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Determination of Thrombotic Genetic Mutations in Thalassemia Intermedia Patients in the West Bank, Palestine

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Determination of Thrombotic Genetic Mutations in Thalassemia Intermedia Patients in the West Bank - Palestine

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Thesis Approval

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Dedication

To my Father and mother

To my brother and sisters

To my teachers

To my colleagues

To whoever inspired me positively

Mohammed Isam Abdulfatah Kurdi

Declaration

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

Signed

Mohammed Isam Abdulfatah Kurdi

Date

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Abstract

Thalassemia is a heterogeneous group of genetic disorders of hemoglobin (Hb) synthesis, all of which result from reduced rate of production of one or more of the globin chains of Hb. The outcome of the disease depends on the severity of the mutation and the companion of other external modifiers that determine the clinical picture of the patient. Thus, thalassemia is classified clinically into three major subclasses, thalassemia major (TM), thalassemia minor or trait and thalassemia intermedia (TI). TI is most commonly caused by genetic defects in one or two of the globin genes: β, α or γ-globin gene. Thalassemic patients suffer from many clinical complications that affect most body organs including severe bone deformities and hepatosplenomegaly. Several complications that were identified in thalassemic patients have been noticed to be unique to thalassemia intermedia over β thalassemia major. Among these complications is the tendency of those patients toward forming venous thromboembolic complications, cerebral, pulmonary or deep vein thrombosis in any other body site.

This study aimed to determine thrombotic genetic mutations in a cohort of TI patients from West Bank region, Palestine that may increase the risk of developing thrombotic manifestations in these patients. For this purpose, four thrombotic mutations were investigated: FV Leiden (G1691A), FII (G20210A), MTHFR (C677T) and platelet GP β3 (T1565C) mutations/polymorphisms.

Eighty-two TI patients, from the West Bank covering all thalassemia care centers in the West Bank region, were included in the study. A matched group of non-anemic normal subjects were used as a control group. Samples were tested for the presence of the four thrombotic mutations using RFLP-PCR, and results were analyzed by the chi-square at α (0.05).

The study showed a gradual increase in the incidence of reporting of TI cases from north to south; with the lowest incidence reported in Jenin (5 cases) and the highest in Hebron (65 cases). The rate of thrombotic manifestation incidences among TI patients was found to be (3.7%). Molecular testing of the thrombogenic mutations/polymorphisms revealed that 1.3% of the TI patients were heterozygous and 2.5% were homozygous for the FII (G20210A) mutation. According to FVL 19.5% of the patients were heterozygous and no homozygous cases were detected. The highest incidence among the four polymorphisms was the MTHFR (C677T); where 39% of the patients showed heterozygosity for the polymorphism, and 6.1% were homozygous. For the platelet GP β3 (T1565C) polymorphism 9.8% of the patients were heterozygous and 1.2% were homozygous. Chi-square analysis

revealed no statistically significant difference in the frequency of all four mutations/polymorphisms analyzed among the TI patients compared to normal control subjects. However, such thrombotic genetic defects when combined with acquired thrombotic risk factors may contribute to the hypercoagulable state in TI patients, as illustrated in three patients among the study samples. Furthermore, our results showed a substantial lower frequency of FII G20210A polymorphism and higher frequency of MTHFR C677T mutations among Palestinians compared to previous reports.

In conclusion, no statistical difference was observed in the frequency of the four thrombotic genetic defects investigated in this study between TI patients and normal control subjects. However, further investigations of TI patients for other thrombotic genetic defects may help to assess their contribution to the hypercoagulable state in such patients.

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Definitions

Beta-thalassemia: (β-thalassemia) are a group of inherited blood disorders caused by reduced or absent synthesis of the beta chains of hemoglobin resulting in variable phenotypes ranging from severe anemia to clinically asymptomatic.

Deep vein thrombosis, or **deep venous thrombosis**, (**DVT**): is the formation of a blood clot (thrombus) in a deep vein, predominantly in the legs. Non-specific signs may include pain, swelling, redness, warmness, and engorged superficial veins.

Embolus: a freely travelling thrombus in the blood vessels.

Hemorrhage: is the loss of blood from the circulatory system, which can occur internally or externally.

Hemostasis: is a process that causes bleeding to stop, and retains blood within a damaged blood vessel and initiates wound healing.

Infarction: death of an organ due to a blockage of the main nourishing artery of an organ because of hypoxia.

Thalassemia: is inherited autosomal recessive blood disorders that originated in the Mediterranean region.

Thrombosis: (Greek: θρόμβωσις) is the formation of a blood clot (**thrombus**; Greek: θρόμβος) inside a blood vessel, obstructing the flow of blood through the circulatory system.

Thromboembolism: is thrombotic complications that occur due to an embolus, or emboli, and includes DVT and PE.

Thrombus: a blood clot that is formed by aggregation of platelets and clotting factors.

Abbreviations

ADP: Adenosine Di-phosphate

APC: Activated Protein C

APCR: Activated Protein C Resistance

APTT: Activated Partial Thromboplastin Time

AT: Anti-thrombin

DHF: Dihydrofolate

DIC: Disseminated Intravascular Coagulation / Coagulopathy

DVT: Deep Vein Thrombosis

EPCR: Endothelial Protein C Receptor

FII: Factor two

FIX: Factor nine

FV: Factor five

FVa: Factor five activated

FVII: Factor seven

FVIII: Factor eight

FX: Factor ten

FXI: Factor eleven

FXII: Factor twelve

FV Leiden: Factor five Leiden

GP αIIβ_3: Glycoprotein alpha-2 beta-3

HA: Hemolytic Anemia

Hb A: Hemoglobin A

Hb A_2: Hemoglobin A_2

Hb E: Hemoglobin E

Hb F: Hemoglobin F / Fetal Hemoglobin

HPFH: Hereditary Persistence of Fetal Hemoglobin

HPVR: Hyper variable Region

MRI: Magnetic Resonance Imaging

MTHFR: Methylenetetrahydrofolate reductase

NATP: Neonatal Alloimmune Thrombocytopenic Purpura.

PAI: Plasminogen-Activator Inhibitor

PCI: Protein C Inhibitor

PCR: Polymerase Chain Reaction

PE: Pulmonary embolism

PEA: Phosphatidylethanolamine

PF3: Platelet Factor 3

PS: Phosphatidylserine

PTP: Postransfusion Purpura

RBC: Red Blood Cell

RFLP-PCR: Restriction Fragment Length Polymorphism-Polymerase Chain Reaction

SAM: *S*-Adenosyl methionine

TAFI: Thrombin-Activatable Fibrinolysis Inhibitor

TFPI: Tissue Factor Pathway Inhibitor

TI: Thalassemia Intermedia

TPFS: Thalassemia Patient's Friends Society

VTE: Venous Thromboembolism

β-TM: Beta Thalassemia Major

Chapter One

Introduction

Since the first description of Thalassemia in five children presented with microcytic hypochromic anemia, splenomegaly and bone deformities by Cooley and Lee in 1925 several studies were conducted in order to investigate the complications of this disease either by testing the etiology of the disease or by observing its clinical complications involving blood cells and other body systems that could be involved in the "thalassemia syndrome" (Cohen et al., 2004; Weatherall and Clegg, 2008).

By the 1940s, the genetic characteristics of the disease were understood; and it was recognized that the first identified cases were homozygotes for the autosomal disease, which was called thalassemia major, while the heterozygous, or the carrier state, which was associated with much milder, or clinically asymptomatic cases, was termed as thalassemia minor or minima. Later on, a clinically significant, but not as severe as the major state, with a broad spectrum of symptoms where named thalassemia intermedia (Weatherall, 2006).

Thalassemia is clinically heterogeneous and patients usually show diverse spectrum of clinical symptoms that presents primarily with hematologic disorders (e.g. hemolytic anemia). If not properly treated, the condition exacerbates and disseminates to affect almost every organ in the body including bones, the skeletal system, the endocrine system, spleen, liver,

lungs, auditory system and cardiovascular system. Thalassemia can also cause disturbances in the hemostatic system leading to thrombosis or infarction either in veins or arteries (Rund and Rachmilewitz, 2005).

This study focuses on the hypercoagulable state and its complications in thalassemia intermedia patients and detection of the genetic risk factors that were proven to be associated with thromboembolic disorders.

1.1 Thrombosis and thrombophilia

There is a delicate balance between the prohemorrhagic state and the prothrombotic state. However, the hemostatic system is highly regulated, and formation of a blood clot (or thrombus) is a very complicated. A blood clot is composed of platelets consolidated with fibrin mesh; this agglomeration is usually formed as a response of the body to an incision or injury in a blood vessel. This could arise in any blood vessel throughout the whole circulatory system. Thus, platelets with many plasma proteins and the endothelium that lines blood vessels integrate together in order to prevent blood loss, due to hemorrhage; which could be either external or internal, and maintain intact vessels in a process called hemostasis (Furie and Furie, 2008).

The hemostatic system is a meticulously controlled process involving six major regulatory systems: (1) Platelets; which are subcellular fragments that form the primary plug and act as the bench plate that provide several factors and proteins and many hemostatic reactions are executed on. (2) The vascular endothelium, which is termed to be non-wettable; that prevents blood loss through its tight endothelial cell-to-cell adhesion and repels any adhesion of platelets or coagulation factors. (3) The procoagulant plasma proteins (i.e. the coagulation factors); act towards the formation of the fibrin mesh, which intensifies the primary plug formed by the platelets, through a set of successive steps which is triggered by certain stimuli to prevent bleeding or blood leakage from blood vessels. (4) The natural anticoagulant proteins (e.g. protein C and protein S) which counteract the action of the coagulation system by inhibiting the formation of a clot. (5) The fibrinolytic proteins, which lyse the fibrin clot. (6) The antifibrinolytic proteins, which stops the action of the fibrinolytic system. Each of

these six hemostatic components must be present in fully functional state, in adequate quantity, and at the proper location to prevent excessive blood loss after vascular trauma and, at the same time, to prevent pathologic thrombosis (Brummel-Ziedins et al., 2004).

Thrombophilia is not a disease per se; it is, rather, a term that expresses the tendency of a person towards developing thrombosis. It is not an expectation of possibility of forming thrombosis, since developing thrombosis is a multifunctional process, but it determines the increased likelihood of developing thrombosis (Bertina and Rosendaal, 1998; Martinelli et al., 1998).

The majority of the thrombophilic manifestations include venous thrombosis, or thromboembolism (VTE), but the minority of cases develop arterial thrombosis; especially if vascular defects were accompanied (Heit, 2007). Other less frequent cases present with visceral or cerebral vein thrombosis, second- or third- trimester pregnancy loss, severe preeclampsia and recurrent fetal loss and some complications of pregnancy (e.g., intrauterine growth restriction, stillbirth, placental abruption), purpura fulminans (neonatalis or adult), superficial or deep vein thrombosis (DVT), pulmonary embolism (PE), warfarin-induced skin necrosis, arterial thrombosis (e.g., stroke or acute myocardial infarction), thrombosis in unusual venous circulations (e.g., cerebral, hepatic, mesenteric, and renal veins; arm, portal, and ovarian veins; retinal vein or artery) and many others (Heit, 2007).

1.1.1. Risk factors of thrombophilia:

Thrombosis is believed to develop because of combinations of several factors and defects together which result in accumulation of a clot at some site in a blood vessel, which was favored due to some reasons. The majority of these risk factors either enhances procoagulant reactions or inhibits anticoagulant mechanisms and results in a hypercoagulable state (Dahlback, 2008b; Rosendaal, 1999a, b; Tripodi, 2003).

The first suggestions of the existence of risk factors for thrombophilia were set in 1854 when Rudolph Virchow, a German pathologist, proposed that thrombosis results from three interrelated factors; (1) decreased blood flow (venous stasis), (2) inflammation of the blood vessels (vascular endothelial injury) and (3) intrinsic alterations in the nature of the blood itself. These factors were known as Virchow's triad (Kitchens, 1985; Seligsohn and Lubetsky, 2001).

Most of the patients with thrombosis were noticed to share variety behaviors, which include some living habits, medications and clinical states (Dahlback, 2008b). These non- genetic risk factors were indicated as environmental or acquired; since, no obvious genetic defect could be determined. Those risk factors include prolonged immobilization, surgery, malignant diseases, inflammatory conditions, trauma, use of oral contraceptives, hormone replacement, pregnancy/puerperium, the presence of anti-phospholipid antibodies, previous history of thrombosis and advanced age (Bertina, 2001; Dahlback, 2008b). Those mentioned factors, and many others, have been recognized as risk factors for venous thrombosis (Table 1.1).

Table 1.1: **Genetic and acquired risk factors for venous thrombosis.**

(Rosendaal, 1997)

Acquired	Inherited	Mixed
Increase in Age	Factor V Leiden	Hyperhomocysteinemia
Obesity	Factor II (G20210A)	High factor VIII levels
Smoking	Antithrombin deficiency	Hyperfibrinogenemia
Long flights	Protein C deficiency	
Previous thrombosis	Protein S deficiency	
Pregnancy and puerperium	Dysfibrinogenemia	
Oral contraceptives		
Hormonal replacement therapy		
Immobilization		
Major surgery		
Orthopedic surgery		
Malignancy		
Myeloproliferative disorders		
Polycythemia vera		
Antiphosphohpid syndromes		
Arteriosclerosis		
Cardiovascular disease		
APCR * unrelated to factor V Leiden		

* APCR; Activated Protein C Resistance

Splenectomy was noticed to be associated with the evolution of thrombosis; this was attributed to the evolution of high numbers of platelets after splenectomy and having abnormal

forms of RBCs circulating in the blood stream (Cappellini et al., 2005; Cappellini et al., 2000; Eldor and Rachmilewitz, 2002). On the other hand, frequent transfusions were noticed to decrease the chance to have thrombosis for splenectomized patients (Cappellini et al., 2000).

Some studies indicated that prothrombotic genetic factors might interact with acquired ones (e.g. smoking) by modifying the stroke phenotype and affecting midterm survival (Oksala et al., 2007).

1.1.1.1. Coagulation factors as risk factors for thrombophilia:

Plasma level of several coagulation factors seems to have an impact on the tendency for thrombophilia. For example, hyperprothrombinemia is one of the known risk factors for thrombophilia, and the identification of a point mutation in the 3′ untranslated region of the gene that leads to over production of prothrombin confirmed the findings (Poort et al., 1996). Similarly, Hyperfibrinogenemia is another considered factor that correlates with thrombophilia (Kamphuisen et al., 1999; Koster et al., 1994). Also, deficiencies of protein C, protein S and anti-thrombin were listed among the risk factors of thrombophilia (Dahlback, 2000; Nicolaes and Dahlback, 2002), with over 100 different mutations have been identified (Table 1.2) (Seligsohn and Lubetsky, 2001).

High level of FVIII is one of the strongly believed risk factors for genetic thrombosis, according to studies that reported Familial clustering of high factor VIII levels in patients with venous thromboembolism (Kamphuisen et al., 2000a; Schambeck et al., 2001), but nobody succeeded to identify any polymorphism in the FVIII gene that is responsible for overexpression of this gene (Kamphuisen et al., 2001; Mansvelt et al., 1998).

FV is homologous to FVIII; both are activated by thrombin, and are inhibited by activated protein C (APC), and they are cofactors for vital steps in the coagulation cascade, moreover, one could conclude that low levels of FV may have an impact in VTE since it acts as a cofactor for APC in the inhibition of FVIII. But no evidence was noticed to correlate neither elevation nor reduction of FV with VTE except the in the case of APC resistance by Factor V Leiden (FVL) (Kamphuisen et al., 2000b).

It was early, proposed that FXI activation is done only by FXIIa. This belief was disproved afterwards (Gailani and Broze, 1991; Naito and Fujikawa, 1991). Levels of FXI is now known to be enhanced by elevated levels of thrombin and down regulation of fibrinolysis by the action of thrombin activatable fibrinolysis inhibitor (TAFI) (Von dem Borne et al., 1997). So, high levels of FXI is strongly advised to be considered as venous thromboimbolism (VTE) risk factor (Meijers et al., 2000; Tripodi, 2003). Similarly, high levels of FIX could play a role in VTE as FIX is the main member of the tenase complex; a key step in the coagulation cascade (Tripodi, 2003; van Hylckama Vlieg et al., 2000).

On the other hand, there is a big debate about the association between FXII deficiency and developing of VTE; where many researches tried to connect the presence of low levels or low activity percentage of FXII with VTE comparing diseased individuals with healthy ones using statistical analysis for these theories. Some results showed positive relationship between FXII deficiency and developing of VTE (Halbmayer et al., 1992), others showed no strong evidence for this relationship (Von Kanel et al., 1992), that's why the role of FXII deficiency is still not considered as a risk factor for thrombophilia and many articles reject the role of FXII deficiency as a risk factors for VTE (Tripodi, 2003).

Also, confusing results were obtained while studying FVII, FX and FXIII as risk factors for thrombosis, and they are still not considered as risk factors for VTE (Balogh et al., 2000; de Visser et al., 2001; Koster et al., 1994; Tripodi, 2003).

1.1.1.2. Hereditary thrombophilia, a historical background:

The first suggestions of hereditary thrombophilia were set in 1965 by Egeberg and Beck; where the former noticed recurrent venous thrombosis in a family whom was diagnosed to have antithrombin deficiency, which is inherited in an autosomal dominant manner, and the latter mentioned a hereditary thrombophilia due to inherited dysfibrinogenemia (Beck et al., 1965; Egeberg, 1965).

In 1981, Griffin and colleagues correlated heterozygous protein C deficiency and venous thrombosis in young adults (Griffin et al., 1981). In 1984, many reports were published showing that thrombosis correlates with protein S deficiency (Berman et al., 1986; Schwarz et al., 1984).

A critical finding made by Dahlback and coworkers in 1993 showed that venous thrombosis is often associated with hereditary resistance to activated protein C (APC) (Dahlback et al., 1993; Svensson and Dahlback, 1994). In 1994, several research labs indicated that resistance to APC could be due to a point mutation in FV (Arg506Gln); which was later referred to as factor V Leiden (Bertina et al., 1994; Greengard et al., 1994; Voorberg et al., 1994).

In 1993, mild to moderate hyperhomocysteinemia was recognized as a risk factor for venous thrombosis (Bienvenu et al., 1993).

In 1996, a single nucleotide polymorphism was indicated in the 3'-untranslated region of the prothrombin gene (G20210A) and linked to familial venous thromboembolism (Poort et al., 1996).

After that, many experiments proved a positive relationship between elevated levels of FVIII in plasma and venous thrombosis, but no genetic factors could be identified (Kamphuisen et al., 2000a; Koster et al., 1995; Schambeck et al., 2001).

1.1.1.2.1. Major hereditary defects:

Among the main genetic defects that correlate with thrombosis are: a point mutation in *F5* gene that cause resistance to the APC; known as FV Leiden, another one is a point mutation in the 3' untranslated region of *F2* gene; that causes hyperprothombinemia, both of which are considered to be the most frequent among the genetic causes of thrombophilia (Table 1.2). Hyperhomocysteinemia and GP β3 pro33 are, also, of the mentioned risk factors that are correlated with high tendency towards developing thrombosis (Iolascon et al., 2001).

Table 1.2: **Genetic defects in inherited thrombophilia.** (Bertina, 1997)

Genetic defect	Prevalence%	Mutations*
Dysfibrinogenemia	1.0	> 11
Antithrombin deficiency	4.3	> 79
Protein C deficiency	5.7	> 160
Protein S deficiency	5.7	> 69
APC resistance	45	1
Increased prothrombin	18	1
Unknown	30	?

1.1.1.2.1.1. Prothrombin gene mutation (G20210A):

The activated form of prothrombin, thrombin (factor IIa, FIIa) is the central protease in the coagulation cascade and one of the most widely studied enzymes. It was described in 1892 by Alexander Schmidt, but the first identification of thrombin was set in 1845 by Buchanan (Siller-Matula et al., 2011).

The gene coding for prothrombin (FII) is located on chromosome 11p11 (Chromosome 11, NC_000011.9: 46,740,743-46,761,056), and is termed as (*F2*) gene. It spans (20,314 bp) consisting of 14 exons separated by 13 introns (Degen and Davie, 1987; Royle et al., 1987) (Figure 1.1).

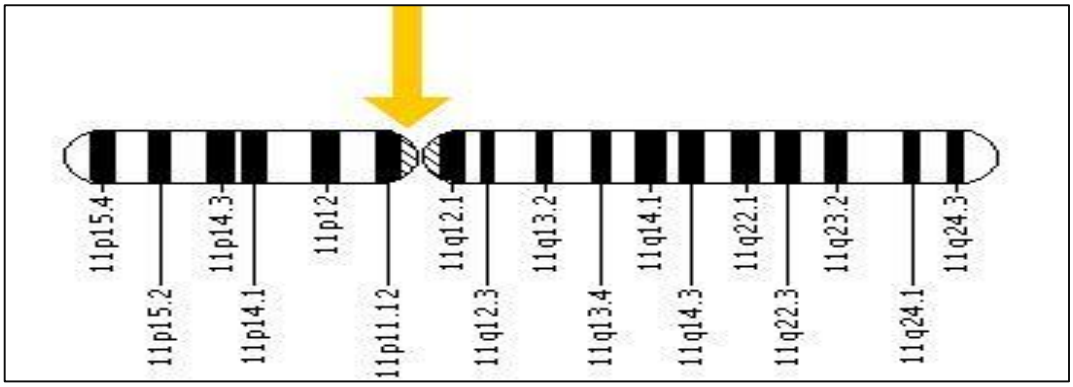

Figure 1.1: ***F2* gene location**.
The *F2* gene is located on the short arm (p) of chromosome 11 at position 11, as indicated by the arrow (Genetics Home Reference, 2008).

Prothrombin is a glycoprotein synthesized by the liver parenchyma cells as a proenzyme with a molecular weight approximately (72 KDa) consisting of (579) amino acid residues. Activation of prothrombin to thrombin takes place by sequential cleavages at two argenin residues (Arg320 and Arg271) by the prothrombinase complex (FXa, FVa and Ca^{++}) on a phospholipid surface. FIIa is a heterodimer consisting of two polypeptide chains with a molecular weight of approximately 37 KDa (Degen and Davie, 1987; Matafonov et al., 2011).

Thrombin is a vitamin K dependent serine protease enzyme, which exhibits a trypsin-like specificity and cuts at specific Arg residues of the substrate. Thrombin acts on twelve known

substrates with the assistance of five cofactors. Upon activation of thrombin in a reasonable amount many vital activities are carried out, termed as (Thrombin burst); which involve the action of thrombin on various lines and systems including coagulation and complement systems, as well as other roles in various vital processes throughout the body tissues (Bode, 2006).

Thrombin converts fibrinogen (FI) into active fibrin monomers (FIa). Then, it stabilizes the fibrin monomers by activating the transglutaminase (FXIII) that cross links the fibrin monomers and produces a clot mesh of fibrin polymers (Di Cera, 2003). Thrombin, also, is a potent activator of the nearby platelets via protease- activated receptors 1, 3 and 4 (PARs1,3 and 4) (Kahn et al., 1998); a single thrombin-activated platelet exposes more than 12,000 copies of GP(αIIbβ3) receptors that can concentrate fibrinogen for efficient fibrin formation (Peerschke et al., 1980). Subsequently, many coagulation factors (i.e. FV, FVII, FVIII and XI) bind to the activated platelet membrane in order for subsequent coagulation steps to take place. Activation of these factors feeds positively on the formation of the prothrombinase complex, which intensifies the thrombin burst action (Lancellotti and De Cristofaro, 2009).

Thrombin, also, activates Thrombin Activatable Fibrinolysis Inhibitor (TAFI), which inhibits plasmin-mediated fibrinolysis, and thus, facilitates plasminogen binding, from partially degraded fibrin (Lancellotti and De Cristofaro, 2009; Pihusch et al., 2001).

In addition to its procoagulant activity, thrombin has an anticoagulant function. Upon binding of thrombin to its receptor on the endothelium, thrombomodulin (TM), it exhibits its anticoagulant role in the coagulation process (Esmon, 1995). Here, the ability of thrombin to cleave fibrinogen and PAR1is suppressed. In contrast, it enhances its affinity towards the zymogen; protein C by more than a thousand fold (Di Cera, 2003). APC cleaves and inactivates FVa and VIIIa; which are two essential cofactors for the action of coagulation factors Xa and IXa, which are required for thrombin generation, thereby, feeds negatively on the progression of the coagulation cascade, and so, on its production (Di Cera, 2003).

Thrombin also activates tissue components represented in the placenta and plays a role in cell adhesion, smooth muscle proliferation, and vasculogenesis. Thrombin also has growth factor and cytokine-like activities that may play a role in atherosclerosis, wound healing, and inflammation (Siller-Matula et al., 2011). Thrombin plays a role in arterial and venous

thrombosis as wells as in the pathogenesis of multiple conditions, e.g. sepsis and disseminated intravascular coagulation (DIC), cancer, inflammatory brain diseases, wound healing and atherosclerosis (Siller-Matula et al., 2011).

Finally, thrombin is irreversibly inhibited at its active site by the serine proteases antithrombin (AT) and protease nexin I which are assisted by heparin and the thrombin-specific heparin cofactor II, that regulates the growth factor and cytokine-like activities of thrombin (Davie and Kulman, 2006; Muszbek et al., 2010; Siller-Matula et al., 2011).

1.1.1.2.1.1.1. Prothrombin deficiency:

Hypoprothrombinemia, or congenital prothrombin deficiency, is a rare autosomal recessive disorder characterized by low thrombin antigenic levels and activity below 10% of the normal patient which lead to severe bleeding manifestations (Lancellotti and De Cristofaro, 2009). Dysprothrombinemia, which is characterized by normal antigen levels but a dysfunctional prothrombin molecule, shows a more variable level in bleeding tendency, and there is often a good correlation between the levels of prothrombin activity and clinical severity. It has been stated that 32 molecular defects in prothrombin were identified (Akhavan et al., 2002; Lancellotti and De Cristofaro, 2009). Complete prothrombin deficiency, or aprothombinemia, is believed to be incompatible with life (Meeks and Abshire, 2008).

1.1.1.2.1.1.2. Hyperprothrombinemia:

A common genetic variation in the 3' untranslated region of the prothrombin gene (G20210A) was noticed to correlate with elevated plasma prothrombin levels and an increased risk of venous thrombosis (Poort et al., 1996) (Figure 1.2). This single base substitution was found in 18% of thrombophilic families, 6% of unselected consecutive patients with deep-vein thrombosis, and 2% of healthy controls (Rosendaal et al., 1997). Moreover, it was found that this mutation was associated with a 4-fold increased risk of myocardial infarction in women (Rosendaal et al., 1997), while among men the risk was increased up to 1.5 folds (Doggen et al., 1998).

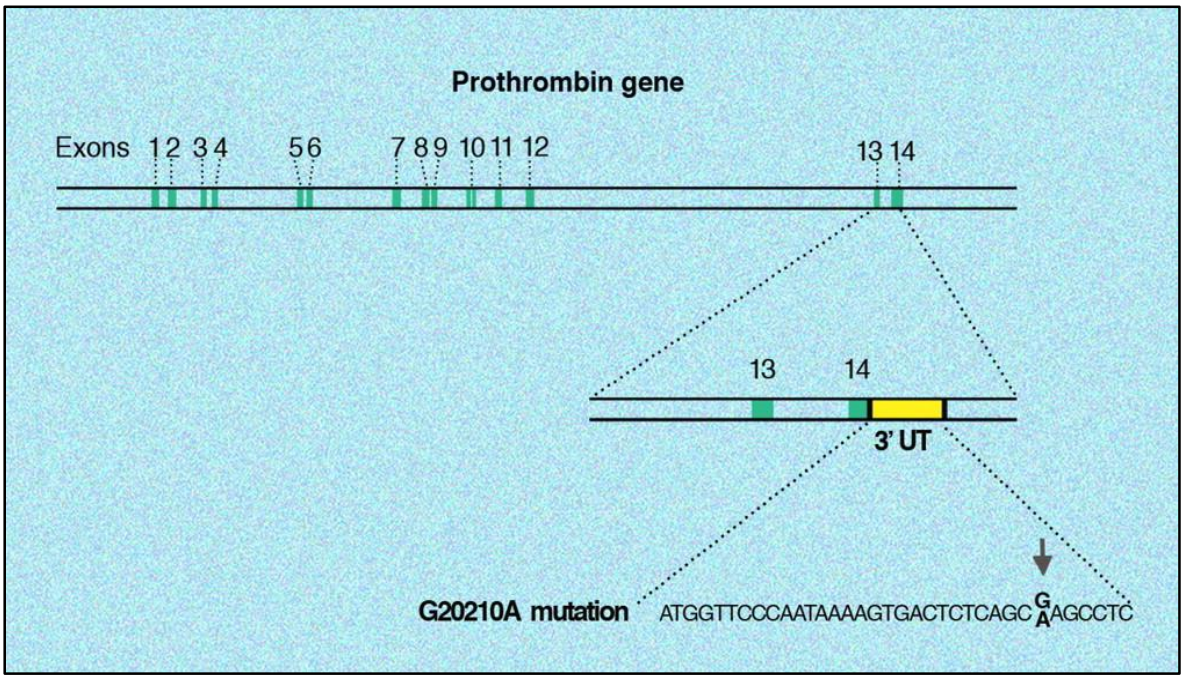

Figure 1.2: **The prothrombin gene (G20210A) mutation**.

The single point G to A mutation at position 20210 affects the 3' untranslated region of the prothrombin gene (F2). Thus, the protein-coding sequence of the prothrombin gene is not affected by this mutation. (Dahlback, 2008a).

1.1.1.2.1.2. Activated Protein C Resistance (APCR):

Most inherited risk factors for venous thrombosis are found in the protein C system. Any inherited or acquired abnormality that interferes with the expression of Activated Protein C APC activity is termed as Activated Protein C Resistance (APCR) (i.e. auto-antibodies and serine protease inhibitors) (Oosting et al., 1993; Zivelin et al., 1999). However, the most common cause of hereditary thrombophilia is a point mutation in the *F5* gene, which is known as FV Leiden (Bertina et al., 1994; Svensson and Dahlback, 1994).

1.1.1.2.1.2.1. Factor V:

Factor V (also called proaccelerin or the labile factor) is encode by the (*F5*) gene which is located on chromosome (1q23), as shown in Figure (1.3), and spans (75,774 bp) consisting of 25 exons (Duga et al., 2004; Galanello et al., 2001). FV is produced as a pre-procofactor protein with (2224 aa) long and weighs (330 kDa) (Tracy et al., 1982).

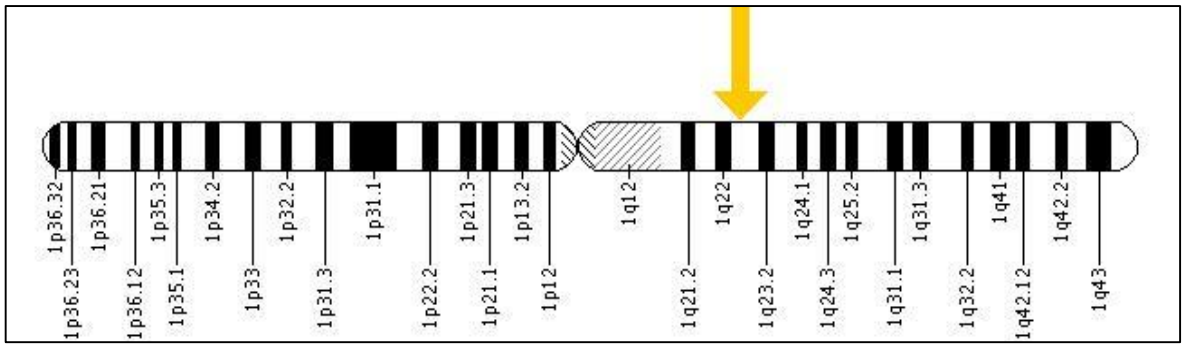

Figure 1.3: ***F5* gene location**.
The *F5* gene location is located on the long (q) arm of chromosome 1 at position 23, as indicated by the arrow (Genetics Home Reference, 2013a).

About 80% of the blood FV is produced and secreted by the hepatocytes and circulates in the plasma at a concentration of about (7 µg/mL) (Tracy et al., 1982). Most of the remaining 20% is produced by the megakaryocytes and is stored, as a partially proteolyzed protein, in the alpha (α) granules of the platelets (about 4600 – 14,000 molecules per platelet) (Gewirtz et al., 1986). Some of this stored FV was noticed to be absorbed from the plasma via endo-

cytosis (Camire et al., 1998). This platelet-derived FV is that responsible for thrombin generation at the primary plug site upon platelet activation at the injury site (Mann and Kalafatis, 2003; Nicolaes and Dahlback, 2002).

Factor V has almost no procoagulant activity until it is proteolytically activated by thrombin or by activated factor X (FXa). FV activation involves proteolysis at three arginine residues (Arg709, Arg1018, and Arg1545) (Guinto and Esmon, 1982; Nicolaes and Dahlback, 2002). Upon activation of FV, activation of thrombin increases by 300,000 folds relative to the rate of the reaction catalyzed by factor Xa acting alone (Mann and Kalafatis, 2003; Nicolaes and Dahlback, 2002).

FV has a dual action depending on the nearby microenvironment; in the presence of FIIa or FXa the precursor of FV is proteolyzed in a way directing the molecule towards its procoagulant fate (FVa) where it is involved in the formation of the prothrombinase complex; which activates prothrombin to thrombin. Alternatively, cleavage by APC may recruit FV towards its anticoagulant fate (FVac) where it acts as a cofactor for APC, which inactivates FVIII (Shen and Dahlback, 1994; Thorelli et al., 1999).

Down regulation of the procoagulant activity of FVa is achieved, mainly, by APC; which involves proteolysis at three arginine residues (Arg506, Arg306, and Arg679). Kinetically, the Arg506 residue is the highest, then Arg306 and, finally, Arg679 residue is the slowest (Dahlback, 1999; Nicolaes et al., 1995). Moreover, the Arg306 site seems to be the most important for FV inactivation in vivo; where it is potentiated 20 folds in the presence of protein S. Whereas Arg506 seems to be more important in the regulation of free FVa (Dahlback, 1999).

The first cleavage, at Arg506, reduces both FVa cofactor activity (by 25 – 40%) and its affinity for FXa, resulting in partial inactivation of FVa. The subsequent cleavage at Arg306 leads to the complete inhibition of FVa, while Arg679 cleavage seems to be with the least importance (Dahlback, 1999; Nicolaes et al., 1995).

Another mechanism for FVa inactivation involves thrombin-mediated cleavage at Arg643 site, in the presence of endothelial cells, which results in the reduction of the affinity between the heavy and the light chains of the molecule (Mann and Kalafatis, 2003).

Down regulation of the anticoagulant activity of FVac is achieved, mainly by thrombin proteolysis at three arginine residues (Arg709, Arg1018, and Arg1545). Moreover, it was found that FVac could be cleaved by thrombin at both Args (709 and 1018) and still work fully as a cofactor to APC, whereas cleavage at (Arg 1545) completely eliminates the anticoagulant activity of FV (Thorelli, 1999).

As FV has a dual role; as procoagulant and anti-coagulant agent, defects in *F5* gene result in either an autosomal dominant form of thrombophilia (i.e. APCR) or in hemorrhagic complications with an autosomal recessive mode of inheritance (Asselta et al., 2006).

1.1.1.2.1.2.1.1. FV mutations:

Twenty-four mutations were identified to result in FV deficiency; a very rare bleeding disorder (1:1,000,000). Deficiency in FV was subdivided into type I; very low or un- measurable antigen level, or type II; normal or mildly reduced antigen levels associated with reduced coagulant activity. Severe type I is termed as parahemophilia or Owren parahemophilia, which is inherited in an autosomal recessive fashion, and the mutation was identified in 1998. Sixteen mutations of the twenty-four give rise to null alleles; either due to nonsense or to frame-shift mutations, which are thought to result in mRNA instability. The other three mutations (Cys472Gly, Val1813Met, and Arg2074Cys) were found to impair FV secretion (Duga et al., 2003; Montefusco et al., 2003; Owren, 1947; van Wijk et al., 2001).

1.1.1.2.1.2.1.2. Factor V Leiden:

In 1994, a point mutation in FV (G1691A) was identified (Bertina et al., 1994). This form was termed FV Leiden, and was described as the most common genetic cause for thrombosis (Bertina et al., 1994; Svensson and Dahlback, 1994). The mutation (Arg506Gln) causes resistance to inactivation of FV by APC as it affects the 506 site, which is the first and the favorable site for proteolysis by APC, and so, interferes with the FVac activity and inactivation of FVIIIa (Bertina et al., 1994).

Thromboembolic episodes associated with FV Leiden are almost exclusively venous in nature. Although rare case reports linked FV Leiden to arterial thrombosis. FV Leiden, has been found in 30 to 60% of cases of familial thrombophilia in patients of various ethnic origins and in 3 to 7% of healthy people in two white populations. A large number of studies

has estimated the increase in risk for venous thrombosis due to FV Leiden to be 7-folds for heterozygous carriers and 80-folds for homozygous carriers of the mutation (Rosendaal et al., 1995; Simioni et al., 2005).

Besides FV Leiden, two additional FV allelic variants have been reported in patients with APC resistance: FV Cambridge and FV Hong Kong, where they affect the second and the most important site for FV inactivation and responsible for the complete loss of FVa pro-coagulant activity in vivo (i.e. Arg306), but the amino acid substitution in both is different, which is (Arg306Thr) and (Arg306Gly) for FV Cambridge and FV Hong Kong, respectively (Norstrom et al., 2002). FV Cambridge was identified in individuals with unexplained APC resistance and seems to be an extremely rare mutation. In contrast, FV Hong Kong is common in certain Chinese populations but does not appear to be a risk factor for thrombosis (Nicolaes and Dahlback, 2002). On the other hand, some articles didn't find any association between those two variants and any increase in the risk for thrombosis at the population level (Duga et al., 2004).

1.1.1.2.1.3. Methylenetetrahydrofolate reductase mutation (C677T):

5,10-Methylenetetrahydrofolate reductase (MTHFR) catalyzes a rate-limiting step in the metabolism of folate within the methionine - homocysteine pathway (Figure 1.4); it catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate for homocysteine remethylation to methionine (Fodinger et al., 2000).

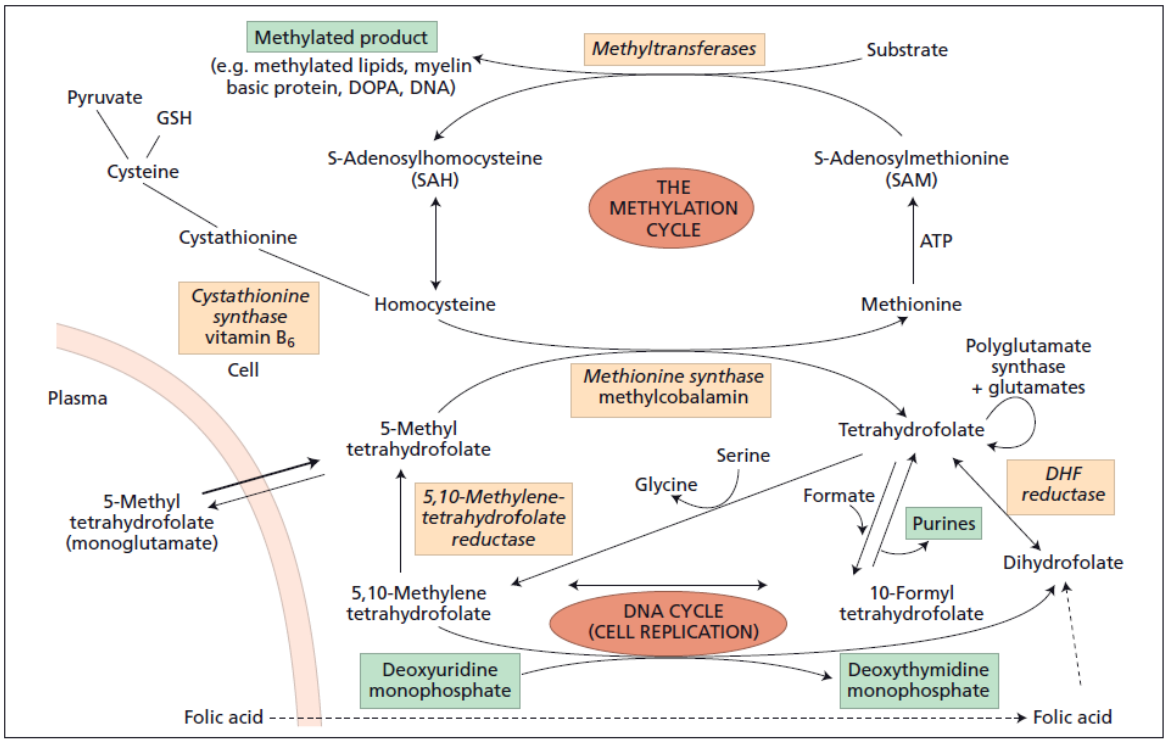

Figure 1.4: **Homocysteine and folate cycle**.

Methylenetetrahydrofolate reductase catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a cosubstrate for homocysteine remethylation to methionine. 5-Methyltetrahydrofolate is used to convert homocysteine (a potentially toxic amino acid) to methionine by the enzyme methionine synthase. Absence or low activity of the MTHFR leads to accumulation of homocysteine in cells which leads to homocysteinemia and homocysteinurea (Hoffbrand, 2011).

The gene encoding the MTHFR is located on chromosome 1p36.3 (Chromosome: 1; NC_000001.10, 11845787-11866160, complement), and is termed as (*MTHFR*) gene (Figure 1.5). It spans (20,374 bp) consisting of 11 exons (Gaughan et al., 2000; Goyette et al., 1998; Goyette et al., 1994).

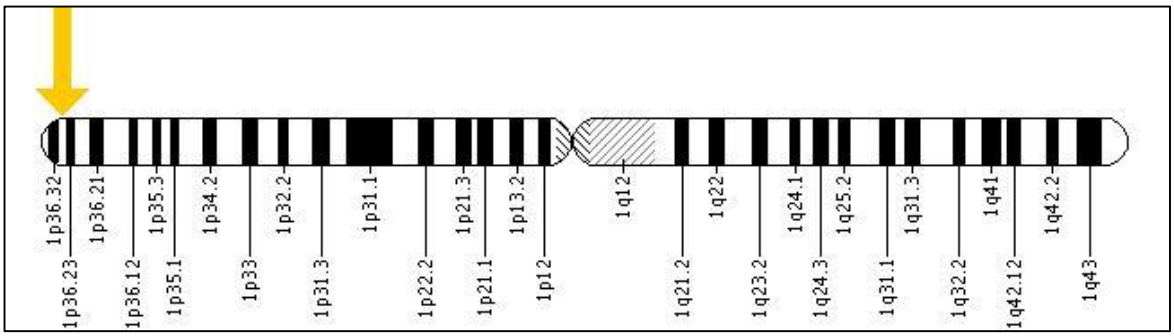

Figure 1.5: **The MTHFR gene location**.

The MTHFR gene is located on the short (p) arm of chromosome 1 at position 36.3, as indicated by the arrow (Genetics Home Reference, 2011).

The mammalian MTHFR has at least two isoforms (70 kDa and 77 kDa), and is composed of an N-terminal catalytic domain and a C-terminal regulatory domain (Tran et al., 2002). The enzyme activity could be regulated by negative feedback inhibition of dihydrofolate (DHF) and S-adenosylmethionine (SAM) (Jencks and Mathews, 1987; Matthews and Daubner, 1982; Miller et al., 1994). In addition, phosphorylation of MTHFR can decrease its activity by 20%, and this allows it to be inhibited more easily by SAM (Yamada et al., 2005). Twenty-four polymorphisms of the MTHFR were described until the 2000; two of the most investigated are C677T (rs1801133) and A1298C (rs1801131) (Sibani et al., 2000; Tran et al., 2002).

1.1.1.2.1.3.1. Hyperhomocysteinemia and MTHFR (C677T):

Hyperhomocysteinemia is a known risk factor for cardiovascular and cerebrovascular diseases (Cattaneo, 1999), atherosclerosis and recurrent arterial and venous thrombosis, usually in the third or fourth decade of life (Rees and Rodgers, 1993; Welch and Loscalzo, 1998), where plasma homocysteine concentration exceeds 15 µM (Guo et al., 2009).

In the 1995 a point mutation (C677T) was identified which results in the substitution of alanine by valine (Ala222Val) and production of a thermolabile form of MTHFR (Frosst et al., 1995), and an enzyme with 30 – 50% of the activity of the wild-type enzyme *in vitro* and is associated with hyperhomocysteinemia (Goyette and Rozen, 2000; Sibani et al., 2000). The wild type form was designated as (667CC), while the homozygous form was designated as (667TT), and the heterozygous (667CT) (Friso et al., 2002). Hyperhomocysteinemia due

to the (C677T) mutation has been suggested as a risk factor for venous thrombosis, heart diseases, strokes, hypertension, preeclampsia, glaucoma (eye disorder), cleft lip and palate, Acute Lymphocytic Leukemia (ALL) and several other diseases by interfering with the cross-linking between the collagen fibers (Boers et al., 1985; Botto and Yang, 2000; Clarke et al., 1991; Dietrich-Muszalska et al., 2012; Fodinger et al., 2000; McLean et al., 2004a; McLean et al., 2004b; Morris, 2003; Robien and Ulrich, 2003; Schmidt et al., 2011; Stampfer et al., 1992; Ulrich et al., 2003).

In 2002, two sisters were described to be homozygous for the (C677T) mutation and heterozygous for three other mutations. The problem was noticed when one of the sisters (27 years) developed pulmonary embolism due to venous thrombosis 8 months after taking oral contraceptives. On the other hand, her sister (26 years) had intellectual retardation and a slow gait. At the age of 5 years, she developed seizures, walking difficulties, and mental retardation. The father developed myocardial infarction at the age of 48 years. The mother had never expressed venous or arterial thrombosis but had been treated with folic acid because of mild hyperhomocysteinemia (Tonetti et al., 2002). In the same year (2002), a case (42 years) man was reported of renal artery thrombosis, who was homozygous for the (C677T) mutation and had a low folate level (Queffeulou et al., 2002). Another group found that both hyperhomocysteinemia due to the (C677T) mutation and factor V Leiden are risk factors for recurrent venous thrombosis (Keijzer et al., 2002). In a comprehensive meta-analysis involving 32 genes involving approximately (18,000) patients and (58,000) controls, a statistically significant association was found between ischemic stroke and the (C677T) substitution (Casas et al., 2004).

In a study, more than 7,000 newborns from 16 areas in Europe, Asia, the Americas, the Middle East, and Australia were examined for geographic and ethnic distribution of the (C667T) polymorphism (Wilcken et al., 2003). Results showed that the (667TT) genotype was common in northern China (20%), southern Italy (26%), and Mexico (32%). There was also some evidence for geographic gradients in Europe (i.e. increases from north to south) and China (i.e. decrease from north to south). The (667TT) genotype frequency was low among newborns of African ancestry, intermediate among newborns of European origin, and high among newborns of American Hispanic ancestry. The findings suggested the existence of selective pressures leading to this marked variation (Wilcken et al., 2003).

Hyperhomocysteinemia also occurs in a rare hereditary disease homocystinuria; due to cystathionine beta synthase deficiency (CBS deficiency), an inherited autosomal recessive disorder, and is associated with multi-systemic disorders of the connective tissue, muscles, neuropathy, and cardiovascular system (Reish et al., 1995).

However, acquired hyperhomocysteinemia in the absence of any mutation or polymorphism may be caused by vitamin deficiencies (e.g. folic acid (B_9), pyridoxine (B_6) or cobalamin (B_{12})), pernicious anemia, renal failure, diabetes mellitus, hypothyroidism, some carcinomas, inflammatory bowel disease, and methotrexate, theophylline, or phenytoin therapy (Guba et al., 1999; Welch and Loscalzo, 1998).

1.1.1.2.1.4. The GP (β3) (T1565C) polymorphism

Platelet adhesion, spreading and aggregation are mediated by a specific receptor on the platelet surface (glycoprotein αIIbβ3) (Bennett, 1990; 2008; Pastinen et al., 1998; Plow and Byzova, 1999).

Glycoprotein (αIIbβ3), also known as GP (IIb/IIIa), is a heterodimer transmembrane (integrin) protein composed of two subunits (α & β) (Fujimura and Phillips, 1983). Each subunit is the product of a separate gene; the (α) subunit or the (αIIb) is encoded by the Integrin αIIb (*ITGA2B*) gene; which locates on chromosome 17q21.32 (Chromosome: 17; NC_000017.10 (42,449,549 - 42,466,872), and the (β) subunit or the (β3) is encoded by the Integrin β3 (*ITGB3*) gene; which is located on chromosome 17q21.32 (Chromosome: 17; NC_000017.10 (45,331,208 - 45,390,077) (Figure 1.6).

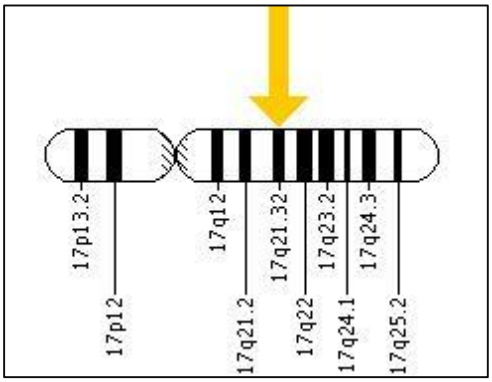

Figure 1.6: **The GP β3 gene location**.
The GP β3 gene is located on the long (q) arm of chromosome 17 at position 21.32, as indicated by the arrow (Genetics Home Reference, 2013b).

Both subunits are synthesized as single glycosylated polypeptide chains; the (*ITGB3*) gene gives the (β3) integrin, which is known, also, as (CD61) polypeptide, while the (*ITGA2B*) gene gives the (αIIb) integrin, which is known, also, as (CD41) polypeptide. Each integrin consists of a large extracellular region, transmembrane region, and a short cytoplasmic tail (Xiao et al., 2004; Xie et al., 2004; Zimrin et al., 1990), as illustrated in Figure (1.7).

GP (αIIbβ3) is the major integral plasma membrane protein in platelets; it represents (3%) of the total platelet proteins and (17%) of the platelet membrane protein mass (Phillips et al.,

1988). Each platelet expresses about (80,000 - 100,000) copies of GP (αIIbβ3) (Niiya et al., 1987; Wagner et al., 1996). The platelet α-granule membranes also contain a GP (αIIbβ3) pool, which could be expressed upon platelet stimulation (Berman et al., 1986; Wencel-Drake et al., 1986).

This protein complex has the capacity to undergo activation (i.e. transformation from low-affinity resting state to a high-affinity active state) upon stimulation by adenosine diphosphate (ADP), epinephrine, collagen, or thrombin, which allows it to act as a fibrin and von Willebrand factor receptor (Calvete, 1999; Prandini et al., 1988; Vickers, 1999). Moreover, it can recognize and bind other ligands (e.g. vitronectin, fibronectin, and thrombospondin) which mediate platelet adhesion to the subendothelial matrix and regulate platelet aggregation (Hynes, 1992; Plow et al., 2000).

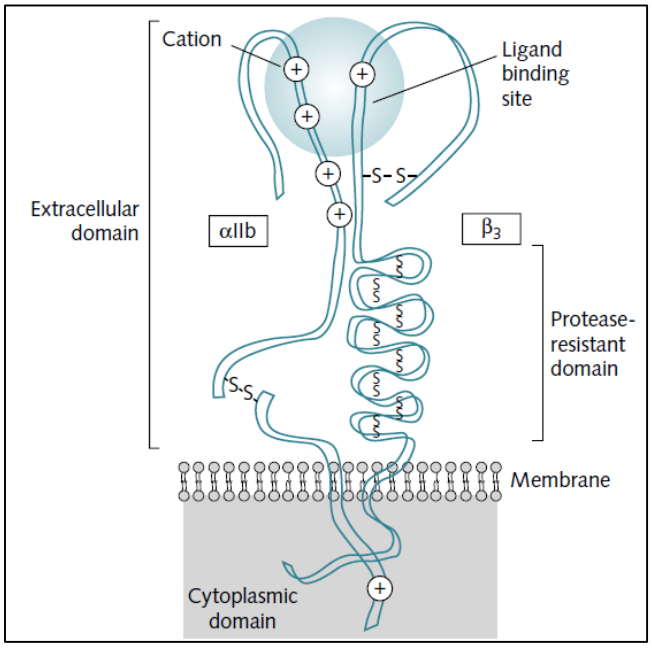

Figure 1.7: **Schematic model of Glycoprotein (αIIbβ3) structure.**
(Downes and McCrae, 2005).

In addition to their physiological role on platelets, surface proteins can express many alloantigenic determinants (Kunicki and Newman, 1986). Both (α) & (β) subunits of the GP (αIIbβ3) are known to bear a number of clinically important epitopes that are responsible for triggering alloimmune response and platelet destruction syndromes; particularly, posttransfusion purpura (PTP) and neonatal alloimmune thrombocytopenic purpura (NATP) (Kunicki and Newman, 1986; Newman et al., 1989). There are at least seven alleles of the GP (β3)

(Newman, 1994), but the most common and clinically important alloantigens are in the Platelet-specific Alloantigen (Pl(A)), which is a part of the external cellular domain of the (β) subunit of the GP (αIIbβ3) (i.e. β3). There are two serologically defined antigens of the Pl(A): Pl(A1) and Pl(A2) (Kunicki and Newman, 1986; Newman et al., 1989). These platelet alloantigens, Pl(A1) and Pl(A2), were shown to associate with (Leu33) and (Pro33) amino acid polymorphism, respectively (Newman et al., 1989). This polymorphism results from a (C1565T) substitution in the exon 2 of the *ITGB3* gene (Newman et al., 1989).

The gene frequency for Pl(A1) in Caucasians is about (85%) and for Pl(A2) it is about (2%) (Newman et al., 1989) and among the Amerindians it is (4.2%); which is lower than that obtained for blacks and whites which were (14.8% and 10%) respectively (Covas et al., 1997). The gene frequency of the (PIA2) allele in Koreans is very low (0.5%). But, no significant differences were found in the gene frequencies of the human platelet antigens in European whites (Kim et al., 1995). Since most of the population carries the Pl(A1) antigen, Pl(A2) homozygotes are at risk of producing anti- Pl(A1) antibodies against paternally inherited Pl(A1) antigens presented on fetal platelets, and are most likely to develop (NATP), or (PTP) after blood transfusion (Newman et al., 1989).

Given its importance in adhesion, spreading and aggregation of the platelets, studying of GP (αIIbβ3) polymorphisms and mutations occupied a large sector of studying and examining their role in bleeding and thrombotic events (2008). Most GP (αIIbβ3) mutations lead to an inherited autosomal recessive bleeding disorder, Glanzmann thrombasthenia; which is a disease characterized by production of defective or low levels of the GP (αIIbβ3) (Kannan and Saxena, 2009). The presence of the Pl(A2) allele was shown to associate with increased platelet activation and aggregability (Feng et al., 1999; Michelson et al., 2000), defective platelet response to arachidonic acid and thromboxane (A2) (Andrioli et al., 2000) and more tendency to bind fibrinogen (Vijayan et al., 2000). It was, also, associated with enhanced thrombin formation and impaired antithrombotic action of aspirin, which might favor coronary thrombosis in the Pl(A2) carriers (Undas et al., 2001). Pl(A2) homozygosity is associated with a three to four-fold risk of ischemic and thrombotic cardiovascular disease and MI in young men (Bojesen et al., 2003a; Byzova and Plow, 2000; Galasso et al., 2010), increased risk for cancers (Bojesen et al., 2003b) and a two-fold risk of hip fracture, mainly confined to postmenopausal women (Tofteng et al., 2007). Furthermore, Pl(A2) positive individuals show significantly shorter bleeding times than Pl(A1) individuals (Bray, 2000). The PlA2

polymorphism also enhances cell spreading, actin cytoskeleton rearrangement and clot retraction (Bray, 2000), and a lowers the threshold for GP (αIIbβ3) activation and α-granules release of the protein (Michelson et al., 2000). Moreover, it was shown that having Pl(A2) antigen might have implications for antiplatelet therapy of patients with MI (Grove et al., 2004).

However, the clinical implications of these in vitro studies and the role of GP (αIIbβ3) polymorphisms in genetic susceptibility to thrombotic diseases remain controversial (Bray, 2000); some data suggest that the Pl(A2) allele may be a weak risk factor for myocardial infarction in young men (Clemetson, 2008). Moreover, many other research groups didn't find any contribution of the Pl(A1)/Pl(A2) polymorphism in developing of coronary atherosclerosis or the genetic susceptibility to premature myocardial infarction (Lagercrantz et al., 2003) or any association with DVT (Renner et al., 2001).

1.2 β-Thalassemia

Beta Thalassemia (βT) is a congenital extra-vascular hemolytic anemia that is caused by a defect in the β- globin gene, which results in a reduction (β^+), or absence (β^0), of the production the β- globin chains, an integral component of the heterodimer ($\alpha_2\beta_2$) adult hemoglobin (Hb A) molecule. This disease affects multiple organs and systems, and is associated with considerable morbidity and mortality around the world (Cohen et al., 2004). Thalassemia is among the most common genetic disorders worldwide; about 4.83% of the world's population carry globin variants, including 1.67% of the population who are heterozygous for α-thalassemia and β-thalassemia (Skordis et al., 1998). It was estimated that the incidence of symptomatic individuals is about 1 in 100,000 throughout the world and 1 in 10,000 people in the European Union (Vichinsky, 2005). The prevalence of β-thalassemia trait among the Palestinian population in the West Bank was estimated to be 3.5% (Darwish et al., 2005).

One of the main problems in β-thalassemia is the imbalance in the production of both globin chains (α and β) which constitute the adult hemoglobin. In β- thalassemia, excess α- globin chains precipitate in the RBCs and mainly on their plasma membranes which results in the reduction of the life span of the cells, and eventually, evolution of hemolytic anemia (Taher et al., 2009; Weatherall and Clegg, 2001).

Classification of βT depends on several factors; such as the clinical picture of the disease, the severity of the anemia and blood transfusion requirement. Depending on these factors and others (i.e. genetic) thalassemia could be classified into β-thalassemia minor, thalassemia intermedia (TI) and β-thalassemia major (β-TM) (Musallam et al., 2012; Olivieri, 1999; Rund and Rachmilewitz, 2005).

1.2.1. β-Thalassemia major (β-TM):

This type was formerly named "Cooley's anemia" after Dr. Thomas Benton Cooley, who was the first to describe this syndrome in 1925 in five young children with severe anemia, splenomegaly, and peculiar bone abnormalities (Cooley and Lee, 1925). It is the most severe case; where the patient expresses the homozygous state (β^0/β^0) having almost complete loss of the β- chain production, and expressing fetal hemoglobin (Hb F) as the main hemoglobin in his RBCs (**Error! Reference source not found.**). Patients present with severe microcytic

hypochromic anemia between 6 and 24 months of age, fail to thrive and become progressively pale. They suffer from severe bone deformities and progressive abdominal enlargement caused by splenohepaotmegaly. Management is achieved by periodic lifelong blood transfusion, with iron chelation and splenectomy in parallel, which, if taken regularly, makes the patient to progress to live beyond 40 years (Galanello and Origa, 2010). Currently, the available way of possibly curing this disease is bone marrow transplantation (Weatherall, 2004). Gene therapy, on the other hand, is a promising strategy for curing thalassemia (Lisowski and Sadelain, 2008; Quek and Thein, 2007; Xie et al., 2007).

1.2.2. β-Thalassemia minor:

Here, patients have a heterozygous state (β^0/β or β^+/β) and show minor loss of β- chain production, and slight microcytic (MCV 50 – 70 fL) hypochromic (Hb 9 – 11 g/dL) anemia. The red cell count is usually normal or elevated, with an increase in hemoglobin A_2 (Hb A_2) fraction (3.5 – 7%) and dominancy of the adult hemoglobin (Hb A) fraction, and the fetal Hb sustains normal with rare elevation above 5%. Patients sustain in a clinically asymptomatic status until they are exposed to a stressful situation or disease where sometimes supporter medication is needed (Weatherall, 2006).

1.2.3. Thalassemia intermedia (TI):

Naming of this type of thalassemia "Intermedia" was put in 1955 by Dr. Sturgeon, which came from the clinical situation of the patients who express symptoms that are too severe to be termed minor and too mild to be termed major with moderate to severe anemia (Musallam et al., 2012; Sturgeon et al., 1955). Most TI patients are homozygotes or compound heterozygotes (β^+/β^+ or β^+/β^0) for one or more beta-thalassemia mutations (Galanello and Cao, 1998). Finding a relationship between genotype and phenotype in TI is so complicated, and many modifiers can affect the outcome of a certain mutation (Galanello and Cao, 1998).

Thalassemia intermedia is not a definite type that is produced due to a defined genetic defect; rather, it is a collection of factors combined together to produce this phenotype (Taher et al., 2009). For example, coexistence of α- thalassemia trait decreases the severity of the disease due to the decrease in the imbalance between α and β chains (Weatherall, 1995). Another

factor, is the coinheritance of a mutation in the γ gene (i.e. γ^G XmnI); which is a polymorphism that results in an over production of the γ- chain and helps to neutralize large proportion of unbound α- chains which produces higher levels of Hb F (Camaschella et al., 1995). On the other hand, the TI phenotype may result from increased production of α-globin chains by a triplicated or quadruplicated alpha genotypes associated with the existence β-thalassemia heterozygosity (Camaschella et al., 1997). Other criteria are followed to differentiate between β-TM and TI as shown in Table (1.3) (Taher et al., 2009).

Table 1.3: **Thalassemia Intermedia vs. β-Thalassemia Major.**

Parameter	β-TM* more likely	TI* more likely
Clinical		
Presentation year	< 2	> 2
Liver/Spleen enlargement	Severe	Moderate to severe
Hematological		
Hb level (g/dL)*	6 – 7	7 – 10
Hb F	> 50	10-50 (may be up to 100%)
Hb A_2	< 3.5	> 3.5
Genetic		
Parents	Both carriers of high Hb A_2 β-thalassemia	One or both atypical carrier: - High Hb F β-thalassemia - Borderline Hb A_2
Molecular		
Type of mutation	Severe	Mild/Silent
Co-inheritance of α-thalassemia	No	Yes
Co-inheritance of extra α-alleles	Yes	No
HPFH*	No	Yes
δβ-thalassemia	No	Yes
γ^G XmnI polymorphism	No	Yes

β-TM: β-Thalassemia Major. TI: β-Thalassemia Intermedia. Hb: hemoglobin. HPFH: Hereditary Persistence of Fetal Hemoglobin.

1.2.3.1. Pathophysiology in TI:

A good and acceptable phenotype scoring system was proposed in 2003 tried to sub-classify TI patients into three separate groups: mild, moderate and severe. The severity of TI was graded according to a number of clinical features; such as age at presentation, severity of anemia, extent of growth retardation, bone marrow hyperplasia, blood transfusion requirements and need for splenectomy (Phadke and Agarwal, 2003).

The disease manifests itself in many aspects; in one hand, hemolysis and ineffective erythropoiesis, due to the precipitation of the unstable α- chains within erythroid precursors in the bone marrow causing membrane damage and cell death, are the cause of the anemia (Pootrakul et al., 2000). On the other hand the bone marrow mass expands due to Hypertrophy of erythroid lineage in medullary and extramedullary sites in order to compensate the loss in the RBC compartment in the peripheral blood. Consequently, characteristic bone deformities and shape changes result in thalassemic patients (Olivieri, 1999). Add to this, the iron overload complications which develop primarily due to increased intestinal iron absorption, in contrast to patients with β-TM, in whom iron loading occurs mainly as a result of the blood transfusion therapy (Origa et al., 2007). Iron deposits in various body tissues causing severe toxicity for many organs; including endocrine disturbances (e.g. diabetes mellitus and hypogonadism) typically by the fourth decade of life (Taher et al., 2009). Furthermore, high incidence of pigment gallstones, bone and joint disease and leg ulcer are observed in these patients (Taher et al., 2009).

Consequently, several complications have been identified in patients with TI, and some of these complications have been noticed to be unique to TI over β-TM (Table 1.4), especially in splenectomized transfusion naive patients (Taher et al., 2006a). Cholelithiasis gallstones are much more common in TI than in β-TM patients because of ineffective erythropoiesis and peripheral hemolysis (Galanello et al., 2001). Moreover, Gilbert's syndrome has been reported to increase gallstone formation in patients with thalassaemia (Borgna-Pignatti et al., 2003; Galanello et al., 2001). Leg ulcers are more common in older patients with TI than younger patients (Taher et al., 2006a). It was noticed that ulcers develop in some patients who are maintained at relatively low Hb levels and have the same amount of Hb F as others whom ulcers do not develop in, due to an unclear reason, and these patients suffer from

fragile, very painful and difficult to cure skin lesions at their extremities (Musallam et al., 2012).

Pulmonary hypertension is considered as a part of the clinical complications for adult β-thalassemia patients, with a frequency ranging from 10% to 74% with higher rate in TI patients (Aessopos and Farmakis, 2005).

Extra-medullary hematopoiesis is a compensatory mechanism that attempts to overcome the chronic anemia of TI patients by forming erythropoietic tissue masses in the spleen, liver and lymph nodes. However, this tissue can cause neurological problems such as spinal cord compression and paraplegia, and intra-thoracic masses (Castelli et al., 2004; Haidar et al., 2010).

Table 1.4) provides some clinical complication that were recorded in TI and β-TM patients, with a focus on some complications that were noticed to be unique to TI patients over β-TM patients (Taher et al., 2009).

Table 1.4: **Prevalence of complications in TI vs. β-TM.**

Complication (% of patients affected)	**TI***		**β-TM***	
	Lebanon (n=37)	**Italy (n=63)**	**Lebanon (n=40)**	**Italy (n=60)**
Splenectomy	90	67	95	83
Cholecystectomy	85	68	15	7
Gallstones	55	63	10	23
Extramedullary hemopoiesis	20	24	0	0
Leg ulcers	20	33	0	0
Thrombotic events	28	22	0	0
Cardiopathy	3	5	10	25
Pulmonary hypertension	50	17	10	11
Abnormal liver enzymes	20	22	55	68
Hepatitis C virus infection	7	33	7	98
Hypogonadism	5	3	80	93
Diabetes mellitus	3	2	12.5	10
Hypothyroidism	3	2	15	11

* β-TM: β-Thalassemia Major. TI: Thalassemia Intermedia.

1.2.3.1.1. Thalassemia and hypercoagulability:

Among the complications that were correlated with different hemolytic anemias (HA) including thalassemias are thrombotic and hypercoagulability states (Eldor and Rachmilewitz, 2002; Taher et al., 2008). Deep venous thrombosis (DVT), pulmonary embolism, leg ulcers and recurrent arterial occlusion have been described in patients with β-TM and TI from many countries (Akar et al., 1998; Cappellini et al., 2000; Taher et al., 2006b). Most cases developed thrombosis spontaneously with no known risk factors, although some cases developed venous thrombosis after splenectomy (Michaeli et al., 1992; Taher et al., 2006b). Evidence of asymptomatic brain damage, including ischemic lesions, has been reported by magnetic resonance imaging (MRI) in patients with TI and it was noticed to be inversely correlated with Hb level and age (Manfre et al., 1999). Defective platelet aggregation in response to adenosine di-phosphate (ADP), epinephrine and collagen was noticed in β-TM patients (Eldor, 1978; Hussain et al., 1979). On the other hand, splenectomy increases the number of circulating platelets, which leads to chronic platelets activation and shortened platelets life span caused by enhanced platelets consumption in β-TM and TI patients (Eldor et al., 1989). Furthermore, increased circulating platelet aggregates was shown in 71% of splenectomized compared to 35% of non-splenectomized patients with TI/hemoglobin E disease (Winichagoon et al., 1981). The existence of chronic platelet activation in thalassemia patients was confirmed by flow-cytometric studies, which demonstrated the presence of an increased fraction of platelets carrying the activation markers CD62P (P selectin) and CD63 (Del Principe et al., 1993; Ruf et al., 1997). Moreover, elevated plasma platelet factor 3 (PF3) was noticed in TI/Hb E patients, which increases platelet aggregation (Opartkiattikul et al., 1992a; Opartkiattikul et al., 1992b).

Several etiologic factors are thought to play a role in the pathogenesis of the hypercoagulable state in thalassemia. Specific changes in the lipid membrane composition of the abnormal thalassemic RBCs and the effect of hemosiderosis may contribute to the activation of the coagulation cascade and the activation of other blood cells, including the platelets, monocytes, granulocytes and the vascular endothelium, which affect the thrombotic process (Eldor and Rachmilewitz, 2002). Moreover, RBCs of thalassemic patients express higher levels of reactive oxygen species and lower levels of intracellular glutathione than do normal erythrocytes, which could be attributed to continuous exposure to oxidative injuries (Visudhiphan

et al., 1994). This leads to the expression of surface anionic phospholipids such as phosphatidylethanolamine (PEA) and phosphatidylserine (PS), and a loss in the cell membrane asymmetry (Manfre et al., 1999). RBCs of patients with β-TM and TI showed enhanced adhesion to cultured endothelial cells (Hovav et al., 1999). They also, were noticed to facilitate thrombin formation (Eldor et al., 1999) and, subsequently, platelets activation in vivo (Ruf et al., 1997). RBCs expressing PS may also contribute directly to the vascular damage observed in thalassemia patients (Manodori et al., 2000). On the other hand, RBCs of β-TM and TI patients showed increased amounts of membrane-bound hemichromes and immunoglobulins. In addition, band 3 protein showed oxidative modifications such as aggregation and a decrease in the sulfhydryl groups (Cappellini et al., 1999; Mannu et al., 1995).

Studies of the coagulation proteins provided strong evidences for the existence of a chronic hypercoagulable state in thalassemia (Michaeli et al., 1992). Many reports recorded changes in the levels of coagulation factors, coagulation factor inhibitors, and components of the fibrinolytic system. Low levels of protein C and protein S have been observed in patients with β thalassemia from a variety of ethnic backgrounds (Cappellini et al., 2000; Shirahata et al., 1992). In Israeli patients, mostly of Kurdish Jewish, Yemenite Jewish or Arabic origin, protein C (antigen and activity) and free protein S were significantly decreased in both adults and children (Eldor et al., 1999). Similar results were obtained in studies of TI patients in Italy (van Teunenbroek et al., 1989).

Currently, it is assumed that the mentioned defects and abnormalities could explain many clinical observations that patients with TI show. Venous thrombosis is more prevalent in TI patients who are not receiving regular transfusions and have undergone splenectomy (Cappellini et al., 2000; Deo et al., 1994). These patients may be more susceptible to thromboembolism because they have more circulating platelets and damaged RBCs. It was noticed that thalassemic patients in less developed countries who have limited transfusion resources showed more thromboembolic manifestations than those in developed countries. This was explained by, having normal (transfused) RBCs decreases abnormal aggregations observed in thalassemic RBCs, which indicates beneficial role of regular blood transfusions for those patients (Chen et al., 1996).

1.3 Literature review

Many studies have investigated the correlation between thrombotic manifestations and β-thalassemia major and intermedia. In a multicenter study in Italy involving nine Italian pediatric thalassemia centers, thromboembolism was investigated in patients with β-TM and TI. The incidence of thromboembolic events was lower (3.95%) in β-TM patients than TI patients (9.61%) (Borgna Pignatti et al., 1998). The same group reported a lower incidence (1.1%) of thromboembolic complications among β-TM patients whom were followed for 37 years (Borgna-Pignatti et al., 2004). In another study, also in Italy, the prevalence of thromboembolic events was 3.3% among β-TM patients compared to 16.2% among TI patients, although 15.3% of these patients (i.e. β-TM and TI) had predisposing congenital or acquired factors contributing to the hypercoagulability (Moratelli et al., 1998). Another group followed 83 TI patients for 10 years for the incidence of venous thromboembolic events (VTEs); pulmonary embolism, DVT, or portal vein thrombosis developed in 29% of them, and recurrent VTEs occurred all splenectomized patients (Cappellini et al., 2000). In a comprehensive study, included 8,860 thalassemia patients (6,670 β-TM and 2,190 TI) in the Mediterranean region (Lebanon, Italy, Israel, Greece, Egypt, Jordan and Saudi Arabia) in addition to Iran, showed 1.65% prevalence of various thrombotic events; which is 4.38 times more frequent in TI than β-TM patients (i.e. 0.9% in β-TM and 4.0% in TI). Furthermore, venous events were more frequent in TI, while arterial events were more frequent in β-TM (Taher et al., 2006b).

A study in Italy examined thrombotic mutations in 580 patients with β-TM. Thirty-three were with history of one or more thrombotic episodes, and 29 were splenectomized. Their mean Hb level was (9.07 ± 0.3 g/dL) and their mean transfusional interval was (22 ± 3 days). The tested mutations were FV Leiden (G1691A), FII (G20210A), MTHFR (C677T), GP (αIIbβ3) (C1565T) and FVII 4 hyper-variable region (HPVR). No significant differences between the thrombotic thalassemic group and the control group for all analyzed polymorphisms were detected except the FVII polymorphism (Iolascon et al., 2001).

A case study article was published in 2004 about a Lebanese patient with S/β° that suffered from pain in the bilateral shoulder and left knee areas. The case was diagnosed initially to

have sickle cell crisis, but duplex scanning revealed a DVT case. The patient showed homozygousity for FV Leiden and heterozygousity for MTHFR mutation. The patient, then, was treated with warfarin and did well (Otrock et al., 2005).

In another study a 24-year-old man with β-TM who expressed three recurrent thrombosis in a short time interval, the first was at the age of 19, so, he was treated on low molecular weight heparin (LMWH) followed by long-term oral anticoagulant with a target International Normalized Ratio (INR) of 2.5. At the age of 22, a DVT of the left popliteal vein occurred. Then the INR was decided to be increased from 2.5 to 3.5, so, no other thrombotic event occurred until the article date. The patient had double heterozygosity for FV Leiden and FII G20210A mutations, which led the authors to suggest that thalassemia patients should be screened for acquired and congenital thrombophilia (Kahn et al., 2002).

1.4 Problem statement

The prevalence of the selected gene mutations that could be associated with hereditary thrombophilia including FV Leiden, MTHFR C677T, FII G20210A and platelet GP (β3) T1565C polymorphism among thalassemia intermedia patients in the West Bank–Palestine, is not known. The incidence of thrombotic manifestations among thalassemia intermedia patients in the West Bank, Palestine and their correlation to genetic thrombotic mutations is not known.

1.5 Goals

1. Investigating the prevalence of thrombosis among Palestinian TI patients in the West Bank.
2. Determine the prevalence of the indicated gene mutations (FV Leiden, MTHFR C677T, FII G20210A and platelet GP (β3) T1565C) among the thalassemia intermedia patients in the West Bank-Palestine.
3. Increasing the awareness against thromboembolic events and their manifestations in thalassemic patients, especially, those who are positive for those mutations and to take care of the environmental risk factors for these patients.

1.6 Justification

The prevalence of β- thalassemia trait among the Palestinian population is reasonable to some extant; and the prevalence of the trait type among Palestinian population is istimated to be 3.5% (Darwish et al., 2005). One of the manifestations of β-thalassemia, especially intermedia, the tendency to have severe thromboembolic manifestations, so screening them for those mutations may serve as a first step in reducing the risk of having thrombosis in future.

1.7 Questions

Our raised questions are:

1. What is the frequency of the thrombotic genetic mutations (FV Leiden, MTHFR C677T, FII G20210A and platelet GP (β3) T1565C) mutations among TI patients in the West Bank-Palestine?
2. What is the frequency of the thrombotic manifestations among TI patients in the West Bank-Palestine?

1.8 Hypothesis

Two hypotheses were presented; one of them will be proven and the other will be rejected; the null hypothesis (H_0) will be returned if no correlation was confirmed between having thalassemia intermedia and having one of the tested polymorphisms. On the other hand, the alternative hypothesis (H_1) will be retained if the H_0 was rejected.

Chapter Two

Methodology

2.1 Materials

Table (2.1) lists all materials that were used in the study.

Table 2.1: **List of materials and instruments used in the study.**

Disposables	
Item	**Manufacturer/country**
EDTA tubes	Greiner bio-one. UK
Needles	Medi-Plus. China
Syringes	Medi-Plus. China
Chemicals and kits	
Item	**Manufacturer/country**
DNA extraction kits from blood	MasterPure™ DNA Purification Kit for Blood Version II, Epicentre, USA
lyophilized PCR master mix	AccuPower® HotSart PCR PreMix, BIONEER, Korea
*Hin*fI	NEW ENGLAND BioLabs. UK
*Msp*I	NEW ENGLAND BioLabs. UK
*Hin*dIII	NEW ENGLAND BioLabs. UK
50 bp leader marker	GeneDirex
TRIS base	MP Biomedicals. USA

Chemicals and kits	
Item	**Manufacturer/country**
EDTA-Na bi-basic	AVONCHEM Ltd. UK
Agarose	Hy.labs. Israel
Ethidium bromide	Hy.labs. Israel
Machines and instruments	
Item	**Manufacturer/country**
Thermal cycler	Esco Healthcare Ltd. USA
Nano-drop machine	Thermo Scientific. USA
Gel electrophoresis system	Scie-Plas. UK
Gel documentation system	BIO-RAD GEL DOC 2000®, USA

The sequence of the PCR primers are shown in Table (2.2). All PCR primers were obtained from Metabion (Germany).

Table 2.2: **PCR primers used in this study**.

Primer	Sequence (5′ - 3′)	Gene Bank Accetion no.	Gene
FVL S	TCA GGC AGG AAC AAC ACC AT	NG_011806	*FV*
FVL A	GGT TAC TTC AAG GAC AAA ATA CCT GTA AAG CT	NG_011806	FV
FII S	GCA CAG ACG GCT GTT CTC TT	NG_008953	*FII*
FII A	ATA GCA CTG GGA GCA TTG AAG C	NG_008953	*FII*
MTHFR S	TGA AGG AGA AGG TGT CTG CGG GA	NG_013351	*MTHFR*
MTHFR A	AGG ACG GTG CGG TGA GAG TG	NG_013351	*MTFR*
GP β3 S	TTC TGA TTG CTG GAC TTC TCT T	NG_008332	GP β3
GP β3 A	TCT CTC CCC ATG GCA AAG AGT	EU015082.1	GP β3

2.2 Methods

2.2.1. Study subjects:

Our target group was all thalassemia intermedia patients throughout the West Bank – Palestine. List of the patients' names and contact informations were obtained from the patients' registration files at the TPFS – Palestine and the clinical care centers that are supervised by the Ministry Of Health. Then, patients were interviewed to fill a questionnaire and to obtain a blood specimen. The inclusion criteria were: presentation of the disease after 2 years of birth with fetal hemoglobin (Hb F) level (10 - 50%) (Table 1.3). Information concerning disease onset and Hb F level were obtained from the patient files. Patients who refused to participate or could not be reached were cancelled.

A matched group from apparently normal individuals (i.e. normocytic normochromic, Hb $\geq$ 12.0 g/dL for females and $\geq$ 13.5 g/dL for males) was collected from the same geographical places where patients' samples were collected, and were used as a control group.

2.2.2. Questionnaire and consent form:

An interview-based questionnaire was developed for this study (Appendix A). The questionnaire was designed to collect information about the demographic information and medical history of the patient. Patients were contacted by telephone, invited for participation in the study and if they accepted the invitation, they were invited for a personal interview at the nearest clinical care center. During the interview, patients were asked to provide the information needed to complete the questionnaire. After that, they were asked to donate a blood sample and to provide a written consent, and those who were younger than 18 years were accompanied by their guardian and the guardian was asked to sign a consent form.

2.2.3. Specimen collection, transporting and preservation:

One to three milliliters of venous blood was collected in a K_3EDTA tubes from each patient. Specimens were transported from the blood collection site to the lab at Al- Quds university; where they were processed within one week of collection. Specimens were transported in a temperature-insulating box and kept at 6 $\pm$ 2°C from the time of specimen collection until the preparation of genomic DNA.

2.2.4. Preparation of genomic DNA:

Genomic DNA was prepared from the buffy coat using a commercially available kit (MasterPure™ DNA Purification Kit for Blood Version II, Epicentre, USA). Briefly, whole blood was centrifuged at 3,500 x g for 3 – 5 minutes. Then, 600 µL of the buffy coat were transferred into a 1.5 mL tube, mixed well, and separated into two equal portions. This was followed by the addition of 1.2 mL Red Cell Lysis (RCL) buffer to each tube in order to lyse the RBCs. The tubes were, then, mixed by inversion three times with flicking the bottom of the tubes; in order to re-suspend the WBCs. Then, the mixture was incubated at room temperature for 10 minutes with shaking every 5 minutes. After that, precipitation of the WBCs was achieved by centrifugation at 10,000 × g for 25 seconds. This, usually, resulted in a small white pellet that settled underneath a clear reddish supernatant, indicating a good RBC lysis step. If the supernatant was not clear or the pellet contained a reasonable amount of intact RBCs, the RBC lysis step was repeated by adding additional RCL buffer. The supernatant was discarded and the pellet was re-suspended in about 25 µL of the left over. Then, the WBCs were lysed by emulsifying the cells in 600 µL of the Tissue and Cell Lysis (TCL) solution. The highly viscous solution was pipetted several times in order to insure complete cell lysis. The next step was the protein precipitation step; that was achieved by salting out and involved the addition of 250 µL of the MPC solution (a highly concentrated; 6M, NaCl aqueous solution) followed by vigorous shaking of the solution for 15 – 30 seconds. After that, clear clumps would appear indicating the success of the protein salting out process. Retaining a clear DNA solution and getting rid of the protein debris was achieved by centrifugation at 10,000 × g for 10 min. Then, the supernatant was transferred into a new 1.5 mL tube and the former tube was discarded. For precipitation of the DNA from the solution, about two volumes (i.e. 700 µL) of absolute isopropanol were added to each tube, then, tubes were inverted 30 – 40 times. The sample was centrifuged at 10,000 x g for 10 minutes, then, the supernatant was discarded and the pellet was rinsed with 500 µL of 70% ethanol and the pellet was left on (40 °C) in order to get rid of the volatile alcohol. Finally, hydration of the DNA was done by the addition of 100 µL of 1/2 TE buffer with pH 7.5, and the DNA solution was preserved at -20°C and later used for DNA amplification.

2.2.5. Assessment of DNA concentration and purity:

Assessment of the DNA concentration and purity were determined spectrophotometrically by the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific®). Purity and DNA concentration were assessed by measuring the optical densities A_{260}, A_{280}, A_{230}. Samples that gave $A_{260/280}$ ratio greater than (1.8) indicating a good purity and minimum protein interferences of the DNA preparation were used for PCR amplification. All samples gave $A_{260/230}$ ratio greater than (2.0); which infers acceptable salt interferences. All preparations gave higher concentrations than (100 µg/mL); and those with very high concentrations were diluted to (100 µg/mL).

2.2.6. Mutation analysis:

The FVL G1691A, FII G20210A (Huber et al., 2000), MTHFR C677T (Frosst et al., 1995) and GP β3 T1565C (Pellitero et al., 2010) mutations were analyzed by restriction fragment length polymorphism polymerase chain reaction (RFLP-PCR) technique as described earlier. The sequence of PCR primers and PCR amplicon size are shown in Table (2.3). However, the amplification of the FII G20210A fragment was modified by designing a new forward primer to shorten the PCR amplicon to 264 bp, and thus the products of *Hin*dIII restriction digestion (232 bp for mutant amplicon and 264 bp for normal/ wild type amplicon) are easily resolved by agarose gel electrophoresis, compared to the primer pair described earlier (Huber et al., 2000); that produces an amplicon of 506 bp. This amplicon gives 384bp product for mutant amplicon and 407 bp for normal/ wild type amplicon upon digestion with *Hin*dIII, which are poorly resolved by agarose gel electrophoresis.

Table 2.3: P**rimers and mutations' detection parameters.**

Mutation	**Annealing Temp. (°C)**	**PCR amplicon (bp)**	**Restriction Enzyme**	**Restriction Products (bp)**		**References**
				Normal	Mutant	
FVL (G1691A)	55	241	*Hin*dIII	241	209 + 32	(Huber et al., 2000)
FII (G20210A)	55	264	*Hin*dIII	264	232 + 32	Modified from (Huber et al., 2000)
MTHFR (C677T)	56	198	*Hin*fI	198	175 + 23	(Frosst et al., 1995)
GP β3 (T1565C)	56 & 55*	266	*Msp*I	221 + 45	177 + 44 + 45	(Pellitero et al., 2010)

∗ The amplification of the GP β3 gene we the step – down way for primer annealing temperature was used; where 56 ºC was used for 15 cycles followed by 20 cycles at 55 ºC.

2.2.6.1. Gene Amplification:

Gene amplification was achieved by Polymerase Chain Reaction (PCR) technique, where lyophilized master mix kit (AccuPower® HotSart PCR PreMix, BIONEER, Korea) was used. The PCR master mix contained a lyophilized mixture of Hot-start Taq DNA Polymerase, Buffer, $MgSO_4$, dNTPs and the loading buffer. Primers and DNA samples as well as the nuclease-free water were added to the mix (Table 2.4). The PCR components were mixed in the provided (0.2 mL) test tubes and the reactions were run using a thermal cycler (Esco Healthcare Ltd. USA) was used. Thermal cycling programs and the annealing temperatures of the primers are shown in Table (2.5).

Table 2.4: **Components of PCR reaction.**

Reaction component	Volume (µL)
Lyophilized master mix	0
DNA sample (100 µg/mL)	3
Forward Primer (10µM)	0.5
Reverse Primer (10 µM)	0.5
DEPC- Water	16
Total Volume	20

Table 2.5: **Programs used for gene amplification.**

Step	Duration	Temperature (°C)
Initial denaturation	5 min.	94
35 cycles:		
Denaturation	30 sec.	94
Annealing	30 sec.	55 or 56*
Extension	1min.	72
Final extension	5 min.	72

*An annealing temperature of (55 °C) was used for FVL and FII G20210A, and (56 °C) for the MTHFR. A step down annealing temperature was used for the GP β3; (56 °C) for 15 cycles followed by (55 °C) for 20 cycles.

The PCR products were analyzed by running 5 µL of the PCR product on 3% agarose gel at 100 Volts and the band in question was detected according to Table (2.3).

2.2.6.2. Restriction Digestions:

Samples that gave the expected PCR amplicon in the previous section were further subjected to restriction digestion with the appropriate restriction enzyme as shown in Table 2.6). All restriction reactions were incubated at 37°C for at least 4 hours (Table 2.6). All restriction enzymes were obtained from New England BioLabs®, UK.

Table 2.6: **Components of the restriction digestion reaction of PCR amplicons.**

Reaction component	Volume (µL)
PCR product	10
Restriction Enzyme (2000 U)	0.3
Buffer	2
Purified Water	7.7
Total Volume	20

The entire product of the restriction digestion (about 20 µL) was run on a 3% agarose gel at 100 V for about 45 minutes, or until the DNA fragments are resolved. After that, the gel was viewed and photographed using a gel documentation system (BIO-RAD GEL DOC 2000®, USA) in order to detect and discriminate between normal, heterozygous and homozygous bands (Table 2.3).

2.2.7. Data analysis:

Data were analyzed using two softwares; Microsoft Excel 2013® and SPSS 17®. Drawing tables and building figures were executed using the Excel, while the association analyses were done using the SPSS. The test for association was used to test the significance of the relationships between the variables was the Chi-square, and a *p*-value less than 0.05 was considered significant.

Chapter Three

Results

3.1 Study population

The exact number of thalassemia patients or their classifications into major, intermedia or minor is not precisely known in Palestine. A patient who is registered in one health care center is not necessarily registered in the TPFS, and vice versa. Therefore, the registration files of those patients are not identical between the two sources that cooperated with us; TPFS and the health care clinics that are subordinated by the Ministry of Health. Many files did not contain any information about the medical history or diagnosis of the patient, except his/her personal information. Some patients were classified as β-TM in some files while others considered them as TI. Therefore, we went over the files and made our classification according to the criteria mentioned in Table (1.3), and those who met the TI criteria were included in the study.

One hundred forty-eight patients have fulfilled the criteria of TI and thus were approached to participate in this study. Then we tried to reach all of them by phone, but many refused to participate or didn't attend on the assigned dates to meet them at the health care center close to his/her residence place. In total, we have succeeded to recruit 82 TI patients. These 82 patients provided all the information needed to complete the study questionnaire and donated blood samples for the study. In Hebron there was a good response from TI patients compared to other cities, where 48 out of the 65 TI patients participated in this study. The

lowest response was in Ramallah; where 10 out of the 48 TI patients (21%) were included in the study. This low rate of participation observed among TI patients from Ramallah can be attributed to several factors. Three of the 38 patients, who were not included in this study, couldn't be reached neither by phone nor by a specific address, moreover, they didn't follow their medication for several years. Thirteen patients were residing abroad during the period of the study (10 in the United States of America and 3 in Jordan), three refused to participate, one was in the Israeli prison and the rest promised to participate but they didn't come on the assigned dates. Finally, five TI patients were registered in Jenin and all of them participated in this study.

Table (3.1) shows the distribution of all TI patients registered at the TPFS and the clinical care centers among cities of West Bank, as well as the distribution control group. This classification is consistent with their residence place in most cases with some exceptions; where some patients are registered in a health care center (or a branch of the TPFS) and are resident at a different place.

Table 3.1: **Distribution of TI patients among the different cities in the West Bank region.**

Thalassemia Care Centers	**Patients**		**Control subjects**
	No. registered	**No. participated in this study**	
Hebron	65	48	48
Ramallah	48	10	10
Nablus	20	12	12
Tulkarm	10	7	7
Jenin	5	5	5
Total	148	82	82

The highest proportion of the thalassemia intermedia cases in the West Bank was reported in Hebron (44%), while the lowest number of cases was reported in Jenin (3.4%) (Table 3.1). However, the number of patients reported in each city showed a gradual decline from the south to the north (Figure 3.1 and Table 3.1).

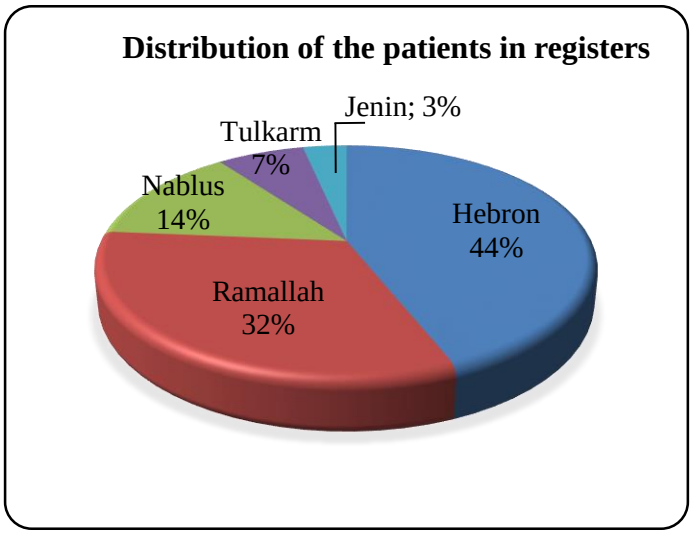

Figure 3.1: **Distribution of thalassemia intermedia patients among the different care centers / cities in West Bank.**

About 55% (82 of 148) of the whole registered patients were included in our study; with a highest response from Jenin area (100%), but with the lowest percentage was from Ramallah (21%). However, the highest number of thalassemia intermedia participants who responded to the study was from the Governmental Hospital of Hebron (representing 58% of study samples). Patients from the northern area; Jenin, Tulkarm and Nablus composed 6%, 9% and 15% of the total study sample, respectively. While patients from Ramallah represented 12% of the total study samples (Figure 3.2).

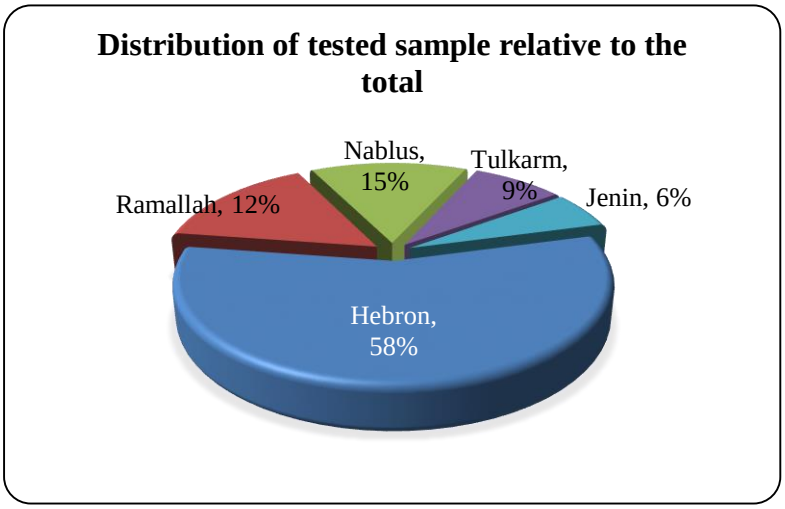

Figure 3.2: **Distribution of TI patients who participiated in this study according to their residence place**.

3.2 Genetic thrombotic mutations

FII (G20210A) polymorphism was detected by RFLP-PCR. The PCR product was digested with *Hin*dIII enzyme and analyzed by agarose gel electrophoresis. A 264 bp fragment indicates normal allele while detection of 232 bp plus 32 bp (this small fragment was not detected on agarose gel) indicates the presence of a mutant allele (Figure 3.3).

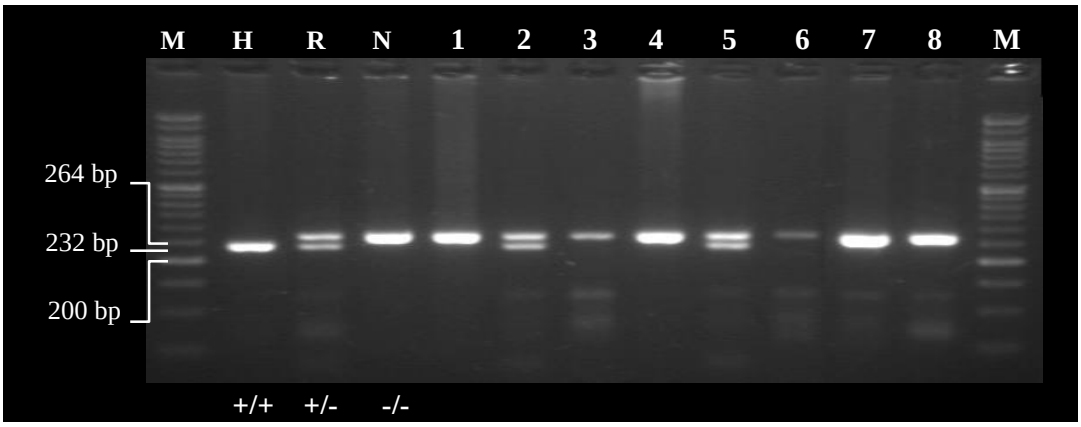

Figure 3.3: **FII (G20210A) polymorphism pattern.**
M: 50 bp DNA ladder. (-/-) Normal (N). (+/+) Homozygous mutant (H). (+/-) Heterozygous (R). Samples 1, 3, 4, 6, 7 and 8 are normal, samples 2 and 5 are heterozygous.

FVL (G1691A) mutation was detected by RFLP-PCR. The PCR product was digested with *Hin*dIII enzyme and analyzed by agarose gel electrophoresis. Detection of a 241 bp fragment indicates normal allele while detection of 209 bp plus 32 bp (this small fragment was not detected on agarose gel) indicates the presence of a mutated allele (Figure 3.4).

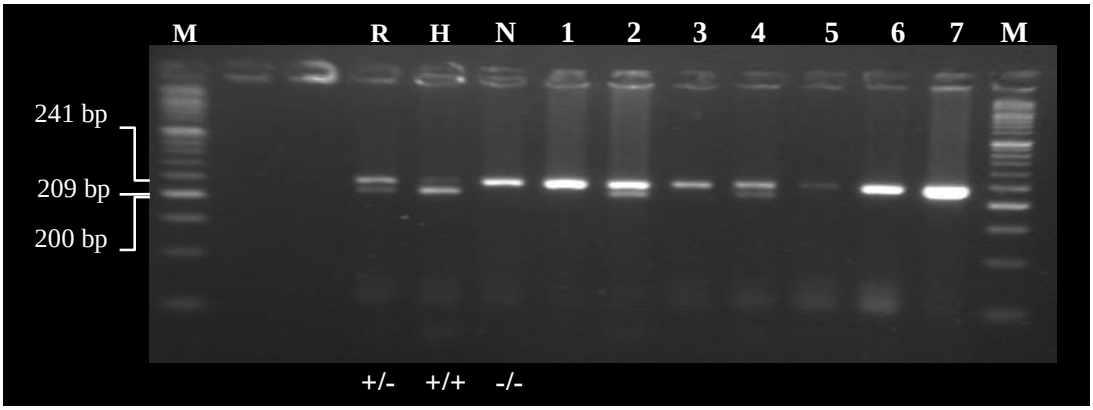

Figure 3.4: **FVL (G1691A) mutation pattern.**
M: 50 bp DNA ladder. (-/-) Normal (N). (+/+) Homozygous mutant (H). (+/-) Heterozygous (R). Samples 1, 3, 5, 7 and 8 are normal, samples 2 and 4 are heterozygous.

The MTHFR (C677T) mutation was detected using RFLP-PCR. The PCR product was digested with *Hin*fI. Analysis of *Hin*fI restriction fragments by agarose gel revealed 198 bp fragment for the wild type or normal allele, and 175 bp and 23 bp fragments for the mutant allele (Figure 3.5).

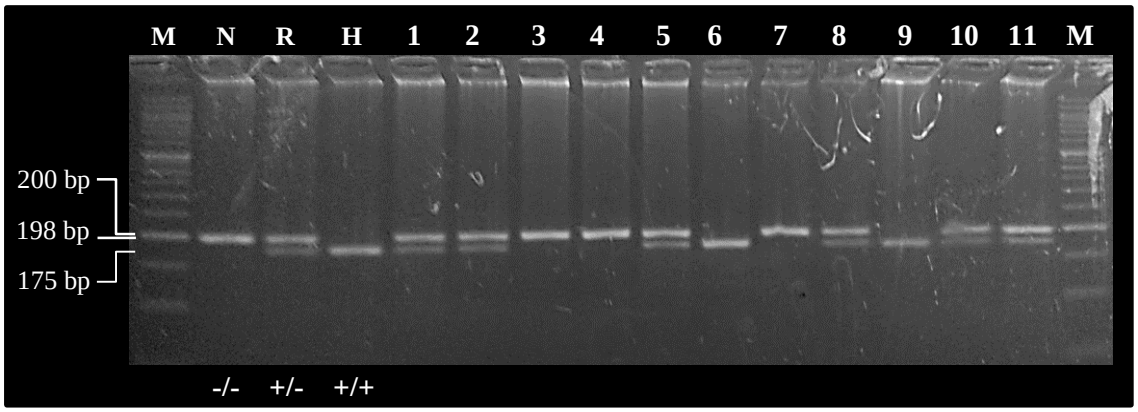

Figure 3.5: **MTHFR C677T polymorphism pattern.**
M: 50 bp DNA ladder. (-/-) Normal (N). (+/+) Homozygous mutant (H). (+/-) Heterozygous (R). Samples 1, 2, 5, 8, 10 and 11 are heterozygous, samples 3, 4 and 7 are normal, and samples 6 and 9 are homozygous mutant.

The GP (β3) T1565C mutation was detected by RFLP-PCR. The PCR product was digested with *Msp*I. Agarose gel electrophoresis of the *Msp*I restriction fragments showed a 221 and 45 bp fragments for the wild type allele or T allele, and 175, 44 and 45 bp fragments fo the mutant or the C allele (Figure 3.6).

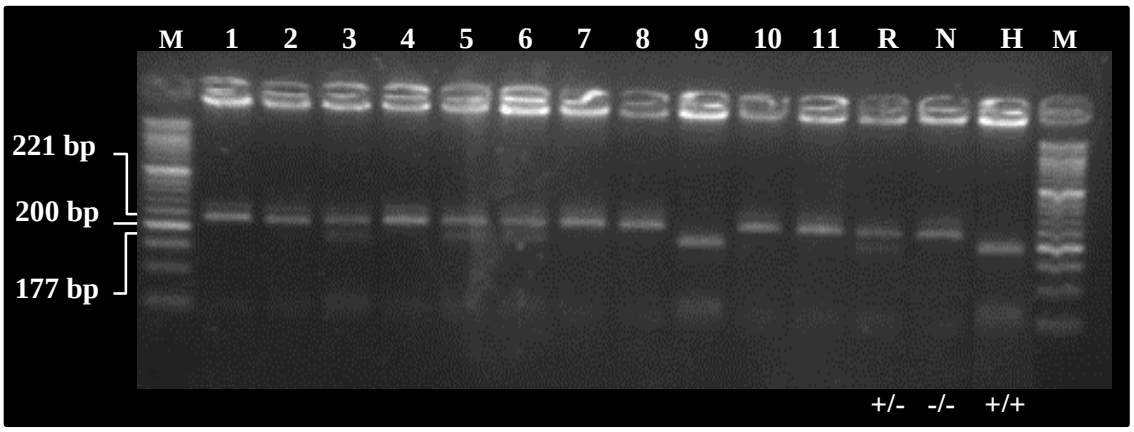

Figure 3.6: **GP (β3) T1565C mutation pattern.**
M: 50 bp DNA ladder. (-/-) Normal (N). (+/+) Homozygous mutant (M). (+/-) Heterozygous (R). Samples 1, 2, 4, 7, 8, 10 and 11 are normal, samples 3, 5 and 6 are heterozygous and sample 9 is homozygous mutant.

The frequencies of thrombotic mutations among the study group are summarized in Table (3.2). Each genotype was categorized into three sub groups; wild type (or normal, N), heterozygous (R) and homozygous mutant (H). The frequencies of each genotype analyzed are show in Table 3.2 Table (3.2). For each mutation, eventually, allele frequency was set according to the relative occurrence of each allele in each sample population (i.e. wild type or mutated). The purpose of doing this type of grouping is to assess the frequency of each allele among the study subjects.

Table 3.2: **Prevalence of thrombogenic mutations in thalassemia intermedia patients and in the normal control group.**

District	Pt/Nc (n)	FII (G20210A)			FVL (G1691A)			MTHFR (C677T)			GP β3 (T1565C)		
		N	R	H	N	R	H	N	R	H	N	R	H
Hebron	Pt (48)	46	0	2	39	9	0	22	23	3	46	2	0
	Nc (48)	47	1	0	38	10	0	21	23	4	37	11	0
Ramallah	Pt (10)	10	0	0	6	4	0	7	1	2	8	2	0
	Nc (10)	10	0	0	8	2	0	2	7	1	8	2	0
Nablus	Pt (12)	11	1	0	11	1	0	11	1	0	9	2	1
	Nc (12)	11	1	0	8	4	0	5	6	1	7	4	1
Jenin	Pt (5)	5	0	0	3	2	0	3	2	0	3	2	0
	Nc (5)	5	0	0	4	1	0	4	1	0	3	2	0
Tulkarm	Pt (7)	7	0	0	7	0	0	2	5	0	7	0	0
	Nc (7)	7	0	0	3	4	0	1	5	1	7	0	0
Total	Pt (82) (%)	79 (96.2)	1 (1.3)	2 (2.5)	66 (80.5)	16 (19.5)	0 (0)	45 (54.9)	32 (39.0)	5 (6.1)	73 (89.0)	8 (9.8)	1 (1.2)
	Nc (82) (%)	80 (97.5)	2 (2.5)	0 (0.0)	61 (74.4)	21 (25.6)	0 (0.0)	33 (40.2)	42 (51.3)	7 (8.5)	62 (75.6)	19 (23.2)	1 (1.2)

Pt: Patients. Nc: Normal controls. N: Normal (wild type polymorphism). R: Heterozygous. M: Homozygous. FII: Thrombin.

FVL: Factor V Leiden. MTHFR: Methylene Tetrahydropholate Reductase. GP β3: Glycoprotein (β3) subunit.

Homozygosity for FII (G20210A) polymorphism was detected in two patients from Hebron, and those were the only homozygous cases that were detected in the study (Table 3.2). None of the normal group were homozygous for the FII polymorphism.

Sixteen TI patients (19.5%) and 21 (25.6%) subjects of the normal control group showed heterozygosity for FVL, while no case showed homozygosity in both groups. Moreover, the results showed dominancy of the FVL mutation in the southern area (i.e. Hebron); where about (11.0%) of the patient and (13.4%) of the normal group were positive for the FVL mutation, respectively (Table 3.2).

Analysis of the data showed that 32 (39%) of the TI patients were heterozygous and 5 patients (6.1%) were homozygous for the MTHFR C677T mutation (Table 3.2), compared to 42 subjects (51.3%) of the control group were heterozygous and 7 subjects (8.5%) were homozygous for MTHFR C677T mutation (Table 3.2). The frequency of MTHFR C677T mutation was the highest in this study compared to the other 3 mutations / polymorphisms analyzed (Table 3.2). Hebron contained the highest frequency of MTHFR C677T polymorphism (54% and 65%) for the patients and the normal groups, respectively.

Analysis of the results showed that eight cases were heterozygous and one case was homozygous mutant for the GP (β3) T1565C mutation among the IT patients. While 19 cases were heterozygous and one case was homozygous mutant for the GP (β3) T1565C mutation among the normal control group (Table 3.2).

The frequency of the four mutations / polymorphisms was, also, tested in different way, by calculating the frequency of mutated allele in the population (Table 3.3).

Table 3.3: **Frequency of the mutant and normal alleles among the study populations**

		FII (G20210A)		**FVL (G1691A)**		**MTHFR (C677T)**		**GP β3 (T1565C)**		***p*- value**
		G	**A**	**G**	**A**	**C**	**T**	**T**	**C**	
Pt	(n)	159	5	148	16	122	42	154	10	0.05
	(%)	(97.0)	(3.0)	(90)	(10)	(74.4)	(25.6)	(93.9)	(6.1)	
NC	(n)	162	2	143	21	108	56	143	21	
	(%)	(98.8)	1.2)	(87)	(13)	(65.9)	(34.1)	(87.2)	(12.8)	

Pt: patients, Nc: normal control group.

The total number of the alleles of the four mutations / polymorphisms for the 82 TI patients is 164, and gives the same for the normal control group.

FII mutant allele (G20210A) or A allele was found in 5 out of 164 chromosomes (about 3%) of the TI patients and in 2 out of 164 chromosomes (about 1.2%) of the normal control subjects (Table 3.3). This showed that the frequency of the A allele is rare among the TI patients (3%) as well as among the normal control subjects (1.2%).

Analysis of the FVL allele (A allele) among the study population revealed that the FVL allele was found in 16 out of 164 chromosomes (about 10%) of the TI patients and in 21 out of 164 chromosomes (about 13%) of the normal control group (Table 3.3). Statistical analysis using Chi-square test of the difference of A allele frequency between IT patients and normal control group showed no significant difference.

The frequency of the T allele of MTHFR was observed in 42 out of 164 chromosomes (25.6%) among the TI patients compared to 56 out of 164 chromosomes (34.1%) in the normal control group (Table 3.3). Statistical analysis showed no significant difference between the frequency of MTHFR alleles between TI patients and normal control group (Table 3.3).

3.3 Acquired thrombotic risk factors

Information about the acquired risk factors were obtained from patients' files and the questionnaire filled by direct contact with them before sample collection.

The acquired risk factors analyzed here included previous thrombotic manifestations, surgery (including splenectomy), prolonged immobilization, pregnancy, oral contraceptives administration and smoking (Table 3.4). These factors can predispose patients to thrombosis. Three out of the 82 TI patients suffered from thrombotic manifestations at least once in his/her life. Twenty-six out of the 82 patients under went at least one surgical operation, twenty-three of them underwent splenectomy. Four women gave births (ranged from 1 to 12 births). The frequency of the other screened risk factors are listed below (Table 3.4).

Table 3.4: **Prevalence of acquired risk factors among Thalassemia intermedia patients vs. frequency of heterozygosity among patients.**

Acquired Risk Factors	**Number of cases**
	Total
Thrombotic manifestations	3
Surgery	26
Splenectomy	23
Splenectomy and other surgeries	
Other than splenectomy	14
Prolong immobilization	1
Pregnancy	4
Oral contraceptives	1
Smoking	7

Hz: heterozygous for one mutation, DHz: doubled heterozygous, THz: tripled heterozygous, QHz: quadrupled heterozygous.

Since it has been shown that splenectomy contributes to hypercoagulability (Cappellini et al., 2005; Eldor and Rachmilewitz, 2002), patients were subcategorized into splenectomized (Sp.) and nonsplenectomized (NSp.) groups (Table 3.5) to investigate the effect of thrombotic genetic mutations on splenectomy. Chi-square analysis of the frequency of thrombotic mutations revealed no statistically significant difference between splenectomized and

nonsplenectomized, as well as between nonsplenectomized patients and normal control group (Table 3.5).

Table 3.5: **Frequency of the mutant and normal genes among the study populations.**

		FII (G20210A)		**FVL (G1691A)**		**MTHFR (C677T)**		**GP β3 (T1565C)**	
		N	**M**	**N**	**M**	**N**	**M**	**N**	**M**
NSp. Pts.	(59)	56	3	49	10	30	29	55	4
	(%)	94.9	5.1	83.1	16.9	50.8	49.2	93.2	6.8
Sp. Pts.	(23)	23	0	17	6	14	9	19	4
	(%)	100	0.0	73.9	26.1	60.9	39.1	82.6	17.4
NCs.	(82)	80	1	61	21	33	49	63	19
	(%)	97.6	2.4	74.4	25.6	40.2	59.8	76.8	23.2

Sp: splenectomized, NSp: nonsplenectomized, NCs: Normal controls.

The frequency of the FII G20210A mutation was more prevalent in nonsplenectomized patients (5.1%) than that in the normal controls (2.4%), and all the nonsplenectomized patients were free of the mutation. Comparable results were obtained for the FVL mutation between splenectomized patients (26.1%) and normal controls (25.6%). In addition, relatively, comparable results were obtained for the MTHFR C677T mutation among splenectomized patients (39.1%), nonsplenectomized patients (49.2%) and normal controls (40.2%).

However, results of the GP β3 T1565C polymorphism showed higher frequency in the normal control group than the TI patients groups, either whom undergone splenectomy (17.4%) or the nonsplenectomized patients (6.8%).

Chapter Four

Discussion

Analysis of the frequency of TI patients among the different cities showed a gradual increase in the incidence of reporting of TI cases from north to south; where Hebron encountered the highest incidence (65 patients out of 148) and Jenin the lowest (5 cases out of 148). Although Hebron has the highest number of population among all cities of West bank, but other factors could also contribute to this finding such as the high rate of consanguinity, which is more prevalent in the southern area. In support of the later argument and upon analysis of the family names of TI from Hebron, it was observed that 14 patients were from one family and another 11 patients were from another family, although not all of these cases participated in this study. However, since an accurate estimation of the number of TI cases in Palestine is not available, this makes it difficult for us to judge on how much our study sample is representative of the TI population. Additionally, since the clinical severity of thalassemia intermedia varies widely, many patients may be not well diagnosed and the number of TI patients most probably is underestimated.

Our results revealed that 3 out of the 82 TI patients (3.7%) experienced at least one thrombotic manifestation throughout their lives. This result matches the results of some earlier international studies and disagrees with others; especially those that surveyed Mediterranean populations for a correlation between TI and tendency towards developing thrombotic manifestations; where Borgna Pignatti et al. (1998) found that the incidence of thromboembolic events among 52 Italian TI patients was 9.61%, and Taher et al. (2006b) reported a 4.0%

prevalence of various thrombotic events among 2,190 TI patients from the Mediterranean region.

The frequency of heterozygous and homozygous mutant genotypes among TI patients were 1.3% and 2.5% for the FII G20210A; 19.5% and 0% for FVL G1691A; 39% and 6.1% for MTHFR C677T and 9.8% and 1.2% for the GP β3 C1565T, respectively. While the frequency of heterozygous and homozygous mutant genotypes among the normal control group were 2.5% and 0% for the FII G20210A; 25.6% and 0% for FVL G1691A; 51.3% and 8.5% for MTHFR C677T and 23.2% and 1.2% for the GP β3 C1565T, respectively. Hussein (2012) reported the frequency of FVL G1696A, FII G20210A and MTHFR C677T mutations among a cohort of 303 university students from An-Najah University at 20.1%, 9.1% and 13.8%, respectively. Thus, the frequency of FII G20210A reported in this study is lower than that reported by Hussein (2012). But, the frequency of FII G20210A polymorphism among the study population was consistent with that reported earlier in other populations such as in Danish (2.1% heterozygotes & 0.01% homozygotes; (Weischer et al., 2010), in Turkish (3.5% heterozygotes and homozygotes; (Yokus et al., 2009) and in whites (2-3% heterozygotes; homozygotes are rare; (Fogerty and Connors, 2009). The frequency of FVL reported here is similar to that reported by Hussein (2012) but the frequency of MTHFR C677T reported here is higher than that reported by Hussein (2012). The difference between the two frequencies can be attributed partially to the different sample size, and thus further studies are needed to give a more accurate estimate of the frequency of these mutations. However, the frequency FVL G1691A and MTHFR C677T mutations among the study population was higher than that reported in other populations (Kujovich, 2011; Yokus et al., 2009). Yokus et al. (2009) reported that the frequency of FVL G1691A and MTHFR C677T mutations among a population of young adults presenting with thrombosis to be 20% (with 18% being heterozygotes) and 20.9% (with 10% heterozygotes). While Kujovich (2011) reported that the frequency of FVL G1691A mutation (heterozygotes and homozygotes) among several European populations is 3-15%, among Jordanians 12.2% and among Lebanese 14%.

The frequency of mutant alleles of the FII (G20210A), FVL (G1691A), MTHFR (C677T) and GP β3 (T1565C) mutations/polymorphisms among the TI patients was 3%, 10%, 25.6% and 6.1%, respectively. While the frequency of mutant alleles of the FII (G20210A), FVL (G1691A), MTHFR (C677T) and GP β3 (T1565C) mutations / polymorphisms among the

normal control group was 1.2%, 21%, 34.1% and 12.8%, respectively. Statistical analysis of the difference in frequency of mutant alleles between TI patients and normal control group revealed no significant difference in all studied alleles. However, this study included about 55% of all TI patients (n=148) in the West Bank, and given that the actual number of TI is most probably underestimated, so, generalization of these results must be carefully handled.

In addition, analysis of the correlation between genetic thrombotic mutations analyzed in this study and acquired thrombotic risk factors and in particular in patients who have experienced previous thrombotic manifestations suggest that, at least in these subjects, genetic thrombotic mutations may play a role in thrombotic manifestations observed in these patients.

In 2005, a study conducted in Palestine among 148 β-thalassemia patients to identify the causative β- globin gene mutations. The most frequent mutations were the IVS-I-6 (T>C), IVS-I-110 (G>A), IVS-I-1 (G>A) and codon 37 (G>A) (Darwish et al., 2005). However, patients' files did not include the molecular diagnosis of the patients, except one case whose file contained a positive report for IVS-I-110 (G>A) mutation. So, more work and efforts are needed to improve the diagnosis of thalassemia intermedia patients in Palestine.

In conclusion, this study reported the frequency of four thrombotic genetic mutations FVL G1691A, FII G20210A, MTHFR C677T and GPβ C1565T mutations/polymorphisms, among TI patients and a normal control group. No statistically significant difference was observed in the frequency of these mutations between TI patients and normal control group. These results indicate that the hypercoagulable state present in our TI patients may not be affected by these thrombotic genetic mutations. Although these mutations / polymorphisms may contribute to the hypercoagulable state in TI when present in combination with other acquired or environmental risk factors. Additionally, our results indicate a high frequency of three thrombotic genetic mutations namely FVL G1691A, MTHFR C677T and GPβ C1565T mutations compared to other populations. The latter finding warrants further investigation of these mutations in terms of their clinical significance.

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Appendices

Appendix A

استبـــانة

أنا الطالب – محمد عصام الكرد – طالب ماجستير في برنامج العلوم الطبية المخبري / مسار علم دم في دائرة العلوم الطبية المخبرية – كلية الدراسات العليا – جامعة القدس.

أقوم بعمل بحث بعنوان "تحديد الطفرات الجينية المسببة للخثار الدموي في مرضى الثلاسيميا في الضفة الغربية في فلسطين" و الذي يهدف إلى تحديد مدى انتشار طفرات مسببة للخثار الانصمامي و الوريدي بين مرضى الثلاسيميا وبالأخص الثلاسيميا الوسيطة في الضفة الغربية؛ وهذه الطفرات هي:

MTHFR C677T, FV Leiden, FII G20210A, and platelet GP β_3 T1565C polymorphism

حيث سيتم سحب عينات دم من المرضى الذين يعانون من الإصابة بمرض الثلاسيميا في الضفة الغربية و من ثم سيتم عمل الفحوصات الوراثية اللازمة للكشف عن الطفرات المذكورة.

لذلك فإن العينات المسحوبة و الفحوصات التي ستجرى عليها و النتائج التي سيتم الحصول عليها سوف تعامل بسرية تامة و لغرض البحث العلمي فقط ولن يتم الإفصاح عن أي نتيجة إلا بموافقة المريض نفسه أو ولي أمره راجيا من المرضى التعاون لإتمام البحث و الحصول على النتائج المرجوة مما قد يساهم في تحسين وضع المريض هو و غيره أو يساعد في تلافي بعض الأخطار و المضاعفات في المستقبل .

وتقبلوا الاحترام

محمد الكرد
دائرة العلوم الطبية المخبرية
كلية المهن الصحية
جامعة القدس

Questionnaire

I am - Mohammad Essam Kurd - a master student in Medical Laboratory Sciences program / Hematology track at the Department of Medical Laboratory Science - Faculty of Graduate Studies – Al-Quds University.

I am doing a research entitled "Determination of Thrombotic Genetic Mutations in Thalassemic Patients in West Bank-Palestine" which aims to determine the prevalence of mutations causing thromboembolic manifestations in patients with thalassemia, especially thalassemia intermeda in the West Bank; and these mutations are: MTHFR C677T, FV Leiden, FII G20210A, and platelet GP β_3 T1565C polymorphism.

I need to obtain blood samples from patients who suffer from Thalassemia in the West Bank and then will perform genetic tests needed to detect the mutations mentioned above.

Therefore, the obtained samples, the tests that will be conducted and the results that will be obtained, will be treated in strict confidence and for the purpose of scientific research only. Therefore no result will be disclosed for anyone but with the consent of the patient or guardian's, I request that patients cooperate to complete the research and get the desired results, which may contribute to improving the status of the patient and other patients or assist in avoiding some risks and complications in the future.

Please accept my respect

Mohammad Kurd

Department of Medical Laboratory Sciences
Faculty of Graduate Studies
Al-Quds University

Code

استبـــانة

Questionnaire

Personal information **المعلومـــات الشخصية**

Name : ..... : الاســـــــم

City : ..... : المدينـــــــة

Phone : ..... : الهـــــاتف

Date of Birth : ..... : تاريـــخ الميلاد

ID : ..... : رقـــــم الهوية

Gender : ☐ Male ذكر ☐ Female أنثى : الجنـــــــس

Hight (m) : ..... : الطـــــول (م)

Weight (Kg) : ..... : الـــوزن (كغم)

Hematologic Information **معلومات متعلقة بالدم**

ABO and Rh: ..... الزمرة الدموية:

Type of thalassemia: ..... نوع الثالسيميا:

Name of Mutation (if known): ..... اسم الطفرة (إن كانت معروفة):

Age of presentation of thalassemia (in years): ..... : عمر ظهور الأعراض (بالسنوات)

First transfusion age (in years): ..... عمر أول نقل دم (بالسنوات):

Frequency of transfusions (/years): ..... معدل مرات نقل الدم (سنويا):

Average Hb (before transfusion) (g/dl): ..... معدل تركيز الخضاب (غم/هل):

Ferritin conc. (ng/mL): ..... معدل تركيز الفرتين (نغم/هل):

Requirement of chelation: □ No لا □ Yes نعم هل يوجد حاجة للاستخالب؟

(Brand name ..... اسم المنتج)

(Frequency ..... التكرار)

(Dose ..... الجرعة)

Splenectomy: □ No لا □ Yes نعم هل أجريت عملية استئصال للطحال؟

(At which age? ..... في أي عمر؟)

Do you take anti-thrombotic drugs? □ No لا □ Yes نعم هل تتناول مواد مميعة للدم؟
(Mention .....)

Clinical examination **الفحص الطبي**

Pallor □ No لا □ Yes نعم شحوب

Facial deformities □ No لا □ Yes نعم تشوهات في الوجه

Splenomegaly □ No لا □ Yes نعم تضخم في الطحال

(Degree ..... الدرجة)

History **التاريخ المرضي**

Thrombotic manifestation: □ No لا □ Yes نعم هل سبق أصيبت بجلطة ما؟

(Age ..... العمر)

(Localization ..... العضو)

Risk Factors **عوامل الخطورة**

Ever did surgery? ☐ No لا ☐ Yes نعم هل سبق أن أجريت عملية جراحية ما؟

(mention :……..……..) (اذكرها :

Prolonged immobilization? ☐ No لا ☐ Yes نعم هل تمكث فترة طويلة دون حراك؟

(How long? ……..…… ?ما المدة)

Ever Pregnancy? ☐ No لا ☐ Yes نعم هل سبق أن حملت؟

(How many? ……..…… ?كم مرة)

Oral contraceptive intake? ☐ No لا ☐ Yes نعم هل تأخذ أدوية مانعة للحمل؟

Smoking? ☐ No لا ☐ Yes نعم هل تدخن؟

(Daily number of cigarettes ……..) (معدل السجائر في اليوم

Declaration **إقـــــــرار**

التوقيع في المستطيل التالي من قبل المريض – أو ولي أمره – يقتضي بقبول سحب عينة دم من المريض لإجراء الفحوصات اللازمة لغرض البحث العلمي و الاطلاع على ملف المريض من قبل الباحث – إن لزم الأمر – لأخذ أي معلومات قد تفيد البحث، و له جزيل الشكر.

Signing in the following rectangle by the patient or guardian entails the permission for withdrawal of a blood sample from the patient to conduct the necessary tests for the scientific research and investigate the patient's file by the researcher - if necessary - to take any information that may help the research, and we are very thankful.

Signature with thanks **التوقيع مع الشكر**

الملخص

تحديد الطفرات المسببة للتجلط في مرضى الثلاسيميا المتوسطة في الضفة الغربية – فلسطين

إعداد الطالب: محمد عصام عبد الفتاح كردي

مشرف أول: الدكتور محمود عبد الرحمن سرور

مشرف ثاني: الأستاذ خالد رجا اليونس

الثلاسيميا مجموعة غير متجانسة من الاضطرابات الوراثية تؤثر على تصنيع الخضاب (الهيموغلوبين)، يؤدي كل منها إلى انخفاض معدل الإنتاج واحد أو أكثر من سلاسل الكريين (الغلوبين) من الخضاب (الهيموغلوبين). تعتمد صورة المرض الناتجة على شدة الطفرة و العوامل الخارجية المرافقة الأخرى التي تحدد الصورة السريرية للمريض. يتم تصنيف الثلاسيميا سريريا إلى ثلاثة أقسام فرعية رئيسية: الثلاسيميا العظمى(TM)، الثلاسيميا الطفيفة و الثلاسيميا الوسطى (TI). وتتسبب الثلاسيميا الوسطى عادة نتيجة خلل في واحد أو أكثر من الجينات الكريين: α، β و γ. مرضى الثلاسيميا يعانون من مضاعفات سريرية كثيرة، و التي تؤثر على معظم أجهزة الجسم بما في ذلك تشوهات العظام شديدة و تضخم الكبد و الطحال. وقد لوحظت العديد من المضاعفات التي تم تحديدها في مرضى الثلاسيميا الوسطى أكثر من مرضى الثلاسيميا العظمى. بين هذه المضاعفات هو الميل نحو تشكيل مضاعفات الانسداد التجلطي الوريدي، الرئوي و المخي أو جلطة في أي موقع أخرى من الجسم.

هدفت هذه الدراسة إلى تحديد الطفرات الجينية الخثارية في مجموعة من مرضى الثلاسيميا الوسطى من منطقة الضفة الغربية في فلسطين، و اللائي قد تزيد من خطر الإصابة بالأعراض التجلطية في هؤلاء المرضى. و لهذا الغرض تم البحث عن أربعة طفرات خثارية هي: طفرة العامل الخامس لايدن (G1691A)، طفرة البروثرومبين (G20210A)، طفرة مختزل رباعي هيدروجين الفولات (C677T) و طفرة برتين الصفائح الدموية السكري (β_3) (T1565C).

اثنان وثمانون مريضا بالثلاسيميا الوسطى، من مرضى الضفة الغربية تم جمعهم من مراكز رعاية مرضى الثلاسيميا في منطقة الضفة الغربية و تضمينهم في الدراسة. بالإضافة إلى تضمين مجموعة مطابقة من المتطوعين الطبيعيين غير المصابين بأي فقر الدم في الدراسة لتستخدم كمجموعة ضابطة محددة لمجموعة المرضى. تم فحص العينات لوجود

الطفرات الأربعة باستخدام طريقة تعدد أشكال القطع المقطوعة للتفاعل التسلسلي المتعدد (RFLP – PCR)، و تم تحليل النتائج وفقا لمربع كاي α (0.05).

أظهرت الدراسة زيادة تدريجية في معدل حالات الإصابة بالثلاسيميا الوسطى من الشمال إلى الجنوب؛ حيث كان المعدل الأدنى في جنين (5 حالات)، والأعلى في محافظة الخليل (65 حالة). و قد بلغ معدل حدوث مظاهر الجلطات بين مرضى الثلاسيميا الوسطى نسبة (3.7٪). وكشفت الاختبارات الجزيئية عن أن 1.3٪ من مرضى الثلاسيميا الوسطى يحملون الطفرة و 2.5 ٪ كانت متماثلة اللواقح بالنسبة طفرة البروثرومبين (G20210A). وفقا لطفرة العامل الخامس لايدن (G1691A) 19.5 ٪ من المرضى كانوا متخالفي اللواقح، و لم يتم الكشف عن أي حالات متماثلة اللواقح. كانت أعلى نسبة بين الأشكال الأربعة طفرة مختزل رباعي هيدروجين الفولات (C677T)؛ حيث أظهر 39 ٪ من المرضى تغاير اللواقح، و كان 6.1٪ متماثلي اللواقح. أما بالنسبة لطفرة برتين الصفائح الدموية السكري (β_3) (T1565C) فكانت نسبة متخالفي اللواقح 9.8 ٪ من المرضى و متماثلو اللواقح شكلوا 1.2٪ من المرضى. و كشف تحليل مربع كاي عن عدم وجود فروق ذات دلالة إحصائية في وتيرة كل من طفرات الأربع بين المرضى الثلاسيميا الوسطى مقارنة مع المجموعة الضابطة المحددة. ومع ذلك عندما تقترن مثل هذه العيوب الوراثية بالمكتسبة يمكن أن تسهم في حالة الميل نحو فرط الخثورية في المرضى الذين يعانون من الثلاسيميا الوسطى، كما تبين في ثلاثة من المرضى ضمن عينة البحث. علاوة على ذلك، أظهرت نتائجنا نسبة متدنية لطفرة البروثرومبين (G20210A) و مرتفعة بالنسبة لطفرة مختزل رباعي هيدروجين الفولات (C677T) بين الفلسطينيين مقارنة مع التقارير السابقة.

على أي حال، في الختام، لم يلاحظ أي فرق إحصائي في وتيرة كل من طفرات الأربع بين المرضى الثلاسيميا الوسطى مقارنة مع المجموعة الضابطة المحددة. ومع ذلك، فإن هناك حاجة لمزيد من الفحوصات المرضى الثلاسيميا الوسطى للبحث عن عيوب وراثية خثرية أخرى قد تساعد على تقييم حالة فرط الخثورية في مثل هؤلاء المرضى.