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Occurrence of Hereditary Hemochromatosis Among β-Thalassemia Intermediate and β -Thalassemia Minor Subjects In Gaza Strip - Palestine

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بسم الله الرحمن الرحيم

(وقضى ربك ألا تعبدوا إلا إياه وبالوالدين إحساناً إما يبلغن
عندك الكبر أحدهما أو كلاهما فلا تقل لهما أف ولا
تنهرهما وقل لهما قولاً كريماً (24) واخفض لهما جناح
الذل من الرحمة وقل رب ارحمهما كما ربياني صغيراً(25)

سورة الإسراء

In The Name of ALLAH ,
The Most Gracious, The Most Merciful

"And your Lord has decreed that you worship none but Him. And that you be dutiful to your parents. If one of them or both of them attain old age in your life, say not to them a word of disrespect, nor shout at them but address them in terms of honour (24) And lower unto them the wing of submission and humility through mercy, and say: "My Lord! Bestow on them Your Mercy as they did bring me up when I was young (25)."

Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree of the university of other institute, except where due acknowledgment has been made in the text.

Signature	Name	Date
Zayed	zayed Mohmmed Harara	Aug-15-2006

Dedication

To my Great Parents, wife and my sons:
Yazan, Karam, Qosay and Khalil

&

Speacial dedication to my best Friend
Khalil and all Thalassemics in Gaza Strip
and all over the world

Acknowledgements

Firstly and finally Iam grateful to **ALLAH** for helping me an givin me what I want.

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Abstract

Hereditary hemochromatosis (HH) is a panethnic autosomal recessive disease that is more frequently observed in males. If untreated HH can cause heart disease, diabetes milletus, liver cirrhosis and impotence among men.

The Mediterranean anemia “Thalassemia” is one of the chronic inherited diseases characttreized by iron oveload and prevails in the Mediterranean basin countries. The percentage of thalassemia minors in Palestine ranges between 3.0 % to 4.5 %.

This study aims to figure out the occurrence of HH among β–thalassemia intermediate and β–thalassemia minor. The study population consisted of (25) thalassemics intermediate who do not depend on regular blood transfusion, (30) thalassemia minors and (30) normal persons as the control group.

Blood samples collected from the study subjects were tested for serum ferritin, serum iron and total iron binding capacity (TIBC). DNAs extracted from the samples were genotyped for the two *HFE* gene common mutations C282Y and H63D by PCR-RFLP technique.

The results of the study showed that all the control subjects were normal in terms of serum ferritin, serum iron and TIBC and their *HFE* genotype. Biochemical tests of thalassemia minor group showd that 2 (7%) of them have iron overload, while none of them showed HH due to C282Y mutation. Regarding the H63D mutation, PCR-RFLP results showed that 7 (23%) of them are hetreozygous, and that 2 (7%) of them are homozygous. Concering the thalassemia intermediate group, biochemical tests showed that 17 (68%) of them have iron overload, but C282Y mutation was not detected in any of them. The H63D mutation test however, showed that 5 (20%) of them are hetreozygous, and that 3 (12%) of them are homozygous for this mutation.

This study recommends offering the PCR-RFLP technique to all thalasemics, thalassemia minors, and iron overload patients at the early stages of their diagnosis in order to detect or rule out HH due to C282Y and H63D mutations in the *HFE* gene. This can contribute to prescrbing Deferasirox (Exjad®)or Deferoxamine (Desferal®) to those pateints in early stages of the

disease and thus help reducing the future complications of iron overload, besides finding other methods of treatment.

It is worth to mention that this study is the first of its kind in Gaza Strip that investigated the genetic basis of HH and its relation to thalassemia disease.

Key words

Polymerase Chain Reaction (PCR); Restriction Fragment Length Polymorphism (RFLP); Hereditary hemochromatosis (HH); Thalassemia; Gaza.

مدى الاصابة بمرض زيادة الحديد الوراثي عند مرضى الثلاسيميا الوسطي وحاملي صفة الثلاسيميا الصغري في قطاع غزة – فلسطين

الملخص

- يعتبر مرض زيادة الحديد الوراثي من الامراض الوراثية الشائعة حيث ينتشر عند الرجال و في حال عدم العلاج يكون أحد المسببات لامراض القلب والسكر وتليف الكبد والعجز الجنسي ، وكذلك تعتبر أنيميا البحرالمتوسط (الثلاسيميا) من الامراض الوراثية التي تنتشر في دول حوض البحر المتوسط حيث تبلغ نسبة حاملى الصفة في فلسطين (3 – 4.5%) .

- تهدف هذه الدراسة الى معرفة مدى الاصابة بمرض زيادة الحديد الوراثي عند مرضى الثلاسيميا الوسطي وحاملي صفة الثلاسيميا الصغري وشملت هذه الدراسة على (25) مريض ثلاسيميا وسطي ممن لا يعتمدون على نقل الدم المستمر و(30) مريض ثلاسيميا صغري و(30) شخص كعينة ضابطة (أشخاص طبيعين).

- تم جمع عينات الدم اللازمة و فحصت باستخدام تقنية PCR - RFLP والفحوصات البيوكيميائية مثل فحص مصل الفرتين ومصل الحديد ومجمل كثافة إرتباط الحديد وكذلك نسبة تشبع الخلايا بالحديد.

-خلال الدراسة لم يسجل أي شخص من العينة الضابطة بمرض زيادة الحديد الوراثي وذلك بناءا على نتائج الفحوصات البيوكيميائية و تقنية PCR - RFLP لتحديد الطفرات الوراثية C282Y و H63D . بينما تم تسجيل 2 (7%) من مرضى الثلاسيميا الصغري لديهم زيادة حديد بناءا علي الفحوصات البيوكيميائية، في حين لم تسجل أية حالة مصابة بمرض زيادة الحديد الوراثي الناتج عن الطفرة الوراثية C282Y و أما بالنسبة للطفرة الوراثية H63D فتم تسجيل 7 (23%) شخص من هذه العينة يحملون هذه الصفة لمرض زيادة الحديد الوراثي، بينما تم تسجيل 2 (7%) مرضى بزيادة الحديد الوراثي . أما بالنسبة لمرضي الثلاسيميا الوسطي فقد تم تسجيل 17 (68%) لديهم زيادة حديد بناءا علي الفحوصات البيوكيميائية، في حين لم تسجل أية حالة مصابة بمرض زيادة الحديد الوراثي الناتج عن الطفرة الوراثيهC282Y و أما بالسبة للطفرة الوراثية H63D فتم تسجيل 5 (20%) مريض يحملون هذه الصفة لمرض زيادة الحديد الوراثي، بينما تم تسجيل 3 (12%) مرضى بزيادة الحديد الوراثي.

- توصي هذة الدراسة بإجراء تطبيق تقنيةPCR - RFLP لتحديد الطفرات الوراثية C282Y و H63D والخاصة بمرض زيادة الحديد الوراثي علي جميع مرضى الثلاسيميا العظمي و الوسطي و الصغري في فلسطين منذ تشخيص المرض حتي يتسني تقديم علاج طرح الحديد (إكسجيد) أو (الديسفرال) في مراحل مبكرة وإعطاءة بالطرق الصحيحة و كذلك إيجاد طرق أخري للعلاج.

- يشار إلي أن هذه الدراسة هي الاولي والتي تجري في قطاع غزة للكشف عن مدي الاصابة بمرض زيادة الحديد الوراثي وعلاقتة بمرض الثلاسيميا.

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Abbreviations

bp: Base pair

DNA: Deoxyribonucleic acid

dNTPs: Deoxyribonucleotide triphosphates

EDTA: Ethylenediaminetetraacetic acid

g: gram

GEL: Gelatin liquifaction

HH: Heraditary Hemochromatosis

IDA: Iron Deficiency Anemia

µl: MicroLiter

mg: Milligram

$MgCl_2$: Magnesium chloride

PCR: Polymearse Chain Reaction

RFLP: Restriction Fragment Length Polymorphism

rpm: round per minute

rRNA: Ribosomal RNA

S. F: Serum Ferritin

S. I: Serum Iron

TfR: Transferrin Receptor

TIBC: Total Iron Binding Capicty

Tris: Hydroxymethyl aminomethane

TS: Taransferrin Saturation

WHO: World Health Organization

Chapter 1
Introduction

The term hemochromatosis was originally used by Recklinghausen in 1889, to describe tissue injury caused by the increased level of iron **(Adams P. et al, 2000).** There are two types of this disease : Hereditary (primary) hemochromatosis, and acquired (secondary) hemochromatosis

1.1. Hereditary Hemochromatosis

Hereditary hemochromatosis is inherited in an autosomal recessive manner **(Powell I. et al, 1998)**. Iron overload in hereditary hemochromatosis is attributable to excessive dietary iron absorption. Increased absorption leads to accretion of toxic levels of iron in multiple organs, which causes organ damage and dysfunction and as a result it leads to hepatomegaly, congestive heart failure, evidence of gonadal dysfunction, loss of body hair, arthritis, or any combination of these manifestations **(Bulaj ZJ. et al, 1996)**. There are four types of hereditary hemochromatosis.

In 1996, the *HFE* gene was idintified as a candidate gene for hereditary hemochromatosis type 1 **(Feder N. et al, 1996).**

The most common mutation described is a G to A transition at nucleotide 845, which substitutes a tyrosine for a cystenie at amino acid position 282 in the *HFE* gene **(Bomford A., 2002),** hence the name C282Y mutation.

Two other mutations H63D and S65C have been described in some cases of hemochromatosis. The H63D mutation is a C to G transversion at nucleotide postion 187, which substitutes an aspartic acid for histidine at amino acid 63 **(Tannapfel A. et al, 2001).** The S65C mutation is an A to T substitution at nucleotide postion 193, resulting in cysteine replacing serine at amino acid 65 **(Mura C. et al, 1999).**

Homozygosity for the C282Y mutation is the most important of the *HFE* mutations, although the H63D and S65C mutations can contribute to disease (but substantially less than C282Y). Carriers of a single copy of either gene

have a very slight risk of hemochromatosis when other factors contribute, but are otherwise healthy.

The screening tests for confirming hemochromatosis include the followings:

1- Serum iron concentration
2- Total iron Binding Capacity (TIBC).
3- Transferrin Saturation which is calculated as the ratio of Serum Iron concentration to TIBC.
4- Serum ferritin.
5- Genotyping for detection of C282Y and H63D mutations **(Girouard J. et al, 2001).**

1.2. Acquired Hemochromatosis

This is an intestinal abnormality accompanying other diseases, such as thalassemia.

1.2.1. Thalassemia

Thalassemia is a heterogeneous group of disorders in which there is a genetically determined reduction in the rate of synthesis of one or more types of normal hemoglobin polypeptide chains **(Weatherall D., 1996a)**. If the reduction is in α–chain synthesis, the resulting condition is termed **α-** thalassemia, but if the reduction involves β–chain synthesis then the resulting condition is called β–thalassemia **(Weatherall D., 1991)**.

Thalassemias are very common in the Mediterranean and the equatorial or near the equatorial regions of Africa and Asia, and they are found in a broad belt extending from the Mediterranean shores and Arabian peninsula to India and South eastern Asia **(Schwartz & Benz, 1995).**

In Gaza Strip we have 290 confirmed β-Thalassemia patients **(Thalassemia center, 2005)** who can be divided into two groups: the first group consists of 245 thalassemia major who are dependent on regular blood transfusions and the second group of 45 individuals constitutes thalassemia intermediate, 20 of them rely on irregular blood transfusion (about 4-9 times per year), while the remaining 25 have not been on blood transfusion for up to ten years. In addition, about (3.0 - 4.5 %) of the total

population in Gaza Strip are carriers for β-thalassemia (thalassemia minor) who do not receive transfusion at all **(Sirdah M. et al, 1998).**

β- thalassemics (all the Major and some of the Intermediate) take daily DESFROXAMIN (Desferal®) to excrete the overloaded iron from their bodies **(Eleftheriou A., 2000a).** Many of them suffer from complications like: hepatosplenomegaly, congestive heart failure, diabetes mellitus, erectile dysfunction and hypogondism **(Lewis M., 2001).**

Thalassemics intermediate rarely depend on blood transfusion but they take Desferal daily and have a very high ferritin level and so do thalassemia minor subjects.

Many of the thalassemia minor and all of the thalassemia intermediate who are not on blood transfusion in Gaza Strip are suspected to have iron-overload **(Thalassemia center, 2005)**.

1.3. Objectives of the study

This study was conducted in order to investigate the nature of hemochromatosis in a group of thalassemia intermediate and thalassemia minor subjects.

The specific objectives are:

- Confirm diagnosis of hemochromatosis.
- Apply PCR – RFLP in diagnosis.
- Distinguish hereditary hemochromatosis from acquired.
- Draw attention of healthcare providers to the genetics of hemochromatosis.
- Contribute to the treatment options.

Chapter 2
Literature Review

2.1. Iron

Iron is the most plentiful heavy metal in the earth's crust (**Monsen E., 1988**), it is required by almost all organisms (**Roy & Enns, 2000**). In the human body, iron is required by and present in all cells and has several vital functions (**Pipard M., 1996**). Iron functions as a carrier of oxygen from the lungs to the tissues in the form of hemoglobin (Hb), as a facilitator of oxygen utilization in the muscle tissues as myoglobin, as a transport medium for electrons within the cells in the form of cytochromes, and as an integral part of heme (a protoporphyrin ring that contains an iron atom) enzymes that catalyze fundamental chemical reactions in different tissues (**Center for Disease Control and Prevention, 1998**).

2.2. Body iron and distribution

Iron is an essential nutrient for human. The human body normally contains an average of approximately 3.8 g in men and 2.3 g in women of iron **(Bothwell T., 1995)** which is equivalent to 50 mg/kg body weight for healthy 75 kg adult man and 42 mg/kg body weight for healthy 55 kg adult female **(Bothwell T. *et al,* 1979; Bothwell & Charlton, 1981).**

Iron is present in the human body in the form of iron containing-compounds and consequently iron could be classified into functional iron and storage or transport iron according to the biological role of the iron-containing compound. The higher percentage > 70 % of iron may be classified as functional iron, while the remaining is storage or transport iron **(Pipard M., 1996).**

2.2.1. Iron storage

Ferritin and hemosiderin are the two main protein compounds that serve the functions of iron storage, they contain about 20 % of body iron and are involved in the maintenance of iron homeostasis. The iron storage compounds, ferritin and hemosiderin, are present primarily in the liver, the reticuloendothelial cells, and erythroid precursors of the bone marrow.

2.2.1.1. Ferritin

Ferritin is the primary iron storage protein and provides a reserve of iron. Ferritin is found in almost all cells of the body. However, in the hepatocytes and the macrophage system of the bone marrow, ferritin provides a reserve of iron readily available for formation of hemoglobin and other iron-containing proteins and enzymes **(Burtis & Ashwood, 1994).**

Ferritin is found not only inside the cells, but also is circulating in plasma. Although its function is unclear, however, plasma levels of ferritin have been used as an index of iron storage deficiency **(Bender & Bender, 1997).**

2.2.1.2. Hemosiderin

Hemosiderin is another iron storage complex. Its molecular nature remains poorly defined, but it is always found within cells (as opposed to circulating in blood) and appears to be a complex of ferritin, denatured ferritin and other materials. It arises by partial enzymatic hydrolysis of ferritin in lysosomes **(Brock J., 1989).**

The iron within deposits of hemosiderin is, at best, very poorly available when needed. At normal levels of iron in the liver and spleen (around 9 μmol/g tissue), ferritin is the predominant iron storage protein, and there is little or no haemosiderin. However, as the iron level increases above 18 μmol/g tissue, the percentage of hemosiderin increases **(Bender & Bender, 1997).**

2.2.2. Iron Transport Compounds

Transferrin and the transferrin receptor are the other two proteins in this category and they are involved in the transport, delivery and regulation of iron uptake to the different tissues **(Pipard M., 1996).**

2.2.2.1. Transferrin

Transport of iron from one organ to another is accomplished by a plasma transport protein known as transferrin.

Transferrin is a monomeric glycoprotein of molecular weight about 80,000 Dalton **(Duffy T., 1996),** with the capacity to bind two ferric ions very tightly, but reversibly, concomitantly with two carbonate anions. As the iron loading of transferrin is largely random, transferrin circulates as diferric, monoferric, or apo-transferrin with no iron bound **.**

The extent to which transferrin is saturated with iron provides a sensitive index of iron nutritional status, and is reflecting the pool of iron that is available and being transported between storage and utilization sites **(Bender & Bender, 1997).** Serum transferrin provides a translocative function, transporting iron from sites of absorption and storage to sites of utilization. Transferrin is also found within the cytosol of many cells and may serve as an intracellular iron transport protein **(Hirose M., 2000).**

2.2.2.2.Transferrin Receptor

Transferrin receptor (TfR) is a transmembrane glycoprotein dimer 185,000 Dalton that serves as a gatekeeper in mediating and regulating cellular uptake of iron from the plasma glycoprotein, transferrin **(Punnonen K., 1997).**

The TfR assists iron uptake into the cells through a cycle of endo- and exocytosis of the iron transport protein transferrin. Iron uptake from transferrin involves the binding of iron loaded (diferric) transferrin to TfR, internalization of transferrin within an endocytic vesicle by receptor-mediated endocytosis, and the release of iron from the protein by a decrease in the endosomal pH **(Testa U. et al, 1993; Ponka & Lok, 1999).**

After iron release from transferrin within endosomes, iron passes through the endosomal membrane via the endosomal Fe^{2+} transporter, known as the divalent cation transporter1 (DCT1) **(Fleming & Andrews, 1998).**

After its release from endosomes, iron is transported to intracellular sites of use and/or storage, and the iron-free transferrin (apo transferrin) that remains receptor-bound returns to the cell surface where apotransferrin is released from the cells (at extracellular pH) and replaced by diferric transferrin from serum **(Lawrence C. et al, 1999).** With the exception of mature erythrocytes and some other terminally differentiated cells, TfR are probably expressed on all cells, but their levels vary greatly with immature erythroid cells showing very high densities of TfR. Erythroid heme synthesis, which uses more than 80% of iron leaving plasma, is critically dependent on iron uptake mediated by high levels of TfR in erythroid cells. In addition to immature erythroid cells, TfR is present in highest concentration on cells with high iron requirements, including the placenta, and on rapidly dividing cells both normal and malignant **(Ponka & Lok, 1999).**

2.3. Iron Metabolism

Body iron is normally vigorously conserved and utilized. Our bodies have limited ability to absorb and excrete iron. In specific contrast with other body constituents, the control of iron content is imposed by limiting its entrance into the body rather than by increasing the excretion of any excess. Iron absorption must therefore be regulated in a way to balance the small unavoidable losses (approximately 1 mg /day mainly from exfoliation of skin and gastrointestinal cells), and the increased physiological requirements associated with growth, menstruation, and pregnancy, meanwhile avoid any accumulation or overload of iron from dietary sources **(Monsen E., 1988).**

A distinctive feature of iron metabolism is the very small amount of iron that is lost daily from the body once it has been assimilated. The quantity of body iron is normally maintained at each stage of growth and development, mainly by regulation of iron intake. The primary physiologic objective in regulating intake is to keep pace with the fluctuations in iron requirements at different developmental stages **(Nathan & Osaki, 1993).** In the embryonic stage

regulation is accomplished by the placenta, however, after birth regulation of iron absorption is accomplished by the intestinal mucosa **(Dallman P. et al, 1996; Andrews G., 2000a).**

Because most of the body iron is contained in the hemoglobin of the RBCs, recovery from senescent RBCs is very essential. The efficiency of iron absorption is normally regulated in accord with body iron status **(Gavin et al, 1994; Wood & Han, 1998).** The daily requirements of iron for the formation of new RBCs (erythropoiesis) in healthy individuals is about 20 mg. Almost all required iron for erythropoiesis is provided through macrophage ingestion of aged red cells and by the catabolism of their hemoglobin and the reuse of the released iron **(Andrews N., 2000b).**

2.4. Iron absorption

Iron is vital for all mammalian cells, because it is essential for multiple metabolic processes particularly the production of erythrocytes hemoglobin and the production of cellular energy **(Andrews N., 2000a).** However, excessive iron is toxic to all tissues because it produces free radicals that lead to tissue damage. Therefore, tissue iron concentration must be strictly regulated **(Conrad & Umbreit, 2000; 2002).**

Interestingly, the regulation of body iron and maintenance of its homeostasis occurs at the sites of entrance of iron into the body. Only 1-2 mg of iron are normally absorbed through specific mucosal receptors located in the intestinal epithelial cells of the gastro-duodenal junction, the absorbed amount of iron is balanced by iron output from shed cells **(Andrews N., 2000b).** Intestinal absorption is the primary mechanism regulating iron concentrations in the body of adults. Signals to alter iron absorption are received from the body by cells of the intestinal crypt, which in turn migrate up the villus and differentiate into mature absorptive enterocytes **(Anderson G., 1996).**

2.5. Disorders of Iron Metabolism

The disorders of iron metabolism could be classified into two categories: iron deficiency anemia (IDA) and hemochromatosis (iron overload) **(Andrews N., 1999a).**

2.5.1. Iron Deficiency Anemia

IDA is the most common nutritional deficiency worldwide **(WHO, 1992).** It occurs when body iron stores become inadequate for the needs of normal erythropoiesis. Iron deficiency results from an imbalance between iron intake and iron loss **(Monsen A., 1998).**

2.5.2. Hemochromatosis

The term iron overload (Hemochromatosis) refers to any condition in which body iron stores greatly exceed the normal level, which is generally about 1 g in adult men and somewhat less in women, approximately 300 mg **(Mazza J., 2002).**

The first known report of an individual with probable hemochromatosis was published in 1865 in a discussion of diabetes mellitus. The second known case of hemochromatosis was reported in 1871 in a discussion of bronze melanin skin pigmentation of the face and dorsa of hands. The constellation of bronzed skin, hepatic cirrhosis, and diabetes mellitus was referred to as pigmentation cirrhosis in 1882 and as bronzed diabetes in 1886 **(Richared L. et al, 1993).** While the term hemochromatosis was originally used by Von recklinghausen in 1889, to describe tissue injury caused by increased level of iron **(Adams P. et al, 2000).** A modern definition of hemochromatosis describes it as an autosomal recessive disorder of iron metabolism, characterized by inappropriately high absorption of iron by the gastrointestinal mucosa, leading to excessive storage of iron (particularly in the liver, skin, pancreas, heart, joints and testes) and ultimately resulting in impaired organ structure and function **(Kowdley K. et al, 2003).** There are two types of this disease:

1-Hereditary (primary) hemochromatosis: is a hereditary disease where the intestines lack the normal ability to keep out the available but unneeded dietary iron. Patients suffering from this disease take in the iron,

but have problems excreting it. The excess is therefore placed into storage. It has been shown numerous times, that with an increase uptake from the diet of 1 – 3 mg of iron, in a period of 40 – 50 years, the iron will be accumulated in the body. This accumulation especially occurs in the liver and heart, and will eventually lead to necrosis and cardiopathy.

2- Acquired (secondary) hemochromatosis: is an intestinal abnormality occurring with acquired disease, some of those diseases are:

- Anemia and ineffective Erythropoiesis : these diseases may result when a patient receives blood transfusions, but receives them for too long and the iron begins to build up.
- Liver diseases : if a patient has a liver disease, he will not be able to control the iron uptake from the liver and the iron will begin to accumulate.
- High intake of iron : many people are given supplements that they do not need and the iron in their body increases.

Types of Hereditary Hemochromatosis:

Hemochromatosis can be due to mutation in a number of genes *(e.g. HFE, TFR2, ferroportin1/lREG1/MTP1, and H-ferritin)* but in terms of prevalence and phenotype, the most important type is that due to mutations in *HFE*, termed type 1 or hereditary hemochromatosis **(Bomford A., 2002).** Table 2.1 summarizing the four types of hereditary hemochromatosis.

1- Hemochromatosis type 1:

Classic hereditary hemochromatosis is an autosomal recessive disorder of iron metabolism, is caused by mutation in a gene designated *HFE* located on chromosome 6p21.3.

2- Juvenile Hemochromatosis:

Hemochromatosis type 2 *(HFE2),* is a rare autosomal recessive disease, with clinical symptoms appearing in the second and third decades of life, characterized by cardiomyopathy and hypogonadism **(Anderson L.G. et al, 2001)**. One form, designated HFE_2A, is caused by mutation in the gene enoding hemojuvelin which maps to 1q21. A second form, designated HFE_2B,

is caused by mutation in the gene encoding hepcidin antimicrobial peptide which maps to chromosome 19q13.

3- Hemochromatosis type 3:

An autosomal recessive disorder, it is phenotypically similar to type 1 **(Hoffbrand A.V. et al, 2005).** but is due to mutations in the gene enoding transferrin receptor-2 *(TFR$_2$)* which maps to chromosome 7q22.

4- Hemochromatosis type 4:

An autosomal dominant disorder, is caused by missense mutation in the *SLC11A$_3$* gene which encodes ferroportin and maps to chromosome 2q32.

Table 2.1. Types of hereditary hemochromatosis

Type	Gene	Inheritance and phenotype	Severity	Incidence
1	HFE	AR parenchymal, IO	Highly variable	Common
2	Hemojuvelin	AR parenchymal, IO	severe	Rare
3	TFR2	AR parenchymal, IO	severe	Rare
4	Ferroportin	AD parenchymal, IO	variable	Rare
AR: Autsomal Recessive, AD: Autsomal Dominant, IO: Iron Overload				

2.5. 2.1. Hereditary Hemochromatosis type 1

Classic hereditary hemochromatosis (HH) is an autosomal recessive disorder of iron metabolism associated with the human leukocyte antigen (HLA)-A3 complex characterized by increased iron absorption and deposition in the liver, pancreas, heart, joints, and pituitary gland. Iron accumulation leads to injury and organ failure, the most common presenting feature is fatigue. Other clinical features include arthritis, cardiac arrhythmias or heart failure, diabetes mellitus, hepatic cirrhosis, hyperpigmentation, hypothyroidism, hypogondism, and less commonly, hepatocellular carcinoma **(Elaine L. et al, 2001).** Without treatment death may occur from cirrhosis, liver cancer, diabetes, or cardiomyopathy **(Pietrangelo A., 2004).** The clinical disease of hereditary hemochromatosis is usually caused by a homozygous autosomal recessive mutation in the *HFE* gene.

HH was associated with the human leukocyte antigen (HLA)-A3 complex. Subsequently, HH was linked to HLA-A on the short arm of chromosome 6 **“** Figure 2.1**”**. In 1996, *HFE*, the gene for HH, was mapped on the short arm of chromosome 6 (6p21.3)**.** The model of the *HFE* protein based upon its homology with major histocompatbilty complex (MHC) class I molecules. It is a single polypeptide chain with three extracellular domains analogous to the α_1, α_2 and α_3 domains of other MHC class I molecules. In contrast to other members of the MHC class I family, the α_1 and α_2 domains in the *HFE* protein are non-polymorphic. β_2-microglobulin is a separate protein which interacts with the *HFE* protein in a noncovalent manner in the α_3 homologous region. The approximate locations of the Cys282Tyr and His63Asp mutations are indicated. **(Feder J. et al,1996).**

The *HFE* protein is a 343 amino acid residue type I transmembrane protein that associates with class I light chain beta$_2$-microglobulin **“** Figure 2.2**”**. The *HFE* protein product binds to the transferrin receptor and reduces its affinity for iron-loaded transferrin by 5- to 10-fold. The *HFE* gene consists of 7 exons.

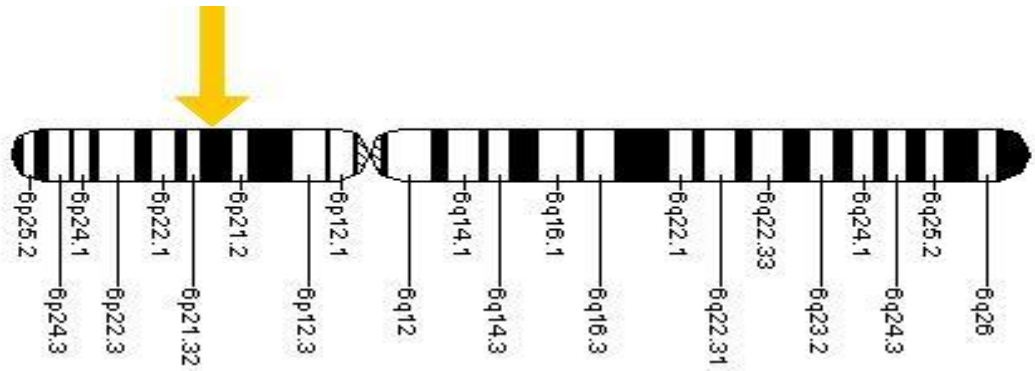

Figure 2.1: The HFE gene is located on the short (p) arm of chromosome 6.

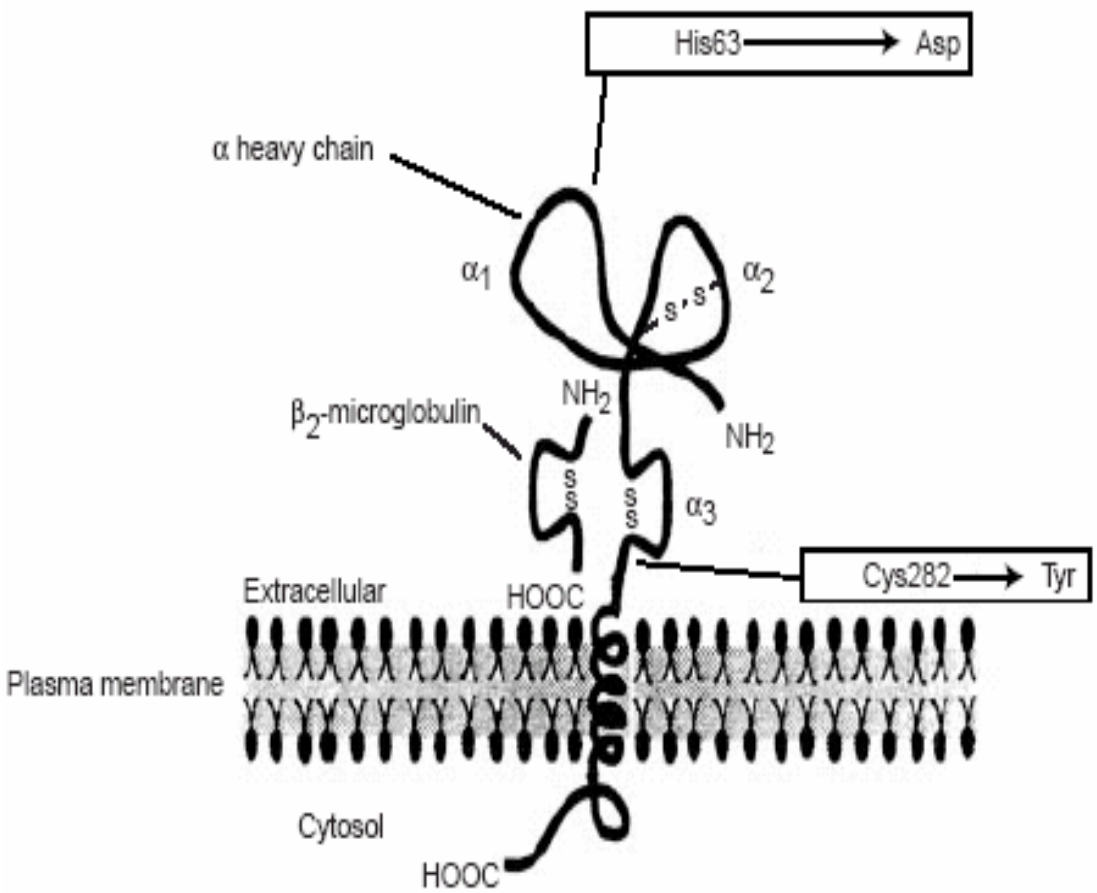

Figure 2.2 : Model of the *HFE* protein based upon its homology with MHC class I molecules.

Approximately 60% to 90% of the cases are the result of defect in exon 4 in the form of a single missense mutation (G-to-A transition) at nucleotide 845 (G845A), which substitutes a tyrosine for a cysteine at amino acid position 282 (C282Y). The C282Y mutant *HFE* protein is unable to bind to beta$_2$microglobulin, with the result being unregulated transferrin receptor-mediated iron uptake in the gut **(Zoller H. et al, 1999).** The prevalence of the homozygous C282Y mutation ranges from 1 in 200 for whites to 1 in 4,000 for those of African-American heritage, the allele frequency in the Caucasian population is 0.063, while was not found in the middle east area **(Alsmadi OA. et al, 2006)**. The association of C282Y homozygosity with hereditary

hemochromatosis is dependent on the population **(Elaine L. et al, 2001). "**Figure 3.3**"** illustrates the frequency of C282Y mutation in various countries.

A second mutation has been found in exon 2 at position 63, described as a C-to-G transition at nucleotide 187 (C187G), where histidine is replaced by aspartate (H63D). The H63D mutation, while able to bind to transferrin receptors, appears to lack the normal high degree of inhibitory effect on the transferrin receptor. Persons homozygous for the H63D mutation and those who are compound hetreozgotes (with the C282Y mutation) have a low rate of phenotypic expression, accounting for approximately 5% and 15% cases of hereditary hemochromatosis respectively **(Robert B., 2001).**

The third mutation has been localized in exon 2 in the vicinity of H63D, described as a A-to-T transition at nucleotide 193 (A193T), where serine has been replaced by cysteine (S65C). It represents 1% to 4% of the cases **(Mura C. et al, 1999).**

The symptoms and signs in patients with symptomatic hemochromatosis are very common in the family practice setting. Common symptoms include weakness, fatigue, arthralgias and arthritis, intermittent abdominal pain, loss of libido, and impotence. The most common symptoms, however, are arthralgias and fatigue **(Powell I. et al, 1998).** Physical and laboratory findings include skin hyperpigmentation, hepatomegaly, evidence of heart failure, testicular atrophy, elevated liver enzymes, hyperglycemia, low testosterone levels and hypothyroidism.

Figure 2.3 : Frequency (%) of the C282Y mutation in various countries or regions in Europe and Algeria.

The symptoms usually begin to appear in the third to sixth decade of life. The hepatic fibrosis resulting from HH as a causative factor is approximately 3% of cases of hepatic cirrhosis and 10% to 30% of cases of hepatocellular carcinoma.

Cardiac involvement occurs in 5% to 50% of the cases. Diastolic dysfunction, dilated cardiomyopathy, atrial and ventricular arrhythmias and conduction abnormalities can also occur **(Robert B., 2001).**

Traditionally, transferrin saturation and serum ferritin have been used to diagnose HH clinically. Measurement of serum iron and total iron binding capacity on a fasting, morning specimen should be performed as the initial test. Transferrin saturation is expressed as the ratio of serum iron concentration and total iron binding capacity. A serum ferritin assay should be performed to evaluate the total body iron content. In the absence of other potential causes of iron overload, persistently increased transferrin saturation (TS ≥50% for premenopausal females and ≥55% for males and post menopausal females) and serum ferritin (SF>200 µg/l for premenopausal females and >300 µg/l for males and post menopausal females) are suggestive of hereditary hemochromatosis **(Burke W. et al, 1998).** A serum TS of 55% has a sensitivity of about 90% in detecting homozygous C282Y males, although most authors recommend TS >60% as a threshold. Although similar in sensitivity to TS, the SF concentration is less specific because increased SF is seen in other disorders as well as in hemochromatosis. In the past, iron overload was confirmed by the detection of increased hepatic iron in a liver biopsy specimen **(Elaine L. et al, 2001).**

Genotypic testing has the advantage of providing a result which is the same at any stage of iron accumulation and is not influenced by dietary intake or tissue damage. However, it is not certain that the majority of people homozygous for the C282Y mutation will eventually develop the clinical condition. People heterozygous for both the C282Y and H63D mutations may also accumulate iron, but the risk of clinical hemochromatosis is much less. Some recommend that testing should be confined to the C282Y mutation, but hetreozgotes should also be tested for the H63D mutation. A brief diagnosis of hereditary hemochromatosis is shown in the following diagram **"**Figure 3.4**"**

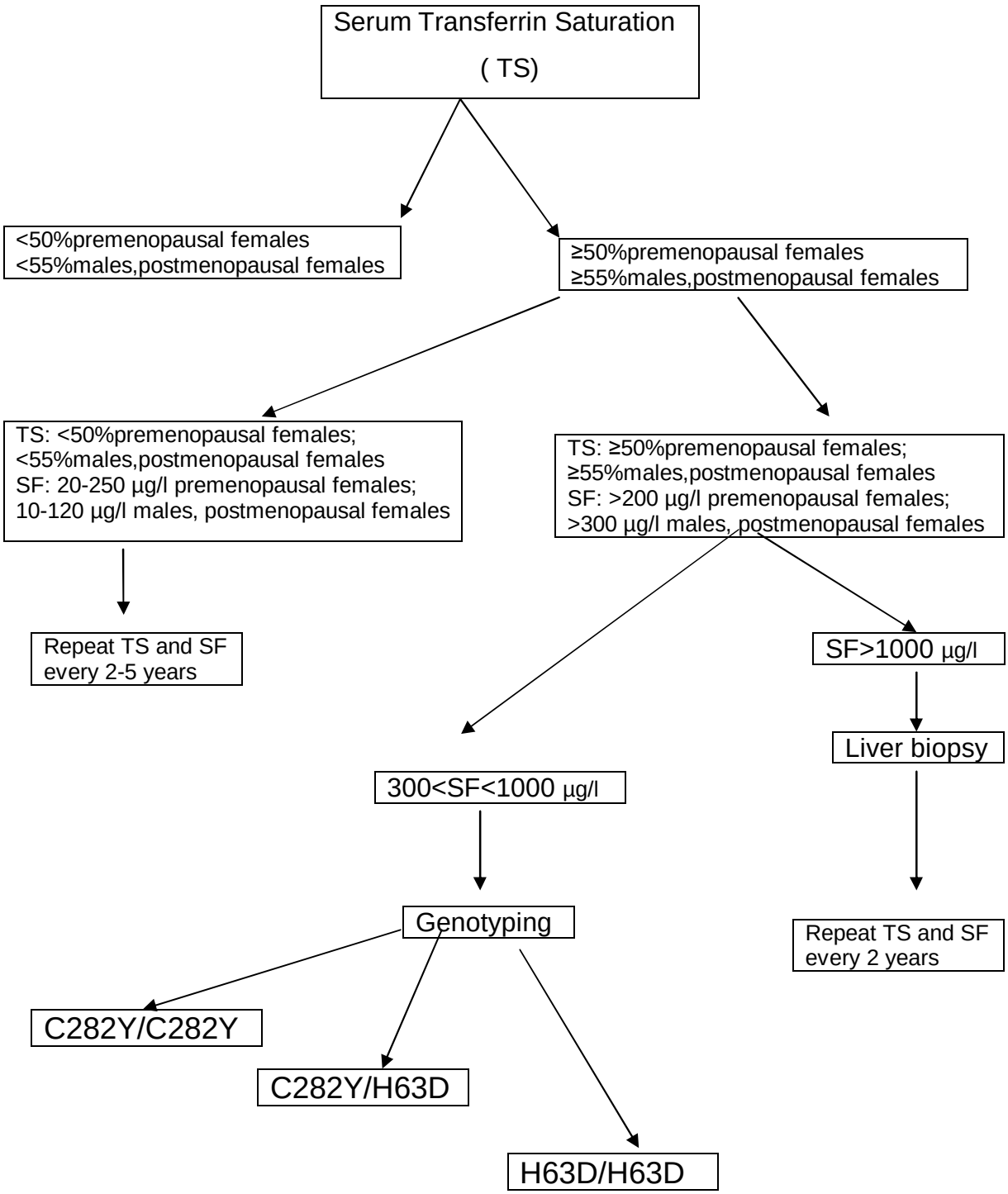

Figure 2.4: Algorithm for screening and diagnosis of hemochromatosis.

Benefits of early treatment include increased survival and prevention of complications **(Witte D. et al, 1996).** Treatment for both symptomatic and presymptomatic patients consists of therapeutic phlebotomy. Initial treatment for symptomatic patients consists of weekly or biweekly phlebotomies of up to one unit (450 mL) of blood, if tolerated. Iron depletion is marked by early iron deficiency anemia with microcytosis and decreased serum TS and SF concentrations. SF concentrations usually normalize before TS does. Iron depletion may require up to 1 to 2 years, depending on the initial iron load. Maintenance therapy consists of phlebotomy at intervals of 2 to 6 months, depending on SF concentrations.

A small proportion of HH patients may have severe anemia resulting from liver failure with bone marrow suppression or unstable cardiac angina that prevent them from tolerating aggressive phlebotomy therapy. These individuals require a different method to treat their iron overload. Chemicals that bind iron and make a water soluble complex can be used for this purpose. Compared to phlebotomy, treatment with iron chelators is slower in the rate of iron depletion and is associated with risks of side effects.

2.6. Thalassemia

The thalassemia syndromes are heterogeneous inherited disorders that arise from mutations in the globin genes that reduce or totally abolish synthesis of one or more of the globin chains. They result in hypochromia and microcytosis and, in the more severe forms, anemia. As a group, they compromise the most common single gene disorders known **(Mazza J., 2002).** If the reduction is in α–chain synthesis, the resulting condition is termed α-thalassemia, but if the reduction involves β–chain synthesis then the resulting condition is called β–thalassemia.

The clinical syndromes can be classified and grouped by simple criteria. Affected individuals are either heterozygous or homozygous for the β-thalassemia gene **(Hillman R., 1985).** Homozygous or compound heterozygous β-thalassemia can either cause dangerously severe anemia (thalassemia major) or cause moderate anemia (thalassemia intermediate). Heterozygotes for each of these types of β-thalassemia have (thalassemia

trait) or (thalassemia minor) or, may be clinically undetectable (thalassemia minima) **(Eleftheriou A., 2000b).**

Whenever both parents carry the abnormal gene for thalassemia, there is a 25% risk in each pregnancy that the child will receive the abnormal gene, and have the severe form of the disease. When only one parent is a carrier of thalassemia, there is no danger of having a sick child. There is, however, a 50% chance that any one of the children will also be a carrier of thalassemia **(Eleftheriou A., 2000b).**

Thalassemia occurs most commonly in people of Mediterranean origin: Turkey, Italy, Greece, Cyprus, Syria, Lebanon and Palestine “Figure 2. 5”.

Types of β-thalassemia:

1- Thalassemia Major is the homozygous or compound heterozygous state for β-thalassemia, affected infants are well at birth. Anemia usually develops during the first few months of life and becomes progressively sever. The first signs of the progressive anemia are pollar, listlessness, and general failure to thrive, and anorexia, diarrhea, loss of body fat, hepatosplenomegaly causes abdominal distention and discomfort, and symptoms due to pressure on surrounding organs, feeding problems, gastrointestinal disturbances, and bouts of fever afflict the patient. As the child grows older, β-thalassaemia major patients are treated by, frequent blood transfusion, splenectomy, iron chelation therapy and bone marrow transplantation. The increase in body iron is due to the combination of frequent transfusions and increased intestinal absorption resulting from ineffective erythropoiesis and chronic anemia. Pancreatic hemosiderosis may cause diabetes and cirrhosis may result from iron deposition in the liver. Haemosiderosis of cardiac muscle leads to arrhythmias, heart block, and chronic congestive heart failure **(Firkin F. et al, 1995).**

2- β-thalassemia intermediate can be produced by at least one dozen genotypes, and consequently the clinical spectrum is broad. All of the clinical misfortunes that happen to patients with β-thalassemia major may occur. β-thalassemia intermediate is treated by, irregular blood transfusion splenectomy, and iron chelation therapy. Iron overload occurs even in untransfused thalassemia intermediate patients because of ineffective

Figure 2.5 : Geographical distribution of β-thalassemia. Colored areas indicate the countries where thalassemia is prevalent.

erythropoiesis, peripheral red cell breakdown and increased intestinal iron absorption. Iron loading secondary to these causes is less accelerated than that associated with transfuional iron overload in transfusion dependent thalassemia patients **(Lewis M. et al, 2001).** Older patients may have the same risk for iron induced hepatic, cardiac and endocrine dysfunction as in patients with thalassaemia major **(Eleftheriou A., 2000b).** Iron chelation therapy with iron chelating agents should be started when the ferritin test result is high.

3. β-thalassemia Minor the carrier or heterozygous state of β-thalassemia. This state is characterized usually by mild anemia, with Hb level falling below 12g/dl. The blood transfusion is not indicated in this trait patients **(Firkin F., 1995).** In many regions, β-thalassemia trait and Iron deficiency are both endemic problems. Females should be forewarned that β-thalassemia minor women became somehow more anemic during pregnancy. Then use of iron therapy in those cases and that they are predisposed to iron overload should be warranted **(Lichtman M. et al, 2003).**

2.6.1. Thalassemia in Gaza Strip :

Palestine is one of the Mediterranean basin countries in which thalassemia disease is prevalent. The average incidence of thalassemia trait in the Gaza Strip and West bank is (3.0 – 4.5%) **(Sirdah M., et al, 1998).** While the number of thalassemia patient's is (750) distributed as (290) patients in Gaza Strip and (460) patients in the West Bank **(Thalassemia Center, 2005).** Concerning patients in the Gaza Strip; they are distributed in all governorates in different rates as shown in the following diagram “Figure 2. 6”.

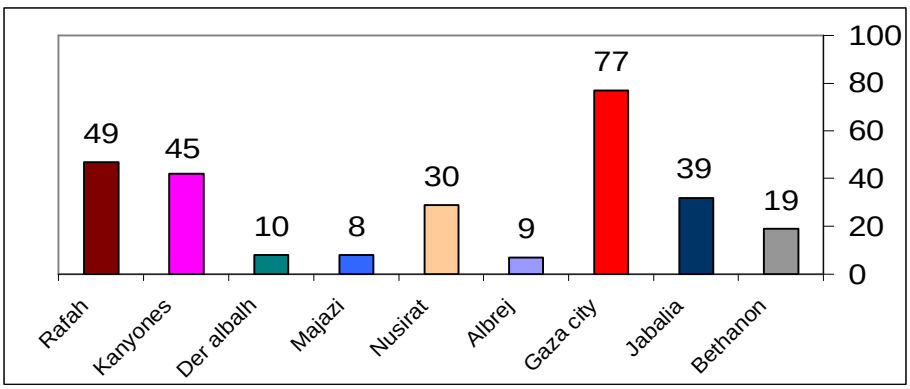

Figure 2.6: Distribution of Thalassemics in Gaza Strip governorates.

Thalassemics get their treatment and health care in three medical centers in Gaza Strip. Thalassaemics living in both Rafah and Khan-Younis are treated at the European Hospital regardless of their age. Adult thalassemics living in Gaza City, Northern and Middle governorates are treated at Al–Shifa Hospital in Gaza City while young thalassemics under the age of (12) are treated at Al-Nasser pediatric Hospital. In Gaza Strip we have 290 confirmed β-thalassemia patients who can be divided into two groups: the first consists of 245 thalassaemia major who are dependent on regular blood transfusion each (2–4) weeks. In order to get rid of iron overload, thalassemics start having *(Desferal)* over the age of (2) years as it is toxic if taken under this age. The second group of 45 individuals constitutes thalassemia intermediate, 20 of them rely on regular blood transfusion (about 4-9 times per year), while the remaining 25 have not been on blood transfusion for up to 10 years.

Chapter 3
Materials and Methods

3.1. Materials

3.1.1. Chemicals

- Agarose Molecular Biology grade (Promega, USA).
- DNA molecular weight marker 100 bp ladder (Promega, USA),
- dNTPs: (dATP, dCTP, dGTP and dTTP) (Promega, USA)
- EDTA disodium salt (Promega, USA).
- Ethidium bromide (Promega, USA).
- Ethanol 70% (Sigma, USA)
- Absolute Isopropanol (Sigma, USA).
- Tris base (hydroxymethyl aminomethane) (Promega, USA).
- Acetic acid (Sigma, USA).
- DNAse, RNAse free Water (Promega, USA).
- $MgCl_2$ (Promega, USA).

3.1.2. Reagent Kits

- Serum iron Kit (Bio-system, Spain)
- Total Iron Binding Capacity (TIBC) Kit (Bio-system, Spain)
- Serum ferritin microparticle enzyme immunoassay kit (Abbott laboratories, USA.)
- Wizard Genomic DNA purification Kit (Promega, USA).
- PCR kits (Promega, USA.)

3.1.3. PCR Primers

Primers were purchased from Operon, USA

1- For C282Y:

The sequence of the primer was as defined by **(Girouard J. et al, 2001).**

Forward **5'- CAATGGGGATGGGACCTACC –3'**

Reverse **5'- CACTGATGACTCCAATGACT –3'**

2- For H63D:

The sequence was deduced from the sequence of *HFE* (NCBI) using primer 3 software (MIT)

Forward **5'- AAGGCCTGTTGCTCTGTCTC –3'**

Reverse **5'- GCTCCCACAAGACCTCAGAC –3'**

3.1.4. Enzymes

- ***Taq Polymerase*** (Promega, USA),
- ***SnaB1*** Restriction enzyme (NEB, USA)
- ***BspH1*** Restriction enzyme (NEB, USA)

3.1.5. 50x TAE Buffer

Composition:

Tris base ……. 242 g

glacial acetic acid ……. 57.1 ml

EDTA ……..18.6 g

H_2O to ……. 1000 ml

pH 8.0

3.1.6. Ethidium bromide (stock solution)

Ethidium bromide 10 mg/ml in water.

3.1.7. DNA loading buffer

Composition:

bromophenol blue 0.25 g

xylene cyanol 0.25 g

glycerine 30 ml

H_2O 70 ml

3.1.8. Instruments and Disposables

The following instruments were used in the present study:

- Abbott Axsym immunoassay analyzer (Abbott Laboratories, USA),
- Alcyon 300 (Abbott Laboratories, USA), immunoassay analyzer.
- Thermal Cycler (Ependorff).
- Centrifuge.
- Water Bath.

§ L.G. Microwave Oven .
§ Hoefer Short wave UV Light Table (Transilluminator)
§ Digital Camera
§ Vortex Mixer.
§ Electrophoresis Tank
§ Power Supply (Bio-Rad)
§ Micro Centrifuge
§ Freezer , Refrigerator
§ Incubator
§ Spectrophotometer UV-Vis
§ Weight Mod.
§ Microscope
§ Microfuge tubes for PCR - thin wall 0.2 mL and 0.5 mL capacity.
§ Microfuge tubes - 1.5 mL capacity.
§ Pipettes (Ependorff).
§ Computer

3.2. Methods

3.2.1. Ethical Consideration

An authorization to carry out the study was obtained from **Helsinki** (Declaration of Helsinki the most widely accepted guideline on medical research involving human subjects) using an agreement letter prepared from the Islamic University of Gaza.

3.2.2. Blood Samples

The blood samples tested in the present study were collected from 85 subjects from both sexes. Venous blood samples (9 mL) from anticubital vein were collected in one EDTA vacuette tubes (3 mL) and two serum vacuette tubes (3 mL each).

The sampling was performed at 8 o'clock in the fasting morning. The blood in the EDTA vacuette tubes was used within 3 – 4 hours of collection for DNA Isolation, and the DNA isolates were stored at 2 – 8 ^{0}C for later PCR amplification.

The blood collected in the serum vacuette tubes was used to determine serum iron, total iron binding capacity (TIBC), and ferritin. The serum was separated 2–3 hours after blood sampling by centrifugation at 3000 rpm for 10 minutes. The separated serum was placed in three plain tubes, sealed and stored at 2–8 ^{0}C to be used in the next day for serum iron, TIBC and serum ferritin analysis. Transferrin saturation was calculated as the ratio of serum iron concentration to total iron binding capacity.

3.2.3. Study population

The study population consisted of three groups

Group 1: All the 25 thalassemics intermediate who are registered at the thalassemia Center and who do not depend on regular blood transfusion. They showed high ferritin level despite having (Desferal), but do not respond to treatment.

Group 2: 30 thalassemia minor cases who do not depend on blood transfusion at all.

Group 3: 30 Healthy subjects (non- thalassemics). They were selected randomly and were considered as the control group.

3.2.4. Serum Iron

Principle of the test is that, the iron in the serum is dissociated from it's Fe (III) transferrin complex by the addition of an acidic buffer containing hydroxylamine which reduces the Fe (III) to Fe (II). Then the addition of the chromogenic agent causes, ferrine to form a highly colored ferric complex that can be measured by spectrophotometery **(Stooky L., 1970, Itano, 1978, Artiss et all,1981).**

Procedure:

- The regents were brought to room temp.
- The blank, sample and standard tubes were prepared by mixing the components shown in Table 3.1

Table 3.1 The components and volumes for serum iron test.

Component	Blank	Sample	Standard
Distilled water	100 µl	-	-
Sample	-	100 µl	-
Iron Standard	-	-	100 µl
Reagent (A)	1.0 ml	1.0 ml	1.0 ml
Reagent (B)	0.2 ml	0.2 ml	0.2 ml

- The test was made using Alcyon 300 (Abbott laboratories, USA.)

The results were analyzed according to the following reference values from the Bio-system Kit:

- Men : 65 – 175 µg/dl
- Women : 50 – 170 µg/dl

3.2.5. Total Iron Binding Capacity (TIBC)

The principle of the test is that, excess ferric iron is added to the specimen to saturate the transferrin. Remaining ferric iron is absorbed by $MgCO_3$ and precipitated by centrifugation. Protein bound iron in the

supernatant is the TIBC which can be assayed in the same manner as in iron determination **(Kunesh and Small, 1970, Tietz N.W., 1995)**

Procedure:

- Combine 1000µl of serum with 2000µl of iron (reagent 1) and the Mixture was incubated for 10 minutes at room temperature.
- Approximately 300 – 350 mg of $MgCO_3$ were then added and the mixture was incubated for 30 minutes at room temperature.
- Then it was centrifuged for 10 minutes at 3000rpm.
- The supernatant was collected.
- The iron concentration was determined as described above.

Calculation

Iron concentration in the supernatant **(**µg/dl) X 3 (dilution factor) = Serum TIBC**(**µg/dl).

The results were analyzed according to the following expected values from the Bio-system kit:

250 – 400 µg/dl

3.2.6. Transferrin Saturation (TS)

TS is calculated from the ratio of serum iron concentration to total iron binding capacity **(Mazza J., 2002).**

Transferrin Saturation = SI/TIBC X 100%

The normal percentage in normal individuals 15% but in hemochromatosis is >50%

3.2.7. Serum Ferritin

In the present study serum ferritin was determined using a Microparticale Enzyme Immunoassay Technology. For this purpose Abbot full – automated Axsym immunoassay analyzer ferritin assay system (Abbott laboratories, USA.) was used.

The obtained results were categorized according to the following expected values:

Adult males (18 – 30 years) : 18 – 323 ng/ml

Adult males (31 – 60 years) : 16– 293 ng/ml

Adult females (Premenopauseal) : 7 – 282 ng/ml

Adult females (Postmenopausal) : 14 – 233 ng/ml

3.2.8. DNA Extraction

After numerical coding of the patient's samples, DNA was extracted from the whole blood samples by using **Wizard Genomic DNA** Purification Kit **(Promega, USA.)** which contains:

1- Cell Lysis Solution.
2- Nuclei Lysis Solution.
3- RNase Solution.
4- Protein Precipitation Solution.
5- DNA Dehydration Solution.

Procedure of Extracting DNA from Blood

- Add 900 µl of cell lysis to 300 µl of whole blood in 2 ml tubes and mixed (5–6) times, incubated for 10 min. and inverted (2–3) times. Samples were then centrifuged at 13.000 rpm for 20 seconds. The supernatant was discarded and the pellet was vortexed for 10 to 15 seconds.
- 300 µl of nuclei lysis solution was added to the tube and mixed by the pipette for (5–6) times till the solution became very viscous.
- Add1.5 µl of RNase solution to the tube, mixed by a pipette (2–3) times. The mixture was incubated at 37 ^{0}C for 15 min. and the tube was cooled at room temp.
- Add 100 µl of protein precipitation solution to the tube, vortexed for (10–20) sec. Then centrifuged at 13.000 rpm for 3 min.
- In fresh 2ml tube 300 µl of Isopropanol was added at room temp.
- The supernatant was added to the tube, gently mixed by inversion until the white threads of DNA were visible.

- The DNA threads were centrifuged at 13.000 rpm for 1 min. as the DNA was visible like a small white pellet.
- The supernatant was discarded by up-downing the tube. 300 µl of 70% ethyl alcohol were added to wash the pellet and DNA was collected by centrifugation at 13.000 rpm for 1 min.
- The supernatant was discarded by up-downing the tube at absorbent paper and the tube was left to dry for (10–15) min at room temp.
-Add100 µl of DNA Rehydration solution to the tube and the tube was incubated at 65 ^{0}C for one hour.
- After confirming the presence of good quality DNA, by agarose gel electrophoresis the sample was stored at (4-8) ^{0}C.

3.2.8.1 Detection and quantation of extracted DNA

3.2.8.1.a. Agarose gel electrophoresis

The quality of the isolated DNA was determined by running 5 µl of each sample on ethidium bromide stained 1.0% agarose gels and the DNA sample was visualized on a short wave U.V. transilluminator and the results were documented by photography.

3.2.8.1.b. Spectrophotometery

The optical density (O.D.) at 260 nm of diluted fractions of the isolated DNA samples was measured by a spectrophotometer and the DNA concentration was calculated by considering 1 $O.D._{(260\ nm)}$ = 50 µg/ml DNA and taking into account the dilution factor.

3.2.9. Polymerase Chain Reaction (PCR) for C282Y

PCR was carried out in a total volume of 25 µl, the reaction component were as described in Table 3.2 below.

Table 3.2 The components and concentration of a PCR reaction for C282Y.

Reagents	PCR reaction mixture		
	Initial concentration	Volume (µl)	*Final concentration*
PCR buffer	10 X	2.5	1 X
$MgCl_2$	25 mM	2.5	2.5 mM
dNTPs	200 mM	0.2	2.0 mM
Primer (1):- 5'- CAATGGGGATGGGACCT ACC –3'	100 µM	0.3	2 µM
Primer (2):- 5'CACTGATGACTCCAATG ACT –3'	100 µM	0.3	2 µM
Taq DNA Polymerase	5 U/uL	0.2	1.0 U
Template DNA		2.0	150 ng
Water		16.95	

Temperature cycling program

The thermal cycler program was set as follows :

Step 1: Denaturation for 15 min at 95 º C

Step 2: Melting for 45 sec at 95 ºC

Step 3: Annealing for 30 sec at 57 ºC

Step 4: Extension for 20 sec at 72 ºC

Step 5: from Step 2 to Step 4 (30 cycle)

Step 6: Final elongation for 7 min at 72 ºC

PCR product (340 bp) was analyzed by electrophoresis on ethidium bromide stained 2.0% agarose gel. The gel was visualized with UV illumination and photographed. DNA molecular size standards (l00 bp ladder, Promega) were included in each agarose gel.

3.2.10. Restriction Fragment Length Polymorphism (RFLP) by *Snab1* for C282Y:

RFLP was carried out in a reaction mixture in a final volume of 30µl containing :-

- PCR product (20 µl)
- Buffer 3X (3 µl)
- BSA (0.2 µl)
- *Snab1* enzyme 2.5 U (0.5 µl)
- Nuclease Free Water (6.3 µl)

After incubation at 37 ^{0}C for 120 min., the digest was run on 3% agarose gel. The non-digested product (340bp), indicate a normal allele whereas digested products (229bp and 111bp) confirm the presence of mutation, as shown diagrammatically in Figure 3.1.

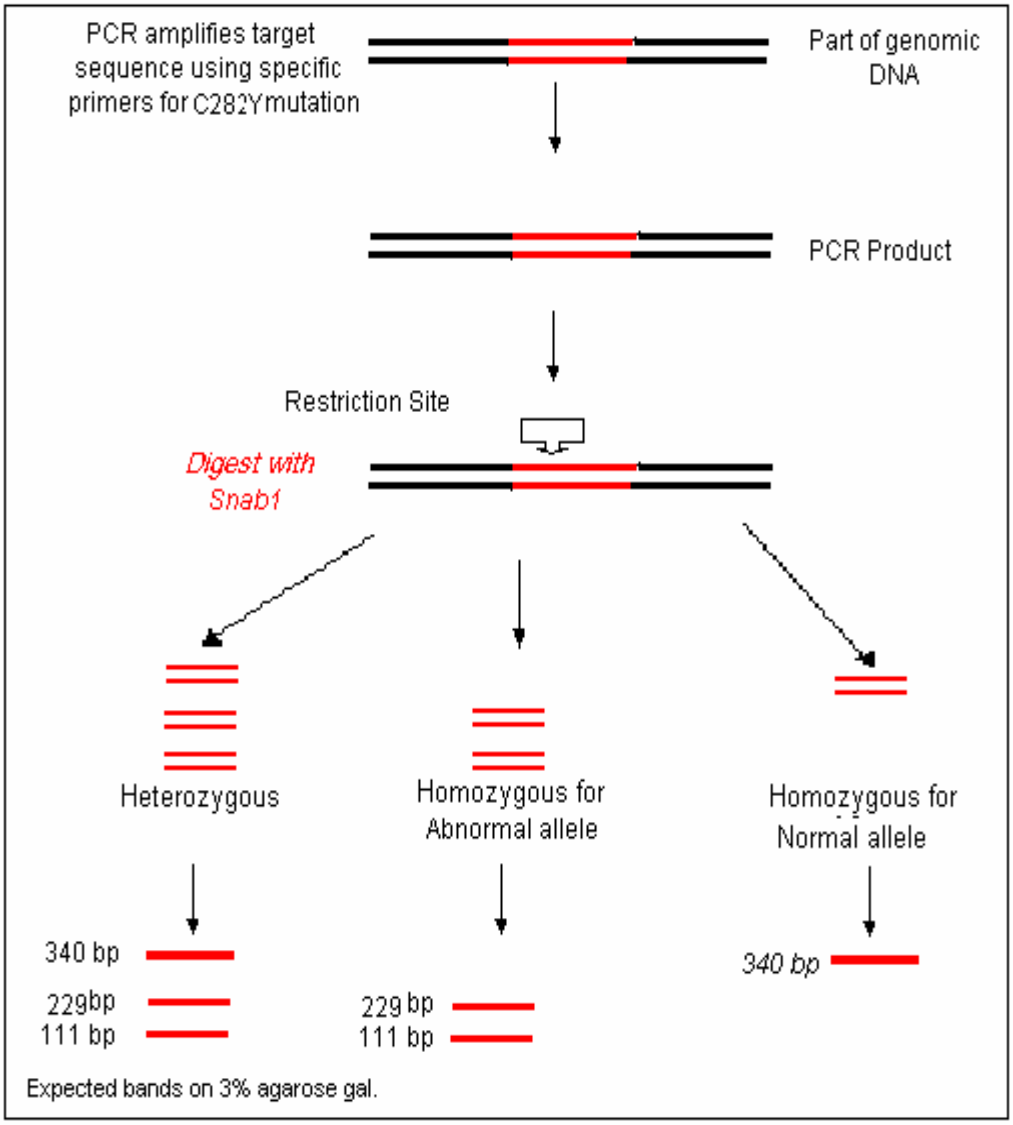

Figure 3.1 RFLP diagram for C282Y.

3.2.11. Polymerase Chain Reaction (PCR) for H63D

PCR was carried out in a total volume of 25 µl, the reaction component were as described in Table 3.3 below.

Table 3.3 The components and concentration of a PCR reaction for H63D.

Reagents	PCR reaction mixture		
	Initial concentration	**Volume (µl)**	***Final concentration***
PCR buffer	10 X	2.5	1 X
$MgCl_2$	25 mM	2.5	2.5 mM
dNTPs	200 µM	0.25	2.0 µM
Primer (1):- 5'CAATGGGGATGGGACC TACC –3'	100 µM	2.0	8 µM
Primer (2):- 5'GCTCCCACAAGACCTCA GAC –3'	100 µM	2.0	8 µM
Taq DNA Polymerase	5 U/uL	0.3	1.5 U
Template DNA		2.0	150 ng
Water		13.45	

Temperature cycling program

The thermal cycler program was set as follows :

Step 1: Denaturation for 15 min at 95 º C

Step 2: Melting for 45 sec at 95 ºC

Step 3: Annealing for 30 sec at 58 ºC

Step 4: Extension for 20 sec at 72 ºC

Step 5: from Step 2 to Step 4 (30 cycle)

Step 6: Final elongation for 7 min at 72 ºC

PCR products were analyzed by electrophoresis on 2.0% (w/v) agarose gel containing ethidium bromide. The gel was visualized with UV illumination and photographed. DNA molecular size standards (l00 bp ladder, Promega) were included in each agarose gel. The expected amplicon is (388 bp).

3.2.12. Restriction Fragment Length Polymorphism (RFLP) by *Bsph1* for H63D

RFLP was carried out in a reaction mixture in a final volume of 30µl containing :-

- PCR product (20 µl)
- Buffer 3X (3 µl)
- *Bsph1* enzyme 2.5U (0.3 µl)
- Nuclease free water(6.7 µl)

After incubation at 37 ^{0}C for 120 min., the digest was run on 3% agarose gel. Normal allele PCR products upon *Bsph1* digestion should yield 250 bp and 138bp fragments, while mutant allele is not digested. Therefore, normal homozygous individuals should produce two fragments 250 bp and 138 bp, heterozygous individuals must show three fragments 388 bp, 250 bp and 138 bp, while mutant homozygous individuals should produce a single 388 bp product, as show in the diagram below (Figure 3.2).

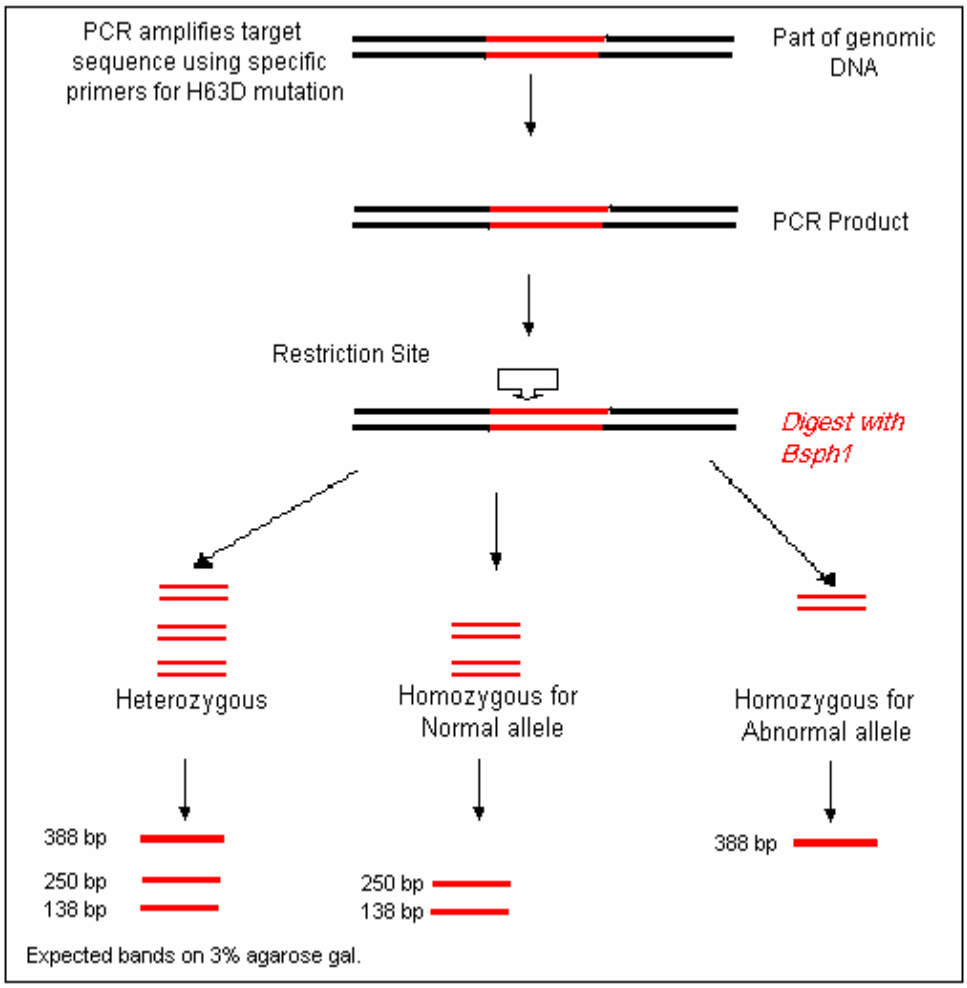

Figure 3.2 RFLP diagram for H63D.

Chapter 4
Results

Our study has focused on occurrence of HH in β-thalassemia intermediate and β-thalassemia minor subjects in Gaza Strip – Palestine, in comparison to normal individuals.

The results of the study can be summarized as follows:

4.1. Biochemical Analysis

4.1.1. S. Ferritin, S. Iron, TIBC and % TS in the Normal Group

The control group consisted of 30 healthy subjects (23 males and 7 females) aged (18–35) years. Table 4.1 illustrates the S. ferritin, S. iron, TIBC, and TS values for the control group.

According to the results presented in Table 4.1, all the control individuals values (S. ferritin, S. Iron, and TIBC) were in the normal ranges and none of the subjects had iron overload.

Correlation analysis of the results in this group, the correlations between (TS and TIBC), (S. ferritin and S. iron), and (S. ferritin and TS) were not significant in control group.

Table 4.1. The Biochemical tests (S.F, S.I, TIBC and TS) results and analysis of variables for the normal group.

No.	Gender	S. ferritin ng/ml	S. Iron µg/dl	TIBC µg/dl	% TS.
1	M	158.1	90.0	215.9	41.6
2	M	61.2	85.0	272.9	31.1
3	F	35.0	39.0	249.0	15.0
4	M	45.6	107.0	273.0	39.1
5	M	58.6	63.0	228.0	27.6
6	M	109.5	73.0	436.4	16.7
7	M	63.8	84.0	263.4	31.8
8	F	1.9	46.0	258.6	17.8
9	F	28.2	90.0	265.8	34.0
10	M	39.6	68.0	306.0	22.2
11	M	3.6	123.0	342.3	36.0
12	M	189.6	98.0	296.7	33.0
13	M	140.0	70.0	364.2	19.2
14	M	59.1	116.4	368.7	31.8
15	M	18.4	163.2	140.1	16.4
16	M	75.4	139.6	332.7	41.0
17	M	69.9	125.3	295.9	42.0
18	M	34.3	78.4	252.0	31.0
19	F	17.8	41.6	461.7	10.0
20	M	46.9	114.0	260.4	44.0
21	M	26.8	158.0	367.3	43.0
22	F	24.2	160.0	357.0	44.0
23	F	91.9	79.0	240.0	32.9
24	M	53.3	108.0	327.0	33.0
25	M	11.4	103.0	396.0	26.0
26	M	56.8	116.0	366.0	31.6
27	F	110.0	40.0	310.0	13.0
28	M	60.0	124.0	323.4	38.0
29	M	43.8	92.0	333.0	27.0
30	M	32.1	122.0	372.0	32.0
Mean		58	97.2	309	30.1
+/- SD		44.8	34.6	68.6	9.89
95% CI for the mean		36.2 – 81.6	84.6 - 109.8	278.7 – 339.6	25.8 - 34.3
Normal Range		7-293	50-175	250-400	<15

4.1.2. S. Ferritin, S. Iron, TIBC and % TS in the β-Thalassemia Minor Group

In this group all the subjects were diagnosed by a qualified physician as β-thalassemia minor. This group consisted of (30) subjects, 22 males and 9

females ranging in age between 20 and 50 years. The results of biochemical tests are presented in Table 4.2. Depending only on the results of TS, only 2 (7%) of the subjects in this group (highlighted in the Table) showed iron overload.

Table 4.2. The Biochemical test (S.F, S.I, TIBC and TS) results and analysis of variables of the β-thalassemia minor group.

No.	Gender	S. ferritin ng/ml	S. Iron µg/dl	TIBC µg/dl	% TS
1	M	350.3	155.0	374.1	41.4
2	F	78.3	91.0	297.3	30.6
3	M	119.7	94.0	321.0	29.2
4	F	40.3	94.0	447.0	13.0
5	M	146.7	75.0	284.1	26.3
6	F	12.7	68.0	344.1	20.0
7	M	135.0	64.0	170.7	37.5
8	M	49.4	70.0	435.9	16.0
9	F	20.1	75.0	163.8	46.0
10	M	120.4	123.0	230.7	53.3
11	M	128.6	82.0	241.2	34.0
12	F	9.1	56.0	376.8	15.0
13	M	60.7	39.1	110.1	35.5
14	F	22.0	31.9	429.9	7.5
15	M	74.3	133.2	257.5	51.7
16	M	37.2	33.0	429.6	7.6
17	M	72.2	89.3	250.8	36.0
18	M	113.1	43.1	373.0	11.5
19	M	30.5	126.4	266.0	47.0
20	M	81.9	110.8	536.8	20.0
21	M	262.1	144.3	331.0	43.0
22	M	69.7	88.0	343.0	36.2
23	M	53.6	70.0	354.0	19.7
24	M	150.1	73.0	354.0	20.6
25	M	53.6	74.0	363.0	20.3
26	F	161.8	121.0	360.0	33.6
27	F	152.7	71.0	369.0	19.0
28	M	130.3	128.0	285.	45.0
29	M	59.0	94.0	390.0	24.0
30	F	5.5	148.0	486.0	30.0
Mean		93.4	88.8	333	29.0
+/- SD		75.6	34.0	95.8	13.1
95% CI for the mean		70.7 – 116.1	76.2 – 101.4	302.1 – 363.0	24.7 - 33.2
Normal Range		7-293	50-175	250-400	<15

Statistical analysis of the results in this group showed negative significant correlation (P < 0.01) between (TS and TIBC). This relation was reached using the t-student test (Figure 4.1).

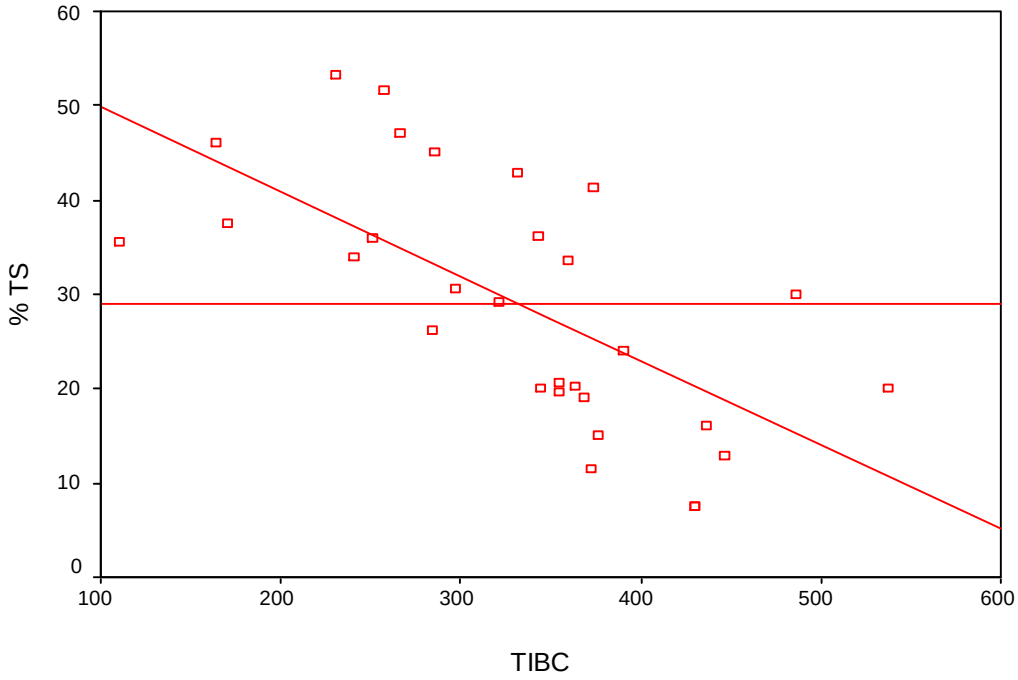

Figure 4.1. Correlation between TIBC and TS in the β-thalassemia minor group.

However, the correlations between (S. ferritin and S. iron), and (S. ferritin and TS) were not significant in this group.

4.1.3. S. Ferritin, S. Iron, TIBC and %TS in the β-Thalassemia Intermediate Group

This group consisted of 25 individuals;15 males and 10 females aged 12 to 38 years. All the subjects in this group were diagnosed by a qualified physician as β-thalassemia intermediate. The subjects in this group however, do not rely on regular blood transfusion.

Table 4.3. The Biochemical tests (S.F, S.I, TIBC and TS) results and analysis of variables of the β-thalassemia intermediate group.

No.	Gender	S. ferritin ng/ml	S. Iron µg/dl	TIBC µg/dl	% TS
1	M	809.1	119.0	248.7	48.0
2	F	1190.6	242.0	309.3	78.3
3	F	237.3	174.0	261.0	66.6
4	F	313.4	62.0	197.3	32.0
5	M	296.4	51.0	242.9	21.0
6	F	147.4	88.0	280.5	31.5
7	M	2.3	57.0	380.6	15.0
8	M	1750.4	159.0	263.7	60.5
9	M	210.6	47.0	361.2	13.0
10	M	265.5	157.0	297.2	59.0
11	M	542.9	165.0	175.8	94.0
12	M	2700.1	266.0	272.7	72.4
13	F	950.6	210.0	282.9	74.5
14	M	64.4	88.0	429.0	19.0
15	F	804.1	175.0	351.0	50.0
16	F	883.8	288.5	400.0	57.0
17	M	1069.0	288.0	294.0	97.0
18	F	857.2	111.0	168.9	65.1
19	M	540. 7	78.8	219.0	26.0
20	M	4075.8	160.1	234.0	68.0
21	M	1172.2	121.8	180.0	67.6
22	M	3957.9	215.0	219.0	98.1
23	M	1558.9	295.0	336.0	87.7
24	F	3296.6	185.0	288.0	64.2
25	F	2228.0	180.0	210.0	86.0
Mean		1197.3	159	276	58.1
SD		1085.0	77.3	70.7	26.4
95% CI for mean		867.8 – 1517	136.1 – 182.6	248.2 – 304.0	50.3 – 65.7
Normal Range		7-293	50-175	250-400	<15

In this group, depending on the TS 17 (68%) individuals showed iron overload. Table 4.4 shows the biochemical tests results for those individuals.

Table 4.4. The Biochemical tests (S.F, S.I, TIBC and TS) results and analysis of variables of the β-thalassemia intermediate group subject with iron overload.

No.	Gender	S. ferritin ng/ml	S. Iron µg/dl	TIBC µg/dl	% TS
1	F	1190.6	242.0	309.3	78.3
2	F	237.3	174.0	261.0	66.6
3	M	1750.4	159.0	263.7	60.5
4	M	265.5	157.0	297.2	59.0
5	M	542.9	165.0	175.8	94.0
6	M	2700.1	266.0	272.7	72.4
7	F	950.6	210.0	282.9	74.5
8	F	804.1	175.0	351.0	50.0
9	F	883.8	288.5	400.0	57.0
10	M	1069.0	288.0	294.0	97.0
11	F	857.2	111.0	168.9	65.1
12	M	4075.8	160.1	234.0	68.0
13	M	1172.2	121.8	180.0	67.6
14	M	3957.9	215.0	219.0	98.1
15	M	1558.9	295.0	336.0	87.7
16	F	3296.6	185.0	288.0	64.2
17	F	2228.0	180.0	210.0	86.0
Mean		1620	199.5	267.2	73.2
SD		1217	57.7	64.1	14.6
95% CI for mean		994.3 – 2245.8	169.8 – 229.2	234.2 – 300.2	65.7- 80.8

Statistical analysis of the results in this group showed a significant correlation ($P < 0.01$) between (S. ferritin and S. iron), (S. ferritin and TS). These relations, as revealed by the t-student test, are illustrated in Figures 4.2 and 4.3.

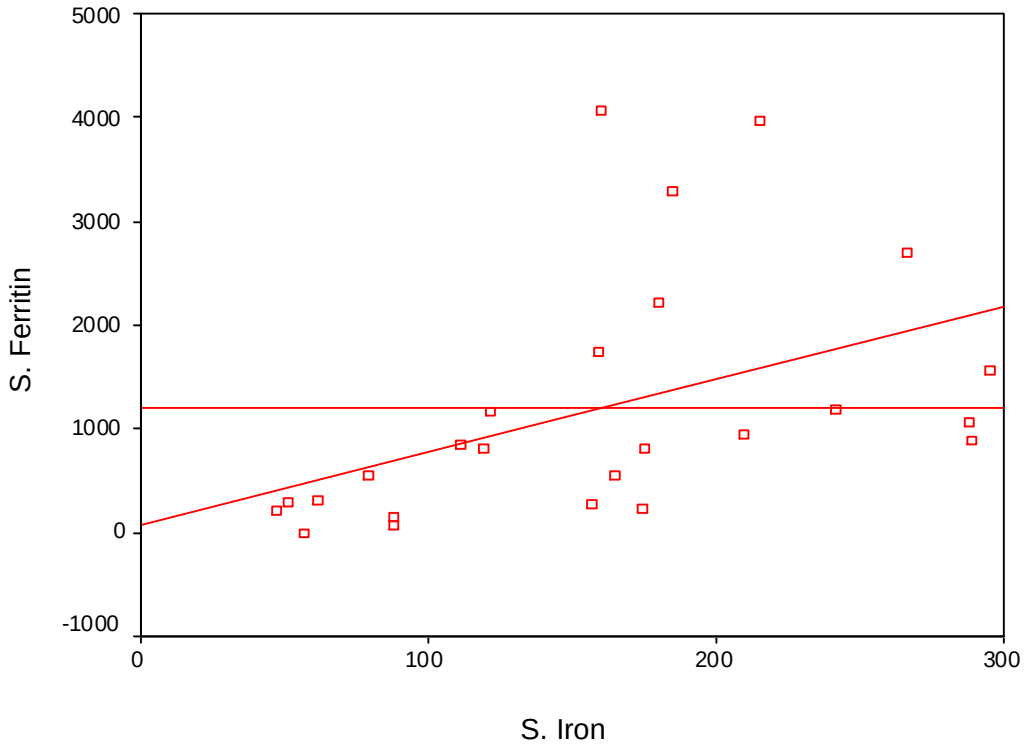

Figure 4.2. Correlation between S. Ferritin and S. Iron in the β-thalassemia intermediate group.

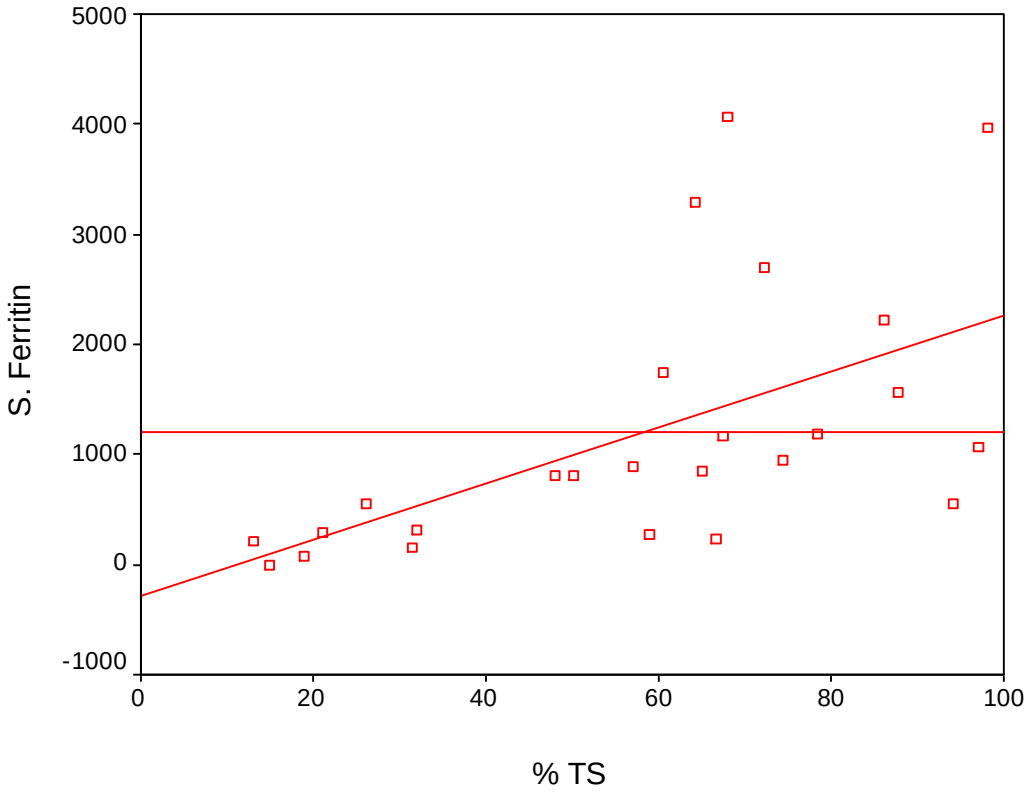

Figure 4.3. Correlation between S. Ferritin and %TS in the β-thalassemia intermediate group.

Table 4.5 summarizes the number and percentage of subjects with hemochromatosis in the three study groups.

Table 4.5. Number and percentage of subjects with hemochromatosis.

Group	No. of hemochromatosis	%
Control	0/30	0
β-thalassemia Minor	2/30	7
β-thalassemia Intermediate	17/25	68

4.2. Genotyping Analysis

4.2.1. DNA extraction

The integrity of the DNA was evaluated by running 5 µl of the DNA extracts on ethidium bromide stained 1% agarose gel, and then visualized on a UV transilluminator (Figure 4.7). DNA concentration was also measured spectrophotometrically at 260nm.

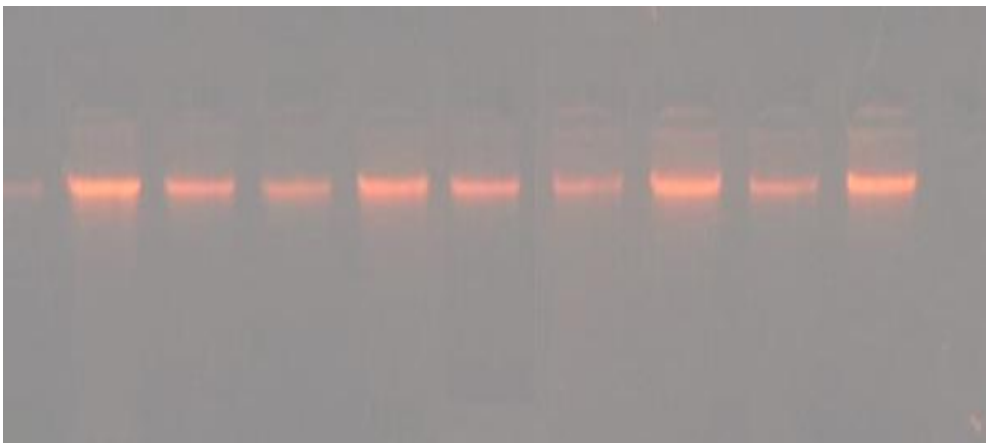

Figure 4.4. A representative photograph of DNA samples extracted from whole blood samples run on ethidium bromide stained 1% agarose gel.

4.2.2. PCR Results

The following figures (**4.8** and **4.9**) represent the PCR results for C282Y, and H63D. The amplicons of 340bp for C282Y, and 388bp for H63D, are indicated in the figures.

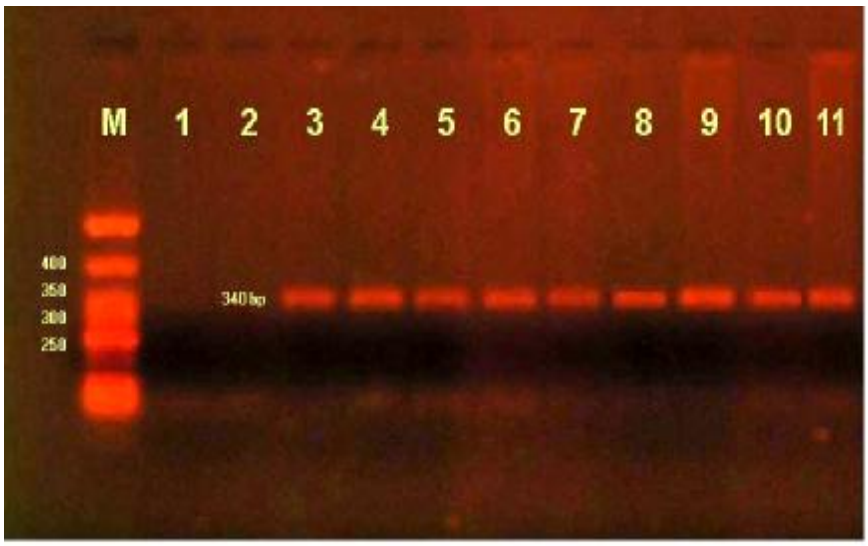

Figure 4.5: A representative photograph of ethidium bromide stained 2% agarose gel showing, the PCR product amplified using the C282Y primers. M = 100bp DNA ladder, (1–2) negative control, (3– 11) PCR products showing the 340bp fragment.

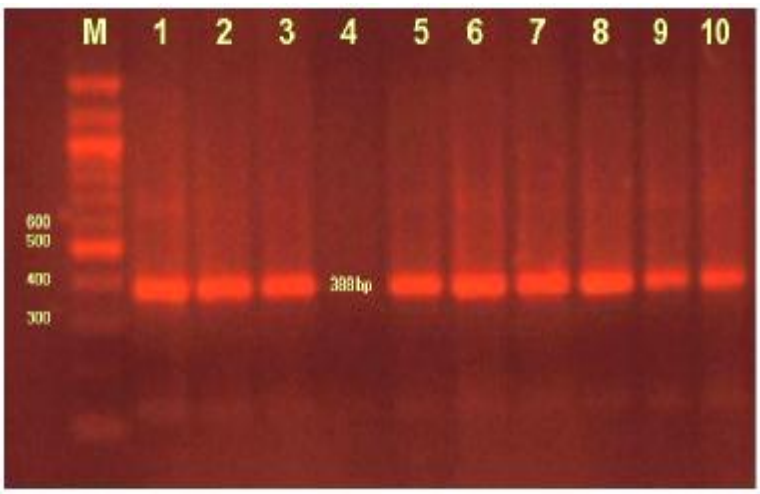

Figure 4.6. A representative photograph of ethidium bromide stained 2% agarose gel showing, the PCR product amplified using the H63D primers. M = 100bp DNA ladder, (1–3 , 5-10) PCR products show 388bp fragments, (4) negative control .

4.2.3. RFLP Results

In this technique two types of restriction enzymes. (*Snab1* for C282Y, and *Bsph1* for H63D) were used. *Snab1* cuts the PCR product (340bp) into 2 fragments in homozygous HH (229bp and 111bp), and 2 fragments in heterozygous HH (229bp,111bp and the band 340bp are present), while in the normal cases the product remains undigested (340bp). *Bsph1* cuts the PCR product (388bp) into 3 fragments in heterozygous HH (388bp, 250bp and 138bp) and 2 fragments in normal (250bp and 138 bp), while in the homozygous HH cases the product is one size (388bp).

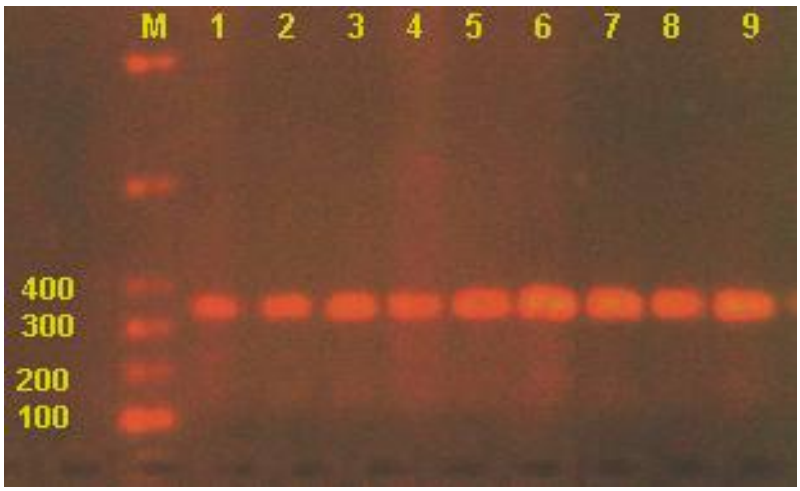

Figure 4.7. PCR – RFLP products for C282Y were run on 3% agrose gel and stained with ethidium bromide. All samples were not digested indicating normal genotypes with respect to C282Y mutation. M = 100bp DNA ladder, (1 – 9) *Snab1* digested samples.

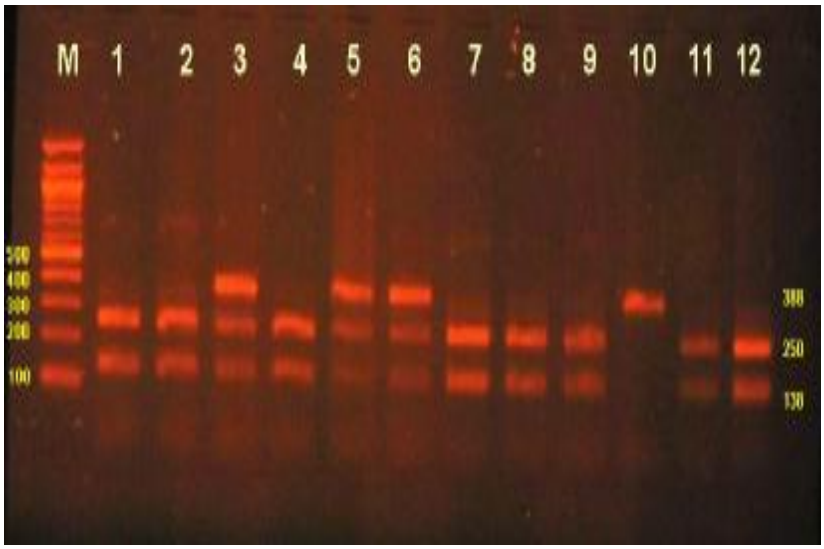

Figure 4.8. PCR – RFLP products for H63D were run on 3% agrose gel and stained with ethidium bromide. M = 100bp DNA ladder, (1,2,4,7,8,9,11,12) indicate normal genotypes, while (3,5,6) are illustrative of heterozygous samples, (10) represents a homozygous mutant sample.

4.2. 4. Genotyping Test Results

4.2.4.1. Genotyping results of the control group

The control group showed no mutant cases (neither for C282Y nor for H63D mutation) .

4.2.4.2. Genotyping results of the thalassemia minor group

No C282Y mutation was detected in the thalassemia minor group, but H63D was found in 7 (24%) of the cases who are heterozygous for the H63D mutation, and 2 (7%) of the cases proved to be homozygous for H63D.

4.2.4.3. Genotyping results of the thalassemia intermediate group

No C282Y mutation was detected in this group, but H63D was found in 5 (20%) of the cases who are heterozygous for the H63D mutation, and 3 (12%) of the cases were homozygous for the H63D .

Table 4.6 Summary of the PCR- RFLP genotyping results for the three groups.

Table 4.6. Summary of the genotyping results for the three groups.

Group	*Normal* + / +	C282Y + / -	C282Y - / -	H63D + / -	H63D - / -
Control	30/30	0/30	0/30	0/30	0/30
β-thala. Minor	21/30	0/30	0/30	7/30	2/30
β-thala. Intermediate	17/25	0/25	0/25	5/25	3/25

The mean, standard deviation and the 95% confidence interval of the mean for the H63D heterozygous and homozygous individuals are summarized in Tables (4.7) and (4.8).

Table 4.7. Analysis of biochemical tests (S.F, S.I, TIBC and TS) results for H63D heterozygous individuals.

Group	**Test**	**Mean**	**+/- SD**	**95% CI for the mean**
β-Thalassemia Minor (n = 7)	**S. Ferritin (ng/ml)**	96.6	62.8	38.3 – 154.5
	S. Iron (µg/dl)	69.9	29.9	42.2 – 97.5
	TIBC (µg/dl)	346.9	60.3	291.1 – 402.7
	% TS	21.5%	10.9%	11.3% - 31.6%
β-Thalassemia Intermediate (n = 5)	**S. Ferritin (ng/ml)**	478.9	414	35.2 – 993
	S. Iron (µg/dl)	102.7	47.1	44.1 – 161.3
	TIBC (µg/dl)	236.6	39.9	188.2 - 285
	% TS	24.5%	22%	14.3% - 70.7%

Table 4.8. Analysis of biochemical tests (S.F, S.I, TIBC and TS) results for H63D homozygous individuals.

Group	Test	Mean	+/- SD	95% CI for the mean
β-Thalassemia Minor (n = 2)	**S. Ferritin (ng/ml)**	106.7	65	74.3 – 120.4
	S. Iron (µg/dl)	55	22.5	68 – 32
	TIBC (µg/dl)	239.5	183	230.7 – 257.5
	% TS	27.2%	11.6%	20 - 30
β-Thalassemia Intermediate (n = 3)	**S. Ferritin (ng/ml)**	739.4	420.7	265.5 – 1069
	S. Iron (µg/dl)	244.5	75.7	56.2 – 432.7
	TIBC (µg/dl)	330.4	60.29	180.6 – 480.1
	% TS	71%	22.5%	15% - 126.9%

4.3. Hemochromatosis and H63D mutation

4.3.1. In the β-thalassemia minor group

According to the biochemical tests results it cames out that the 2 (7%) individuals with iron overload have normal genotypes while the 7 (24%) individuals who are heterozygous for the H63D, and the 2 (7%) individuals who are homozygous for the H63D showed negative biochemical results of iron overload.

4.3.2. In the β-thalassemia intermediate

According to the genotyping group results it emerged that 2 (8%) of the individuals who are heterozygous for H63D and the 3 (12%) individuals who are homozygous for H63D showed positive biochemical tests results of iron overload while 3 (12%) individuals with normal biochemical tests results proved heterozygous for the H63D mutation.

Chapter 5
Discussion

Hereditary hemochromatosis is an autosomal recessive disorder, that leads to excessive dietary iron absorption. Increased iron absorption leads to accretion of toxic levels of iron in multiple organs.

Thalassemia on the other hand is a heterogeneous group of disorders in which there is a genetically determined reduction in the rate of synthesis of one or more types of normal hemoglobin polypeptide chains. The increase in body iron in thalassemia patients is due to the combination of frequent transfusions and increased intestinal absorption resulting from ineffective erythropoiesis and chronic anemia.

In this study we investigated β-thalassemia intermediate and β-thalassemia minor subjects in whom several members have iron-overload. The aim was to examine the role of hereditary hemochromatosis as a cause of the iron overload observed in many β-thalassemia intermediate and β-thalassemia minor individuals. For this purpose three groups: β-thalassemia intermediate (n=25), β-thalassemia minor (n=30) and control individuals (n=30) were enrolled in the study.

5.1. β-Thalassemia Minor Group

The results of our study on the β-thalassemia minor group indicated that only 2 (7%) of the patients in this group showed iron overload. The mean %TS value, the only parameter elevated in this group, was 52.5%. However, this percentage is a little lower than that reported by **(Tilton et al, 1992)** where they reported a mean TS of 55% in their iron overload β-thalassemia minor individuals.

Meanwhile, our finding is nearly concurrent with that of other studies, such as that of **(Lewis M. et al, 2001 and Mazza J., 2002)** where they reported that S. iron, TIBC, % TS and S. ferritin are in the normal range in their β-thalassemia minor group, **(Lichtman M. et al, 2003)** where they showed that S. iron, %TS and S. ferritin values were normal while TIBC was normal or decreased and **(Sirdah M. et al, 1998)** where they reported that S.

iron was normal or decreased while TIBC was normal or increased in their β-thalassemia minor subjects.

In this case (β-thalassemia minor), the S. iron is slightly increased and the increase in TS level was observed increased in two cases only. The S. iron level did not increase to the level that liver can store it, and consequently ferritin did not increase.

Therefore, we can not confirm that S. ferritin, %TS and S. iron could predict the iron overload in cases of β-thalassemia minor.

5.2. β-Thalassemia Intermediate Group

According to the results of the current study in the β-thalassemia intermediate group 17 (68%) of the patients showed iron overload (hemochromatosis).

The mean ferritin level in this group was 1620 ng/ml and the mean %TS level was 73.29%, these levels are higher than those reported by other authors for β-thalassemia intermediate patients with iron overload e.g., **(Old J. et al, 2005)** where they reported a serum ferritin level of 300 ng/ml and a mean TS level of 55%, **(Porter J., 1999)** where they recorded a mean S. ferritin of 1000 ng/ml and, mean TS of 60% in their study population, **(Fargion et al, 1998)** where they showed a mean S. ferritin of 790 ng/ml and a mean TS level of 68% in their iron overload subjects, and **(Cao A., et al, 1997)** where they reported a mean S. ferritin of 500 ng/ml and a mean TS of 70%.

Differences in eating habits, genetic makeup and treatment of iron overload could be the reason behind the differences in the recorded S. ferritin and TS levels in the different studies.

In the β-thalassemia intermediate group, S. ferritin and %TS and S. iron were all significantly higher ($P < 0.01$), than those of the control group. Furthermore, and as indicated in Figures (4.2 and 4.3), linear correlations were obtained between the elevated S. ferritin and TS, and between S. ferritin and S. iron, however, the same parameters did not show such

correlations. These results are consistent with other studies in the field e.g., **(Lewis SM. et al, 2001)** and **(Politou M. et al, 2004)**.

These observed correlations can be explained by the fact that in case of S. iron elevation (such as in the case of the β-thalassemia intermediate), the %TS will increase as a result of the cell saturation of iron, and consequently the iron will be stored in the liver and the S. ferritin will increase. The increase of iron in the serum will lead to its precipitation in other organs as well, and lead to other organ manifestations. In the control group however, the S. iron is in the normal range, therefore the TS is normal and ferritin level will not increase.

The observed results also indicate that the S. ferritin, S. iron and TS are considered the diagnostic criteria in iron overload in β-thalassemia intermediates.

5.3. H63D is the Most Common Mutation in the Studied Groups

The control group showed no mutant cases (neither for C282Y nor for H63D mutation).

In the β-thalassemia minor group the occurrence of C282Y mutation was not observed as well as in other previous studies such **as (Al-smadi OA. et al, 2006, Settin A. et al, 2006 and Goland S. et al, 2004).**

While in the β-thalassemia minor group the H63D mutation was the most common in this group, and the . While 2 (7%) patients with iron overload showed no genetic mutation, H63D mutation was found in 7 (24%) of the cases who were heterozygous for the H63D mutation, and 2 (7%) of the cases proved to be homozygous for H63D in this group. Our percentage is higher than that reported by **(Jazayeri et al, 2003)** in Iran where they reported that 19.4% of their 56 β-thalassemia minor individuals were heterozygous for H63D, and 3.2% of the same group were homozygous for H63D.

In the β-thalassemia intermediate group, 17 (68%) of the patients showed iron overload according to their biochemical parameters. The occurrence of C282Y mutation in this group was not found. This result is

different from that reported by **(Kaur G. et al, 2004)** who studied 81 patients of Asian-African and Middle Eastern origins living in USA and found that 2 patients were positive for C282Y mutation while H63D mutation was not observed.

The H63D mutation was common in β-thalassemia intermediate group, where we found 5 (20%) of the cases heterozygous for the H63D mutation, and 3 (12%) of the cases proved to be homozygous for this mutation. These findings are also different from those reported by **(Politou M. et al, 2004)** where they showed that none of their 25 Greek patients had mutation in the *HFE* gene (neither C282Y nor H63D mutation).

Variations in the prevalence of mutations between studies could be explained by differences pertinent to the study group (e.g., genetic background of the different ethnic groups and founder effect).

In the present study 12 (48%) patients with iron overload showed no C282Y or H63D mutations, only 5 (20%) β-thalassemia intermediate patients with iron overload have H63D genetic mutations (3 homozygous and 2 heterozygous), and 3 (12%) β-thalassemia intermediate patients with normal biochemical tests results proved heterozygous for the H63D mutation. Defects in other genes involved in iron metabolism may explain the absence of C282Y or H63D mutation in the iron overload patients. As a second explanation, mutations in the *HFE* gene other than those tested in this study may be responsible for the iron overload.

In addition, the mutation (H63D in this study) does not seem to be the only factor involved in the disease, since individuals with the mutation had varying degrees of iron overload, some iron overload patients do not have the mutation, and some affected group members, as well as healthy members, did not have the mutation. As discussed above, mutations in other loci and / or other types of mutations in the HFE gene could be possible explanations.

The occurrence of H63D genotypes was highest among β-thalassemia intermediate group and in the patients of this group (who are not on blood

transfusion) iron overload occurs mainly through increased iron absorption. TS values oscillated most in secondary hemochromatosis i.e., (hemochromatosis not explaind by HFE gene mutations) somewhat less than in H63D homozygous and heterozygotes, ferritin levels increased in the β-thalassemia intermediate group H63D homozygotes, than in the β-thalassemia minor group. Ferritin levels increase in β-thalassemia intermediate group H63D heterozygotes, while declined in β-thalassemia minor group H63D heterozygotes

The occurrence of H63D genotypes was observed at a lower frequency in β-thalassemia minor, as compared to the β-thalassemia intermediate patients. Consequently, the H63D mutation alone does not account for the high serum ferritin levels and %TS values in the β -thalassemia minor, and thus, the H63D substitution may be associated with an increased storage of iron. Moreover, the H63D mutation is known for its association with a mild form of the disease **(Tannapfel A. et al, 2001).** This substitution mutation, at least **in vitro**, leads only to a reduced activity of *HFE* protein **(Mura C. et al, 1999)**.

Chapter 6
Conclusions and Recommendations

Iron overload is still a health problem in β-thalassemia patients not only in Gaza Strip, but also worldwide. This study investigated the nature of iron overload in β-thalassemia intermediate and β-thalassemia minor subjects in Gaza Strip using PCR-RFLP and biochemical tests. We hope that the results of this research will contribute to making the diagnosis of inherited hemochromatosis fast and easy and help finding out alternatives for the current treatment and medication.

Conclusions

* All the control subjects were normal in terms of the tested biochemical parameters and the *HFE* genotype.

* Analysis of data according to S. ferritin and TS tests in β- thalassemia intermediate group showed that 68% of them have iron overload, while according to TS test in β- thalassemia minor group 2 (7%) of them have iron overload.

* The occurrence of C282Y mutation was not found in any of the tested β-thalassemia patients.

* The H63D mutation was the most common mutation encountered in the β-thalassemia intermediate individuals, where 5 (20%) of them were heterozygous, and 3 (12%) of them were homozygous for this mutation.

* In the β- thalassemia intermediate group, 12 (48%) patients with iron overload showed no genetic HFE mutation, and 3 (12%) patients with normal biochemical tests results proved heterozygous for the H63D mutation.

* In the β-thalassemia minor group the H63D mutation was also the most common, where 24% of the cases were heterozygous, and 2 (7%) of the cases proved to be homozygous.

* In the β- thalassemia minor group, 2 (7%) patients with iron overload showed no HFE genetic mutation.

* The major cause of iron-overload in the β- thalassemia intermediate group was not due to the presence of the C282Y or H63D mutations in the *HFE* gene.

Recommendations

* we recommend testing all the thalassemics " major and intermediate" for the presence of *HFE* gene mutations, especially those who are about to have a bone marrow transplant, to find out the nature of iron overload in their bodies which will help in the success of the transplant operation.

* Adopt testing the nature of iron overload among thalassemics before providing them with medications to get rid of iron overload in their bodies.

* Test type II Diabetes Mellitus, impotence and heart failure patients to find out whether they suffer from hemochromatosis or not in order to provide them with the appropriate treatment.

* Make screening for inhabitants of the Gaza Strip to find out the prevalence of the different types of hemochromatosis among them as we lack information about this disease in the Middle East area.

* Test for other types of HFE gene mutations in the iron overload patients, who proved negative for the C282Y and H63D mutations.

Chapter 7
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