92.1-93.1)	13 (65%) 7 (35%)	92.1 93.1 93.8* Range (92.1-93.8)	6 (30%) 11 (55%) 3 (15%)
EX 17 (min-max)	90.6 91.2 91.8 92.4 93 Range (88-93)	7 (35%) 3 (15%) 4 (20%) 4 (20%) 2 (10%)	88 90.6 91.2 91.8 92.4 93 Range (88-93)	4(20%) 1(5%) 1(5%) 11(55%) 1(5%) 2(10%)
EX 18 (min-max)	89.7 90.4 91.3+ Range (89.7-91.3)	4(20%) 8(40%) 8(40%)	89.7 90.4 Range (89.7-90.4)	8(40%) 12(60%)

*Shows distinct melting pattern not present in the control

+ shows distinct melting pattern not present in patients

Chapter 5

Discussion

Chapter 5: Discussion

This study investigated the IRIDA phenotype amongst patients with anemia refractory to oral iron in relation of melting temperature for each exon of the matriptase 2 gene of the study population to screen for TMPRSS6 gene rare variants not present in the control, and iron indices. This is the first study in the West Bank to investigate this phenotype based on melting profiles, iron indices and hepcidin concentrations.

5.1 General characteristics of study population

The study population included 20 patients from 14 different families diagnosed with oral iron refractory and iron deficiency anemia, and sex and age matched healthy controls with normal hemoglobin levels. As mentioned previously, these 20 patients did not have any obvious cause for anemia based on their medical history and examination and had normal CRP and blood electrophoresis.

The cases consisted mainly of females in 14:6 ratio. 9 out of the 14 females were adult females and the rest were under the age of 18. All of the males were under 18. This finding confirms that this disease is under-diagnosed and is prevalent under high iron demand situations. Consanguinity did not play a major role in the anemic patients included in this study.

40% (n= 8) of the cases have had recently ended their treatment, all of them under the age of 16. 45% (n= 9) of the cases are having ongoing treatment, all of which are adult females. 15% (n=3) have not had treatment for at least 2years, two of which are males and the third is an adult female who was not aware of her anemia. 15% have had or are having oral iron treatment, one of which is also having vitamin C.

5.2 Biochemical Parameters and Complete Blood Count (CBC) Indices and Hepcidin Hormone among the Study Population

The controls were age and sex matched healthy individuals. They were selected based on their normal Hg levels. None of the controls are having or have recently had iron treatment. Patients with a current or past microcytic hypochromic anemia, not or partially responsive to oral iron with a low TSAT (<15%) were included in the study. CRP test showed that all patients had normal values, eliminating anemia of chronic disease as a cause of iron refractoriness.

There was a statistically significant difference between the values of cases and those of control group for hepcidin hormone, transferrin saturation, TIBC, serum ferritin and all blood indices values ($P < 0.05$) as expected. However, there was no statistical significance difference in serum iron level ($P \geq 0.05$). This observation might have been due to correction of anemia in patients who are having or have recently had parenteral iron therapy.

When comparing cases and control groups according to CBC, biochemical parameters and hepcidin hormone indices, the results showed that the mean difference of serum iron, transferrin saturation, ferritin, Hg, MCV, and MCH were higher in controls compared to cases, as was expected. The mean difference of RBCs and TIBC were higher in cases compared to controls, as was also expected. It was noted in the literature that RBC counts in IRIDA is usually normal to compensate erythropoisis shortage and hypoxia ((Kroot, et al. 2011). TIBC is also expected to be high due to the extremely low transferrin saturation observed in the IRIDA. Meanwhile, hepcidin hormone was also observed to be higher in cases compared to controls. All probands have been shown to have normal values of hepcidin. This observation is not consistent with normal IDA patients, however it is observed in patients with IRIDA due to the defective matriptase 2 gene that results in continuous hepcidin production.

In the initial case series described in Finberg et al (2008), all cases with TMPRSS6 mutations had normal ferritin, low transferrin saturation, marked microcytosis, and high hepcidin levels. In this study, 4 patients had normal transferrin saturation, suggesting correction of anemia with intravenous iron treatment or with age. However, microcytosis persisted in 3 patients out of the 4. 6 patients with corrected Hg levels showed persistent microcytosis and low transferrin saturation. Serum ferritin was also observed to be low-normal in 15 patients and low in 5 patients, whilst serum hepcidin was within normal range in 100% of cases. The 5 cases with low ferritin and normal hepcidin level suggest a phenotype of anemia of chronic disease; however this was ruled out by assessing CRP levels. Also, H. pylori infection and GI endoscopy was assessed in all adult probands and negative results were observed. Meanwhile, single case reports of low ferritin with normal/high hepcidin levels have also been associated with IRIDA (Silvestri et al, 2009; Sato et al, 2011; Lehmberg et al, 2012).

5.3 Comparing cases at diagnosis and cases at time of phlebotomy for this study according to Hg, serum Iron, and ferritin.

When comparing Hg, serum Fe, and ferritin levels between cases at diagnosis and cases at time of phlebotomy for this study, it was observed that the difference was significant for hemoglobin and serum iron levels. The mean difference of hemoglobin and serum iron levels was higher at time of this study than at diagnosis indicating correction of anemia due to parenteral iron therapy. Meanwhile, ferritin was higher at time of diagnosis than at time of this study.

It was observed that patients who recently had or are having parenteral iron treatment, an increase in ferritin level occurred (14/20 patients). A very high ferritin level was presented at diagnosis for one case. However, this case ferritin level was low-normal at time of this study even after having intravenous iron 2 months ago. The two cases on prolonged oral iron treatment had lower ferritin levels than at time of diagnosis. In the evaluation of the cases regarding treatment in previous

studies, a rise in serum ferritin was observed after parenteral iron therapy (Melis et al, 2008; Guillem et al, 2008; Tchou et al, 2009; Khuong-Quang et al, 2013). Once administered, intravenous iron enters reticuloendothelial and iron sequestration takes place (as evidenced by an increase in the serum ferritin level). A portion of the iron probably reaches plasma transferrin despite high hepcidin levels and can be utilized in erythropoiesis; this result in partial correction of anemia and a slight increase in MCV. In their study including 11 children with IRIDA, Akin et al. reported that Hg and serum ferritin levels of the patients increased following i.v. iron administration.

5.4 Association of biochemical parameters and hepcidin according to state of treatment among cases

When testing the association of Hg, S. iron, transferrin saturation, ferritin and hepcidin levels according to state of treatment in the probands, it was observed that there is significance difference in Hg level and state of treatment among cases. The mean difference of hemoglobin was highest in the recently ended treatment and not on treatment groups and lowest in the ongoing treatment group. Higher hemoglobin levels observed in the recently ended treatment group accounts for the release of iron into plasma due to parenteral iron administration. Higher hemoglobin levels in the not on treatment group stands for the correction of anemia with age, mainly with the male probands. Low hemoglobin levels in probands with ongoing treatment compared to the other groups is due to the fact that these probands have recently begun treatment sessions and iron has not yet been sequestered.

There was no significance between S. iron level and state of treatment among cases. The mean difference of S. iron of the cases was highest in patients with the recently ended parenteral treatment group, indicating the release of iron into plasma; and lowest in patients with oral iron

treatment, indicating the unresponsiveness of oral iron therapy due to decreased iron absorption from duodenal enterocytes.

There was no significance between transferrin saturation and state of treatment among cases. The mean difference of transferrin saturation of the cases was highest in the recently ended parenteral treatment group, and lowest in patients with oral iron treatment. Again this indicates the nature of response to parenteral iron therapy in IRIDA patients that is not present when treatment with oral iron is conducted due to a defective iron absorption system.

It was also observed that there is no significance between ferritin and state of treatment among cases. The mean difference of ferritin of the cases was also highest in the recently ended treatment group indicating iron sequestration post parenteral iron therapy. The lowest ferritin level was observed in patients with oral iron treatment, again indicating defective oral iron absorption mechanism.

Finally, there was no significance between hepcidin level and state of treatment among cases. The mean difference of hepcidin hormone of the cases was higher in patients with oral iron treatment (65 ng/ml), recently ended treatment (64.6 ng/ml) and in the ongoing treatment group (63.7 ng/ml) and lowest in patients that are not on treatment (41 ng/ml). This indicates that hepcidin level remained high despite the state of anemia presented in all cases.

5.5 Melting analysis profiles

Melt curves and melt peaks were produced by the Roche Lightcylcler Software for all of the 18 exons of the matriptase 2 gene of each of the study population. A gene variation map was constructed from the control group, and then melt curves of the controls were compared to the patients'. Each melting temperature presented for an exon represents a gene variation in that exon. It was observed that the gene is polymorphic, with only exon 15 presenting with only one melting temperature in the controls. Some of the exons (1, 6, and 17) had as high as 6 different melting profiles in the control group. This variation in the melting profiles within the exons indicates that there is genetic variation between the study populations. It was also noted that distinct melting profiles were observed in the control group only that was not presented in the patients. These distinct melting profiles may also represent a benign gene variation that was not also observed in the patients. Distinct melting profiles were observed in the patients that were not presented in the controls. However, these distinct melting profiles may represent a pathogenic gene variation that may have been responsible for the observed phenotype. It is important to point out that in the cases where siblings were presented with the same phenotype also shared this distinct melting temperature variation; as in probands 1a-c and their cousin proband #2 all shared the same distinct melting temperature in exon 17. These genetic variations presented in the form of distinct melting temperatures can be confirmed by sequencing and their pathogenecity can then be evaluated.

Chapter 6

Conclusions and Recommendations

Chapter 6: conclusions and recommendations

6.1 Conclusions

This study evaluated patients with anemia that have oral iron refractoriness without any obvious reason for the anemic phonotype. A healthy control group that are age and sex matched to the probands were also evaluated. The conclusions drawn from this study will be discussed in the following paragraphs.

It was noted that this disease is under-diagnosed and is prevalent in kids and in under high iron demand conditions. Consanguinity was not significant in the observed phenotype included in this study.

The average levels of serum iron, transferrin saturation, ferritin, Hg, MCV, and MCH were significantly lower in cases compared to controls. In contrast, TIBC was higher in cases compared to controls. The mean level of hepcidin was significantly lower in controls compared to cases. This observation is not consistent with normal IDA patients, however it is observed in patients with IRIDA phenotype.

When comparing iron indices before and after treatment, partial correction of anemia and a slight increase in MCV was observed in the patients following i.v. iron administration. This concludes that these patients require parenteral iron therapy for correction of anemia.

This finding was also confirmed when comparing patients' iron indices and hepcidin levels according to their state of treatment. Hg, S. iron, transferrin saturation, and ferritin levels were higher in the recently ended treatment group and mainly lowest in the oral iron therapy group. In addition, hepcidin level remained normal-high despite the state of anemia presented in all cases.

Serum hepcidin level has a relationship with patients that represent with IRIDA phenotype. Therefore, evaluating hepcidin levels can play an important role in diagnosing anemia among patients with oral iron refractoriness.

It is also concluded that the distinct melting profiles observed in the patients but not in the controls may account for the pathogenecity of the gene variation resulting in the observed phenotype. These findings need to be confirmed by sequencing the exon that contained the distinct melting temperature for that proband/s.

6.2 Recommendations

Serum hepcidin level is sensitive and specific marker of anemia among IRIDA patients, which can provide valuable prognostic information, improve patient care, and may be used to screen for anemia among patients with refractoriness to oral iron.

Administration of parenteral iron supplementation during childhood and in iron demand conditions, such as during pregnancy or surgery is recommended for these patients and for patients with IRIDA phenotype.

Conduction of a cohort study to determine the prevalence of IRIDA among children in the west bank by first supervising an initial oral iron administration among iron deficient anemic children to screen for patients with oral iron refractoriness is recommended. Hepcidin level in these patients can then be assessed.

Considering the heterogeneous nature of polymorphisms and mutations in this gene, it is recommended to screen and sequence all 18 exons including exon-intron boundaries to identify any already described or a novel potentially deleterious mutation in these patients.

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Annexes

Annex 1

برنامج الماجستير في العلوم الطبية المخبرية
موافقة على المشاركة في البحث (Written consent)

عنوان البحث:

Iron refractory iron deficiency anemia caused by mutations in matriptase 2 gene, a negative regulator of hepcidin, in Palestinian subjects

الباحثون: لطيفة عرار و د. يعقوب ظاهر/ جامعة بيرزيت
حيثيات ألبحث: نود مشاركتك في هذا البحث من خلال الموافقة على أخذ كمية قليلة من الدم لعمل فحوصات للبحث عن الجينات التي لها علاقة بفقر الدم الذي لا يستجيب للحديد عن طريق الفم

الغرض من البحث

- تحديد مستويات الحديد والهبسيدين في الدم
- تحديد التغييرات الجينية التي قد تسهم في عدم امتصاص الحديد وتوزيعه وارتفاع نسبة هرمون الهبسيدين.
- امكانية الكشف المبكر بالفحص الجيني لتجنب تناول كميات عالية من الحديد ومباشرة العلاج
- دراسة اثر التغيرات الجينية على النمط الظاهري للمريض

أية مخاطر يمكن توقعها: لا توجد مخاطر عدا عن الحد الأدنى من الراحة أثناء سحب الدم.
كيف سيتم الحفاظ على السرية: لن يتم استخدام اسم المشارك ، سيتم ترميز العينة.
عدد ألمشاركين: 25

ان مشاركتك في هذا البحث سوف تسهم في زيادة المعرفة. ونتائج هذه الدراسة سيكون لها اثر ايجابي في البحث عن وصفات ممكن ان تعالج هذا المرض

اسم المشارك (ة) : ----------
عمر المشارك (ة) : ----------
مكان السكن (الولادة) : ----------
التاريخ : _____

توقيع المشارك (ولي امره) : _____

Primer Alignment for the 18 exons of
TMPRSS6 gene

Exon 1

>chr22:37103442-37103712 271bp CTGAGACCTCCGTCTGTCCTC TGGAAACAGCCTCGCATTTG
CTGAGACCTCCGTCTGTCCTCtgaccatagggaagccagtgggaagcaaa
ggtggggttcttgagccagacccagtccagctctggtgcctgccctctgg
tgcgagctgacctgagatgcacttccctcctctgtgagctgtctcggcac
ccacttgcagtcactgccgcctgatgttgttactcttccactccaaaagg
cagggaagtcctgcttccgtgccccaccggtgctcagcagaggctccctt
gCAAATGCGAGGCTGTTTCCA

Primer Melting Temperatures

Forward: 61.4 C ctgagacctccgtctgtcctc
Reverse: 64.4 C tggaaacagcctcgcatttg
The temperature calculations are done assuming 50 mM salt and 50 nM annealing oligo concentration. The code to calculate the melting temp comes from Primer3.

Exon 2

chr22:37103154-37103548 395bp TGCCGCCTGATGTTGTTACTC GCCTGCTACAGTCACCCCAAG
TGCCGCCTGATGTTGTTACTCttccactccaaaaggcagggaagtcctgc
ttccgtgccccaccggtgctcagcagaggctcccttgcaaatgcgaggct
gtttccaactttggtctgtttccctggcaggatgcccgtggccgaggccc
cccaggtggctggcgggcaggggggacggaggtgatggcgaggaagcggag
ccggaggggatgttcaaggcctgtgaggactccaagagaaaagcccgggg
ctacctccgcctggtgcccctgtttgtgctgctggccctgctcgtgctgg
cttcggcgggggtgctactctggtatttcctaggtaacgttgtgggaccg
cctgggagaggcacctggggaggaCTTGGGGTGACTGTAGCAGGC

Primer Melting Temperatures

Forward: 63.4 C tgccgcctgatgttgttactc
Reverse: 64.3 C gcctgctacagtcaccccaag

Exon 3

>chr22:37098305-37098627 323bp GCAGGAGAAGGCATGGAAGAG TCCCTGTGAATGCTCCAGATG
GCAGGAGAAGGCATGGAAGAGacaggagactttcccacctgcttcctcca
ctaaccctgctggttccttcctgggcagggtacaaggcggaggtgatggt
cagccaggtgtactcaggcagtctgcgtgtactcaatcgccacttctccc
aggatcttacccgccgggaatctagtgccttccgcagtgaaaccgccaaa
gcccagaagatggtaggaaaggatctgggggatgagagggagggaatatg
ggggtgaaaagagaggggtggggtctgatcacatggagccagttggtcaa
ccCATCTGGAGCATTCACAGGGA

Primer Melting Temperatures

Forward: 63.6 C gcaggagaaggcatggaagag
Reverse: 63.5 C tccctgtgaatgctccagatg

Exon 4

>chr22:37096502-37096802 301bp AGTAGGAGCAAAGGGCACCTC GACATGCAGGAAGCCAAGTTC
AGTAGGAGCAAAGGGCACCTCcaggtaggaagtgggcctgtctgccctgc
agagggtcccaggggctgttgtcttcccttctcacagctcaaggagctca
tcaccagcacccgcctgggaacttactacaactccagctccgtctattcc
tttgggtgagttgtccttgcccctgaccagctcctgcaagaagctgagat
tcaaagaatgggaggggcctctgtaggcttctgatgcaatgccttcatgt
ttcaaatggggaaactaaggcatagagaggGAACTTGGCTTCCTGCATGT
C

Primer Melting Temperatures

Forward: 62.0 C agtaggagcaaagggcacctc
Reverse: 62.1 C gacatgcaggaagccaagttc

Exon 5

>chr22:37095838-37096215 378bp CTTCTGCGTGAAGACGGACAG GGCCACACCACAGCTTGTTTC
CTTCTGCGTGAAGACGGACAGcaatggctgaggtgcggaaagtgctcgat
gtgcatgagcaggggtggagctggggccagacctcagaatccttccctgg
cctctctcacttctgcctgccttagggagggacccctcacctgcttcttc
tggttcattctccaaatccccgagcaccgccggctgatgctgagccccga
ggtggtgcaggcactgctggtggaggagctgctgtccacagtcaacagct
cggctgccgtcccctacagggccgagtacgaagtggaccccgagggccta
gtgatcctgggtcagtactgcgagtggaaacgtggggttggcctcatgag
gttggggGAAACAAGCTGTGGTGTGGCC

Primer Melting Temperatures

Forward: 63.4 C cttctgcgtgaagacggacag **Reverse:** 65.2 C ggccacaccacagcttgtttc

Exon 6

>chr22:37095447-37095701 255bp AGACAAGGCTGGCTCCAAGG CCCTGCACACACAACAGAAGC
AGACAAGGCTGGCTCCAAGGgtctcctctaattttattttcattgtattt
tcttttctttttttttttttttgttcttgtttatttgtttgttcatttcc
ttttatcagaagccagtgtgaaagacatagctgcattgaattccacgctg
ggtacgctatttttttttccccctccccatttttccttttgagttggcattt
gtcttgactttgttgtgtatcaggggggacacatgGCTTCTGTTGTGTGTG
CAGGG

Primer Melting Temperatures

Forward: 64.0 C agacaaggctggctccaagg **Reverse:** 64.2 C ccctgcacacacaacagaagc

Exon 7

>chr22:37089439-37090022 584bp AGGCGTGAAGCTCAGTGTGTG CTAGCCGTCCTGTCTCCCAGA
AGGCGTGAAGCTCAGTGTGTGtccattccatgagtgaatgactgaaccaa
tgaacaaatgcatgaatgaggatactgacagggaaagagaaggatggggt
agagcatgtctggctatccccacccggctcccctgcccagcccatcctgc
ctggtggaggaccttgagggacctggctccccagggtcccctccttctgg
ctcacaggaatcaggggctgtgcccctctccccgctccaggttgttaccg
ctacagctacgtgggccagggccaggtcctccggctgaaggggcctgacc
acctggcctccagctgcctgtggcacctgcagggccccaaggacctcatg
ctcaaactccggctggagtggacgctggcagagtgccgggaccgactggc
catgtatgacgtggccgggcccctggagaagaggctcatcacctcgtgag
tccctgggaaggagggcaggagggagggctggaaaagggagtggttgatg
ggggagttgaaagtcacacacagcattcttagacaagggagggtaggacc
ttgggcctgggtaTCTGGGAGACAGGACGGCTAG

Primer Melting Temperatures

Forward: 63.9 C aggcgtgaagctcagtgtgtg **Reverse:** 64.0 C ctagccgtcctgtctcccaga

Exon 8

>chr22:37086161-37086524 364bp GATGTCCAGACTCCCGTCCAC GAATCTTCCCTCTCCCCATCC
GATGTCCAGACTCCCGTCCACcctcccccagcagccgcctgccgccctcc
ctgccactcccctcccagaccccagcccagccttgccacctttctgttct
gccagggtgtacggctgcagccgccaggagcccgtggtggaggttctggc
gtcgggggccatcatggcggtcgtctggaagaagggcctgcacagctact
acgacccccttcgtgctctccgtgcagccggtggtcttccagggtgagagg
tcaggggtccctggggcaggggagggggtggtggtagaatccaagggccct
ccactgggctcactgctcacctttttttgcccaaattgaggatgGGATGGG
GAGAGGGAAGATTC

Primer Melting Temperatures

Forward: 64.2 C gatgtccagactcccgtccac **Reverse:** 63.3 C gaatcttccctctccccatcc

Exon 9

>chr22:37084486-37084943 458bp ATTTGCTGGCAGAGGTGGTAG GGAAACACAGAATCCCAGGTG
ATTTGCTGGCAGAGGTGGTAGgggtggtggggttacaagctgcgccagcc
tgctgggaggtgcagccaggggacccctgtgtaacagctgctctcctggt
ccagcctgtgaagtgaacctgacgctggacaacaggctcgactcccaggg
cgtcctcagcaccccgtacttccccagctactactcgccccaaacccact
gctcctggcacctcacggtgagaccccaccctgcctgcccacctgccctc
tgccgcaagcacactacaggtccctggtgacccgggatgagaggggggcag
tgtcccgcctctgctgaagcgcccacaggctgagccctgggtacacatcc
tgccagggtggagagggctgtgggcgaggtctccctctgtgggtcacagc
aatgcctgtttgttgagtgactgacagactttagcccCACCTGGGATTCT
GTGTTTCC

Primer Melting Temperatures

Forward: 61.6 C atttgctggcagaggtggtag **Reverse:** 61.7 C ggaaacacagaatcccaggtg

Exon 10

>chr22:37084192-37084478 287bp TGTTGTTAGGGAGGTGGGTTCAC GAGATTGGGGACTTGGGCTTC
TGTTGTTAGGGAGGTGGGTTCACcaacctggccacaccccatgggccacc
tgatggcccgctcctccctcccaggtgccctctctggactacggcttggc
cctctggtttgatgcctatgcactgaggaggcagaagtatgatttgccgt
gcacccagggccagtggacgatccagaacaggaggtaccacttcctctcc
tccctctggcttcctttcctccctccccctccctctcttccctcctcaat
agtgacccccctcattgGAAGCCCAAGTCCCCAATCTC

Primer Melting Temperatures

Forward: 64.4 C tgttgttagggaggtgggttcac **Reverse:** 64.3 Cgagattggggacttgggcttc

Exon 11

>chr22:37075063-37075418 356bp AGGGAGAAATCAGGGCAGAGG CCTTGGTGGTTCCAGGGATG
AGGGAGAAATCAGGGCAGAGGaggcacagcgggcgtgcagctccccctgc
ccctctggctgcctgtccttgctctgtctgtgcacgggacccaggagacc
agccagtgcagccctgagtcgtctctgactccccccaggctgtgtggctt
gcgcatcctgcagccctacgccgagaggatccccgtggtggccacggccg
ggatcaccatcaacttcacctcccagatctccctcaccgggcccggtgtg
cgggtgcactatggcttgtacaaccagtcggaccgtgagtatgggcagcc
gggggaacccccctgcagtgactcgctgcctcttggcCATCCCTGGAACCA
CCAAGG

Primer Melting Temperatures

Forward: 64.1 C agggagaaatcagggcagagg **Reverse:** 64.9 C ccttggtggttccagggatg

Exon 12

>chr22:37074428-37074950 523bp GCCACAAGGGTTTGCAGGAAT GAGGCTGCATTGCTGGTCTGT
GCCACAAGGGTTTGCAGGAATacacactcatacatatccacgtgtgtgtt
gtgtattctgtgtgtgtgtctggatatgtatgtctgcttggactgtgtac
acaggtgcccaggaccacgtctgtgggtgcctgtccatgcgcgtgtgagt
gaacaggtgcatgcgtgtctttgtgcgccttcgcggctacgcatggccta
atggcgccctgcctgcctccacggtcccctgtggttttgcagcctgccct
ggagagttcctctgttctgtgaatggactctgtgtccctgcctgtgatgg
ggtcaaggactgccccaacggcctggatgagagaaactgcggtgagtaac
ccgcccgcgcatccctcctctccctgcccatcccttctccttcctcacct
ttcctgctctgagctgagtggagaccccacttctacatgcagcttccatt
atgagcacccaggaagtggggttctctcactgtgccggggtggcaaaatg
agACAGACCAGCAATGCAGCCTC

Primer Melting Temperatures

Forward: 65.5 C gccacaagggtttgcaggaat **Reverse:** 65.1 C gaggctgcattgctggtctgt

Exon 13

>chr22:37073466-37073750 285bp GTGATTGGTAACGTGCAATACAGC
TGAAGCATGTAGCAGGCCTAGA
GTGATTGGTAACGTGCAATACAGCacaccttctacaggcatcgccaagcc
ccggctggctcctctggcttcctcccacctgtcccctctctgtgtcccca
cacagtttgcagagccacattccagtgcaaagaggacagcacatgcatct
cactgcccaaggtctgtgatgggcagcctgattgtctcaacggcagcgac
gaagagcagtgccaggaaggtagggcaggcctagccgagtgtctggaggg
acaccaaaggcagTCTAGGCCTGCTACATGCTTCA

\

Primer Melting Temperatures

Forward: 62.8 C gtgattggtaacgtgcaatacagc **Reverse:**62.3 Ctgaagcatgtagcaggcctaga

Exon 14

>chr22:37070836-37071130 295bp CTCTTCTGGCTCCATCGTTCC TGAGATTTCCCTCCAGCTTCC
CTCTTCTGGCTCCATCGTTCCtgcaccccactttggaaggtttccttggg
gctcagagacccaccttctgtgccctgcccccatcccctctgtcccaggg
gtgccatgtgggacattcaccttccagtgtgaggaccggagctgcgtgaa
gaagcccaacccgcagtgtgatgggcggcccgactgcagggacggctcgg
atgaggagcactgtggtgagccctgcctggctgccggggccctggagctt
gggagggaggggtgcccacagcaGGAAGCTGGAGGGAAATCTCA

Primer Melting Temperatures

Forward: 63.5 C ctcttctggctccatcgttcc **Reverse:** 62.9 C tgagatttccctccagcttcc

Exon 15

>chr22:37070376-37070774 399bp TCTCCCCCTCCATCATTCTCC CCACCACCCTTCCCTCTATCTG
TCTCCCCCTCCATCATTCTCCtgttctctgtctctccatctctttccttt
gcccttcctctctgtctgcttctccccttcccctcctcctctgtccaccc
caccacctgccccccatccccagactgtggcctccagggcccctccagccg
cattgttggtggagctgtgtcctccgagggtgagtggccatggcaggcca
gcctccaggttcggggtcgacacatctgtgggggggccctcatcgctgac
cgctgggtgataacagctgcccactgcttccaggaggacaggtgagcggg
agggtgtgggggcctaggcagtaagagacaagggcagggaaggcccggtg
ggaggtgcactgtgtctgagctctttgCAGATAGAGGGAAGGGTGGTGG

Primer Melting Temperatures

Forward: 64.8 C tctccccctccatcattctcc
Reverse: 64.2 C ccaccacccttccctctatctg

Exon 16

chr22:37068933-37069503 571bp ACCACCAGCTAGGCGACCTTC GCCCAATTTGAATCCCAGCAC
ACCACCAGCTAGGCGACCTTCagggagggaagggagggctgggtgtggag
ggcactgtcccgggcggggatctggctatcttgagggtccctggatgggg
agaggcagcttcctcccacctcacctcaccccaccccaccccaccccacc
ccaccccagcatggcctccacggtgctgtggaccgtgttcctgggcaagg
tgtggcagaactcgcgctggcctggagaggtgtccttcaaggtgagccgc
ctgctcctgcacccgtaccacgaagaggacagccatgactacgacgtggc
gctgctgcagctcgaccacccggtggtgcgctcggccgccgtgcgccccg
tctgcctgcccgcgcgctcccacttcttcgagcccggcctgcactgctgg
attacgggctggggcgccttgcgcgagggcggtgagcagcggggacttgc
ggcgggaggcggagggagaccgtgcggatctgcgccgtaacacctggcct
ggagaagggcggggctgggggtcccggggctccaccccataggccctcta
GTGCTGGGATTCAAATTGGGC

Primer Melting Temperatures

Forward: 65.0 C accaccagctaggcgaccttc
Reverse: 65.9 C gcccaatttgaatcccagcac

Exon 17

>chr22:37066743-37067079 337bp GTGGGCAGAGCAGGAGAGAAG GATGTGAGCAAAGGGCCAGAC
GTGGGCAGAGCAGGAGAGAAGtaggctcctgagatgcaaagggaataatg
ttagggagaatagagaacaggggctccaggctcctgagatctcacttctg
cccttgaccacggacaggccccatcagcaacgctctgcagaaagtggatg
tgcagttgatcccacaggacctgtgcagcgaggtctatcgctaccaggtg
acgccacgcatgctgtgtgccggctaccgcaagggcaagaaggatgcctg
tcaggtgagtcccccgggcatgggagggagagaggagggagaaaggatgc
tgcccacatcaccaggGTCTGGCCCTTTGCTCACATC

Primer Melting Temperatures

Forward: 63.8 C gtgggcagagcaggagagaag
Reverse: 64.3 C gatgtgagcaaagggccagac

Exon 18

>chr22:37065998-37066341 344bp CCCAGTCAATTCCCAACAGTC GAATACTTGTCCCCCTGCTTG
CCCAGTCAATTCCCAACAGTCaggacttaatccctatggctcttcacctg
gaaaaggggtgcccttaccctgcttcagtcctttctccttcccccttt
cagggtgactcaggtggtccgctggtgtgcaaggcactcagtggccgctg
gttcctggcggggctggtcagctggggcctgggctgtggccggcctaact
acttcggcgtctacacccgcatcacaggtgtgatcagctggatccagcaa
gtggtgacctgaggaactgcccccctgcaaagcagggcccacctcctgga
ctcagagagcccagggcaactgcCAAGCAGGGGGACAAGTATTC

Primer Melting Temperatures

Forward: 61.7 C cccagtcaattcccaacagtc
Reverse: 60.9 C gaatacttgtcccccctgcttg

Annex 3

برنامج الماجستير في العلوم الطبية المخبرية

استبيان عن فقر الدم المستعصي للحديد

(جميع الأسئلة الواردة في هذا الاستبيان سرية تماما)

الاسم:----------

العمر (تاريخ الولادة):---------- الجنس:----------

الطول:---------- الوزن:----------

مكان الإقامة :---------- مكان الولادة إذا كان مختلفا:----------

1. يرجى ذكر سبب فقر الدم

---

2. ما هي الفحوصات التي أجريت لتقييم فقر الدم لديك؟ ومتى؟

الفحوصات	التاريخ

3. ما هي نتائج فحوصاتك المخبرية الأخيرة؟ (الرجاء إعطاء التاريخ إن أمكن)

CLINICAL PARAMETERS	RESULTS
RBC	
WBC	
Hg	
MCV	
MCH	
MCHC	
RDW	
Reticulocyte count	
PLT	
Serum Ferritin	
Serum Iron	
Transferrin	

4. هل تأخذ أي أدوية في الوقت الحالي؟ اذا اجبت بنعم، من فضلك وضح.

اسم الدواء	الجرعة	تكرار (كم مرة في اليوم)

---

5. هل تم إدخالك إلى المستشفى من قبل؟ إذا كانت الإجابة نعم، ارجو توضيح السبب.

---

6. هل لديك أي مشاكل صحية (مزمنة) أخرى؟ اذكرها إن وجدت.

---

7. هل تناولت مكملات الحديد عن طريق الفم؟

---

8. هل تم تصحيح فقر الدم بعد تناول مكملات الحديد عن طريق الفم؟

---

9. هل أخذت في أي وقت مضى الحديد عن طريق الوريد؟ إذا كانت الإجابة بنعم، ما الجرعة والتكرار.

---

10. هل تحسن فقر الدم لديك بعد أخذ الحديد في الوريد؟
