بسم الله الرحمن الرحيم

Shendi University

Faculty of Graduate Studies and Scientific Research

Evaluation of Mutations and Changes of Coagulation Profile Associated with Deep Venous Thrombosis in Sudan

A thesis submitted in fulfillment of the requirements of the degree of PhD in Medical Laboratory Sciences (Hematology)

By:

Abdalla Musa Abdalla Mohamed

M.Sc. in Medical Laboratory Sciences (Hematology and Immunohematology), Sudan University of Science and Technology (2009)

Supervisor:

Prof. Babiker Ahmed Mohammed

College of Medicine, Karary University

Co-Supervisor

Dr.Hamdan Sidig Siraj

College of Medicine, Shendi University

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الآية

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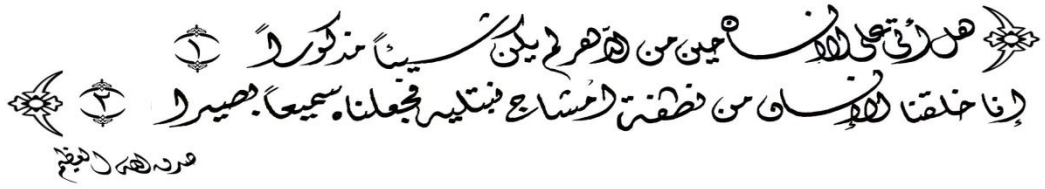

سورة الأنسان الآية ١-٢

DEDICATION

TO ALL OF THEM I DEDICATE THIS WORK, FULFILLMENT AND RECOGNITION.

MY GREAT PARENTS,

WHO ENLIGHTENED MY WAY ALL THE TIME, MAY ALLAH PROTECT THEM.

MY NUCLEAR FAMILY,

WHO ARE PROUD OF ME, AND INSISTED THAT THEIR FATHER COULD ACHIEVE THIS DREAM.

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Abstract

Venous thromboembolism (VTE) includes deep vein thrombosis (DVT) and pulmonary embolism (PE). VTE constitutes the third most common cardiovascular disease, after acute coronary syndrome and stroke. The disease affects individuals over wide range of age although it's prevalence is higher in elderly. The disease is caused by many factors some of which are congenital and others are acquired. In this study we aimed to investigate the relationship between genetic mutations associated with deep venous thrombosis as well as some proteins that may have roles in the development of the disease.

The study was conducted in Khartoum state during the period from December 2014 to May 2018. One hundred and fifty individuals were included in this study. One hundred represented the patient group. Thirty were males and seventy were females; median age 48.14 years; ranging from 19 to78 years. The control group (healthy volunteers) included 29 men and 21 women; median age 32.4 years; ranging from 22 to 47 years). Among study group 25 % (25/100) were post surgery, 6 % (6/100) smokers and 14 % (14/100) were obese.

For genetic mutations we used the conventional polymerase chain reaction (PCR) technique. Proteins C and protein S are measured by enzyme linked immune assay (ELISA). Determination of fibrinogen based on a modified Clauss method. D-dimer measured by nephelometric assay that utilizes antibody coated latex particles. Prothrombin time (PT) and activated partial thromboplastin time (APTT) were measured by clot based technique. Finally for platelets count we used hematology analyzer.

The results of genetic analysis are as follow: plasminogen activator inhibitor-1(PAI-1) has the highest prevalence. In patient group 7 had the 4G/4G allele, 2 had 5G/5G allele and 91 had the 4G/5G allele. The difference between patient group and control group was statistically significant (P= 0.012).

Concerning factor XIII mutation val 34 leu, eight patients had mutant allele (L L). Ninety two had wild type allele (V V). The entire control group had the wild type allele (V V). The difference between study group and control group was significant (P= 0.04).

For fibrinogen G455A mutation one patient had the mutant allele (AA), three patients had the mixed allele (GA) and ninety six had the wild type (GG). Among control group one

participant had the mutant allele, five of them had the mixed type, and forty four had the wild type. The difference was statically insignificant (P = 0.17).

In methylene-tetrahydrofolate reductase (MTHFR C677T) mutation, the mixed allele was found in four patients (C T). Ninety six patients had wild type (C C). No mutant type (T T) in the patient group. No any mutation in the control group. The findings showed no difference between study group and control group (P =0.152).

Regarding prothrombin G20210A mutation, four patients had the mutant allele (A A). The remaining ninety six had wild type (G G). The entire control group had only the wild type. Findings showed no significant difference between study and control group (P = 0.152).

Factor V Leiden showed only the wild type (G G) for both study group and control group. The mutant allele type (AA) and the mixed type (GA) were not found.

Mean of D-dimer was 0.393 microgram/ml in patient group and 0.186 microgram/ml in control group. The difference was statistically significant (P =0.014).

Plasma fibrinogen was higher in study group (mean 474.11 mg/dl) than in control group (mean369.3 mg/dl). But the difference was statistically insignificant (P =0.109).

Protein C showed lower results in patient group when compared with the control group. The mean in patient group was 93.19 mg/dl and in control group was 97.06 mg/dl. In spite of that, the difference is still insignificant (P =0.263).

The mean of protein S in patient group was 75.28 mg/dl while it was 79.27 mg/dl in control group. The difference between patient and control group was statistically insignificant (P= 0.534).

Mean of APTT was 36.79 seconds in patient group and 31.64 seconds in control group. The difference was statistically significant (P= 0.000).

The difference of prothrombin time was statistically significant (P= 0.001). The mean of PT was 13.58 seconds in patient group and 12.78 seconds for the control group. Platelets count was low in the patient group. The mean platelets count was $256X10^9$/Lin patient group while it was $331.4X10^9$/L in the control group. The difference was statistically significant (P=0.000).

In conclusion: The non-significant relations indicated that the investigated polymorphisms are not genuine risk factors for DVT in our population.

Present study revealed that the D-dimer test is useful for the diagnosis of DVT.

Future work should focus on association of other polymorphisms and genes that may be contributing to thrombophilia and DVT in particular.

مستخلص البحث

تشتمل الجلطات الوريدية على جلطات الاوردة العميقة والجلطات الرئوية. تقع الجلطات الوريدية في المرتبة الثالثة من حيث ترتيب امراض القلب وامراض الاوعية الدموية بعد متلازمة الشريان التاجي الحادة والسكتة الدماغية.

هذا المرض يصيب الانسان في فترات عمرية مختلفة غير ان المرض اكثر انتشارا في الفئات العمرية الاكثر تقدما في السن. هناك عدة عوامل تؤدي لحدوث الجلطات الدموية ، منها ماهو مورث وما هو نتاج لمؤثرات مكتسبة.

في هذه الدراسة عمدنا لدراسة الطفرات الجينية المتعلقة بجلطات الأوردة العميقة ودراسة بعض البروتينات ذات العلاقة بتطور المرض.

اجريت هذه الدراسة في ولاية الخرطوم في الفترة مابين ديسمبر ٢٠١٤ ومايو ٢٠١٨ م. تضمنت الدراسة عدد مئه وخمسون شخصا، منهم مئة تمثل مجموعة الدراسة أو المرضى وتضم ثلاثون ذكرا وسبعون انثى. تتراوح اعمارهم ما بين ١٩ و ٧٩ عام بمتوسط عمر ٤٨,١٨ سنة. اشتملت الدراسة على خمسون شخصا اصحاء وسميت مجموعة الضبط وتضم ٢٩ ذكر و٢١ من الاناث. تتراوح اعمارهم ما بين ٢٢و٤٧ بمتوسط اعمار ٣٢,٤ عام.

اشتملت مجموعة المرضى على ٢٥% بعد عمليات جراحية، ٦% مدخنين و١٤ % كانوا بدناء.

لفحص الطفرات الجينية استخدمنا تقنية تفاعل البوليمريز المتسلسل التقليدي (PCR). تم قياس بروتين C وبروتين S عن طريق فحص المناعة الانزيمية (ELISA). تم قياس الفيبرينوجين في البلازما على أساس طريقة كلوس المعدلة. تم قياس D-dimer بطريقة تعتمد على فكره اساسية تسمى(nephelometric) والتي تستخدم جزيئات اللاتكس المغلفة بالأجسام المضادة. تم قياس زمن البروثرومبين (PT) وقياس زمن الثرومبوبلاستين المنشط الجزئي (APTT) بواسطة تقنية تعتمد على تجلط البلازما. وأخيرًا لحساب الصفائح الدموية استخدمنا جهاز عد خلايا الدم.

نتائج التحليل الجيني كانت كما يلي: مثبط منشط البلازمينوجين -١ (PAI-1) لديه أعلى معدل انتشار. حيث اظهرت نتائج الدراسة ان ٧ من المرضى يحملون النمط 4G/4G واثنان بحملون النمط 5G/5G ,والبقية ٩١ يحملون النمط الطبيعي 4G/5G. كان الفرق بين مجموعة المرضى ومجموعة الضبط ذا دلالة إحصائية (P= 0.012).

اظهرت الدراسة الحالية أن الطفرة الوراثية الخاصة بمعامل التجلط (F XIII val 34 leu) ، أن ثمانية من المرضى لديهم النمط المتحول (L L) و اثنان وتسعون منهم يحملون النمط الطبيعي نوع (V V). اظهرت ا لمجموعة الضابطة النمط الطبيعي (V V) فقط. كان الفرق بين مجموعة الدراسة(المرضى) ومجموعة الضبط ذو دلالة احصائية (P= 0.04).

أظهرت الدراسة أن أحد المرضى كان يحمل النمط المتحول (AA) الخاص بطفرة الفيبرينوجين G455A ، وثلاثة مرضى لديهم النمط المختلط (GA) ، و ستة وتسعون من المرضى يحملون النمط الطبيعي (GG). في مجموعة الضبط كان أحد المشاركين لديه الشكل المتحول ، خمسة منهم لديهم النوع المختلط ، و أربعة واربعون يحملون الشكل الطبيعي . حيث اظهرت الدراسة عدم وجود فروقات ذات دلاله احصائية (P = 0.17).

أظهرت النتائج عدم وجود فروق ذات دلالة احصائية فيما يختص بالطفرة (MTHFR C677T) بين مجموعة الدراسة ومجموعة الضبط (P = 0.152) حيث وجد النمط المختلط (C T) في اربع من المرضى و النمط الطبيعي

في ستة وتسعون منهم. لم تظهر الدراسة وجود النمط المتحول (C C) في اي من المرضى. بالنسبة لمجموعة الضبط أظهرت الدراسة وجود الشكل الطبيعي فقط.

أظهرت الدراسة الحالية فيما يتعلق بطفرة البروثرومبين G20210A ، أن أربعة من المرضى يحملون الشكل المتحول (A A) و ستة وتسعون يحملون الشكل الطبيعي (G G) . لم تظهر الدراسة وجود اي طفرات في مجموعة الضبط. خلصت الدراسة على عدم وجود فروق إحصائية بين المرضى ومجموعة الضبط ($P = 0.152$).

أظهرت الدراسة وجود الشكل الطبيعي (G G) فقط للطفرة الخاصة ب (V Leiden) في كل من المرضى ومجموعة الضبط.

أظهرت الدراسة الحالية فروقات ذات دلالة احصائية في قياس.D-dimer ($P=0.014$). حيث كان المتوسط 0.393 ميكروغرام / مل في مجموعة المرضى و ٠,١٨٦ ميكروغرام / مل في مجموعة التحكم

أظهرت الدراسة زيادة الفيبرينوجين في مجموعة المرضى (متوسط ٤٧٤,١١ ملغ / ديسيلتر) مقارنة بمجموعة الضبط (متوسط ٣٦٩,٣ ملغ / ديسيلتر). لكن لم يكن الفرق بين المجموعتين ذا دلالة احصائية ($P = 0.109$).

أظهرت النتائج أن تركيز بروتين C أقل في مجموعة المرضى مقارنة مع مجموعة الضبط. كان المتوسط في مجموعة المرضى ٩٣,١٩ ملغ / ديسيلتر وكان في المجموعة الضابطة ٩٧,٠٦ ملغ / ديسيلتر. على الرغم من ذلك ، لم يكن الفرق ذو أهمية إحصائية ($P = 0.263$).

كان متوسط بروتين S في مجموعة المرضى ٧٥,٢٨ مجم / ديسيلتر بينما كان ٧٩,٢٧ مجم / ديسيلتر في مجموعة الضبط. كان الفرق بين المرضى ومجموعة الضبط لايمثل دلالة احصائية ($P= 0.534$).

بلغ متوسط زمن الثرومبوبلاستين المنشط جزئيا (APTT) 36.79 ثانية في مجموعة المرضى و ٣١,٦٤ ثانية في مجموعة التحكم. الفرق ذو دلالة إحصائية ($P = 0.000$).

كان فارق زمن البروثرومبين (PT) مهمًا من الناحية الإحصائية (P 0.001). كان متوسط PT هو ١٣,٥٨ ثانية في مجموعة المرضى و ١٢,٧٨ ثانية للمجموعة الضابطة.

أوضحت الدراسة انخفاضا في عدد الصفائح الدموية في المرضى المصابين بالجلطات الوريدية (متوسط $256X10^9/L$) بينما كان المتوسط في مجموعة الضبط ($331.4X10^9/L$). كان الفرق بين المرضى ومجموعة الضبط ذو دلالة إحصائية ($P = 0.000$).

يستنتج من هذه الدراسة أن العلاقة غير الجوهرية بين أشكال الطفرات الوراثية التي تم فحصها ومرض الجلطات الاوردة العميقة ليست عوامل خطر حقيقية للإصابة بجلطات الأوردة العميقة في مجموعة البحث.

خلصت الدراسة الحالية ايضا الى أن اختبار D-dimer مفيد لتشخيص الإصابة بجلطات الأوردة العميقة.

يجب أن يركز العمل المستقبلي على الارتباط بين تعدد الأشكال والجينات الأخرى التي يمكن أن تسهم في أهبة التخثر وتجلط الأوردة العميقة على وجه الخصوص.

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Abbreviations

Abbreviations	
ab2GPI	Anti beta 2
aCL	Anti cardolipin
ALL	Acute lymphoblastic leukemia
APC	activated protein C
APCR	Activated Protein C Resistance
aPL	Antiphospholipid antibodies
APS	antiphospholipid syndrome
ARDS	Acute respiratory distress syndrome
AT	antithrombin
BMI	body mass index
COX	cyclooxygenase
CT	cerebral thrombosis
CTEPH	Chronic thromboembolic pulmonary hypertension
CUS	Compression ultrasound
ddNTP	Dideoxynucleotide
DVT	deep vein thrombosis
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ET	essential thrombocythemia
ETP	endogenous thrombin potential
FGR	fetal growth restriction
FMC	fibrin monomer complex
FTA	first trimester abortion
FVL	factor V Leiden mutation
Hcy	homocysteine
HIT	heparin-induced thrombo-cytopenia
INR	international normalized ratio
IUFD	intrauterine fetal death
LA	Lupus antibodies
LIA	Latex immunoassay
LMWH	Low molecular weight heparin
MI	myocardial infarction
MTA	mid-trimester abortion
MTHF	methylenetetrahydrofolate
MTHFR	methylenetetrahydrofolatereductase mutation
OCCP	Oral combined contraceptive pill
PA	placental abruption
PAI-1	plasminogen activator inhibitor-1
PCR	Polymerase Chain Reaction
PE	pulmonary embolism
PMF	primary myelofibrosis
PT	prothrombin time

PTS	Post-thrombotic syndrome
PV	polycythemia vera
PVR	pulmonary vascular resistance
RT-PCR	Real time Polymerase Chain Reaction
SHBG	sex hormone-binding globulins
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
TF	Tissue factor
TG	The thrombin generation
TIA	transient ischaemic attack
TSR	thrombin-sensitive region
UFH	unfractionated heparin
VKA	vitamin K antagonist
VTE	Venous thromboembolism

Chapter one
Introduction and literature review

1.1. Introduction.

Normal hemostasis is controlled activation of coagulation factors and platelets leading to clot formation, with subsequent clot lysis, in a process that stops hemorrhage without excess clotting (thrombosis). Efficient hemostasis is a rapid and localized response to an interruption in vascular integrity (vessel wall injury); such that clots are formed only when and where they are needed [(1)]. There are three possible contributors to formation of an abnormal clot (thrombus): decreased blood flow, vessel injury or inflammation, and changes in the intrinsic properties of the blood. Persistent physiologic changes in any of these three factors (the Virchow triad) are referred to as hypercoagulable state [(2)]. Hypercoagulable or thrombophilia state is an inherited or acquired abnormality of hemostasis that affects the delicate balance between procoagulant and anticoagulant factors, predisposing to thrombosis [(3)].Thrombosis can be venous or arterial[(4)]. Venous thromboembolism (VTE) is a disease that includes both deep vein thrombosis (DVT) and pulmonary embolism (PE). It is a common, lethal disorder that affects both hospitalized and nonhospitalized patients [(5)]. DVT and PE are two clinical presentations of VTE that share the same predisposing factors [(6)]. Deep vein thrombosis can lead to life-threatening pulmonary embolism [(7)].

Multiple complex interactions between genetic and environmental factors occur during the pathological course of thrombosis [(8)]. The three most common genetic thrombophilias known to predispose to venous thrombosis are factor V Leiden mutation (FVL), methylenetetrahydrofolatereductase mutation (MTHFR), and prothrombin gene mutation (FII) [(9)]. During normal clotting activated protein C (APC) inactivates factor Va and VIIIa by cleavage at specific sites. In the presence of the mutation in factor V, the cleavage of this factor is inhibited, leading to enhanced thrombin generation and hence increased clot formation [(10)].The MTHFR enzyme plays a major role in folate metabolism by catalyzing the conversion of 5,10 methylenetetrahydrofolate (5,10-MTHF) to 5-methylenetetrahydrofolate (5-MTHF), which represents the main circulating form of folate, which serves as co-substrate for the methylation of homocysteine (Hcy) to

methionine through the methionine synthase enzyme, in the presence of vitamin B12 as co-factor [11]. The presence of 5MTHF as donor or the metilo group is vital to obtain methionine and maintain the consumption of Hcy in the biochemical cycle, since an increase in the concentration of Hcy in plasma, is associated to vascular damage that can initiate or accelerate thrombotic and atherogenic processes [11]. Prothrombin is the inactive precursor of thrombin, which is the end product of the coagulation cascade. The prothrombotic mutation is a guanine-to-adenine transition at position 20210 in the sequence of the 3'-untranslated region of the gene encoding prothrombin [12]. This mutation is found to be associated with increased prothromhin levels and a 3-fold risk of venous thrombosis [12]. Fibrinogen is an acute phase class II protein that plays a vital role in normal haemostasis by promoting platelet aggregation, clot formation and fibrinolysis. Many gene mutations of fibrinogen have been demonstrated [13].The most common G/A polymorphism have shown in the 455th area of fibrinogen gene. The presence of A allele leads to increase fibrinogen levels [14]. Increased plasma levels of fibrinogen have been associated with an increased risk for vascular events including myocardial infarction, stroke and venous thromboembolism [15].

There are other gene mutations that can affect the balance of hemostais [16]. These mutations include factor Xlll, protein C and protein S system, and plasminogen activator inhibitor-1 (PAI-1) [16]. Coagulation factor XIII (FXIII) is a transglutaminase, which plays a major role in the final stage of blood coagulation. FXIII circulates as a heterotetramer consisting of two active A subunits (FXIII-A) and two carrier B subunits (FXIII-B). The FXIII-A gene is highly polymorphic in which some mutations lead to a deficiency in factor Xlll, while others have been reported to be protective against both arterial and venous thrombosis [17].

Protein C and protein S system are the major regulatory system of hemostasis. Protein C and protein S are vitamin K dependent proenzymes synthesized in the liver [18]. Thrombin- thrombomodulin complex on the surface of endothelial cells is the site for the interaction with protein C and S. Protein C becomes activated (activated protein C) after binding to these complexes. Protein S acts as a cofactor in this process [18]. The findings of a recent genetic study of VTE in northeast Asians indicated that the most frequent

genetic risk factors for VTE in northeast Asians can be attributed to a dysfunction of the protein C anticoagulant system [19].

Impaired fibrinolysis as a result of increased plasminogen activator inhibitor-1 (PAI-1) levels in plasma is a common finding in patients with deep vein thrombosis [19]. A 4G/5G polymorphism in the promoter region of the PAI-1 gene has been reported to influence the levels of PAI-1[19]. The 4G allele was found to be associated with higher plasma PAI-1 activity. Some studies revealed no relationship between the PAI-1 promoter 4G/5G genotype and DVT, nevertheless, some studies showed an association [20].

Acquired thrombophilic conditions include; pregnancy, antiphospholipid antibodies syndrome, immobility, trauma , postoperative state, obesity, oral contraceptives and hormonal replacement therapy, malignancy, myeloproliferative disorders and nephrotic syndrome [21].

This study was aimed, firstly, to estimate the prevalence of mutations associated with deep venous thrombosis and to compare them with local, regional and international findings. Secondly, we aimed to find the correlation between genetic findings and the acquired conditions that may predispose thrombophilia and deep venous thrombosis. Finally, this study aimed, due to genetic heterogeneities, to detect thrombophilic factors in patients with VTE in Sudan to be used as a guide in developing treatment strategies and in establishing prognosis.

1.2. Literature Review

1.2.1. Inherited Risk factors of VTE

1.2.1.1. Factor V

Factor V is a large single-chain glycoprotein that circulates in human plasma. It is also contained in the alpha granules of human platelets, with approximately 18%- 25% of the total factor V found in platelets. Molecular weight of factor V is about 330,000 and plasma half-life of 12 hours with some reports of 36 hours half-life [22, 23]. Factor V is organized in the following pattern of domains; A1, A2, B, A3, C1, and C2. The three A domains have significant homology to the copper-binding plasma protein ceruloplasmin [24]. The C domains have some homology to fat globule proteins [24]. The C2 domain of factor V mediates binding to lipid membranes [24].The A and C domains of factor V are approximately forty percent identical to the homologous regions in factor Vlll [22]. In contrast, the B domains show little homology between the two proteins and are not known to be homologous to any other protein. In factor V, unlike factor Vlll, sequences in the B domain appear to be important in promoting its activation by thrombin [22] .The procofactor factor V is proteolyzed by alpha thrombin to active cofactor Va [25]. Factor Va functions as factor Xa receptor and positive modulator of factor Xa catalytic potential in the prothrombinase complex [26]. Prothrombinase complex consists of serine proteinase factor Xa, the protein cofactor Va, calcium ions, and a phospholipid or cell membrane surface [24]. Factor V deficiencies, based in bioassay data, are well documented and have been shown to occur with liver dysfunction and with acquired factor V inhibitors [23]. It has been postulated that some individuals with combined factor V and factor Vlll deficiency, lack an inhibitor of activated protein C and suggested that these individuals may be devoid of factor V activity but not factor V antigen [23]. Factor V a is inactivated by protein C at a number of sites, including Arg 506 and Arg 306 [27].

1.2.1.1.1. Activated Protein C Resistance (APCR) and Factor V Leiden

In 1993, a poor anticoagulant response to activated protein C (APC) was reported as a common cause of familial thrombophilia [28]. In at least 95% of cases, resistance to APC is caused by a single point mutation in the factor V gene. A transition (Guanine to Adenine) at nucleotide 1691 in exon 10 results in the synthesis of a variant factor V molecule known as factor V Leiden with the substitution of Arganine to Glutamine at

amino acid position 506 [28]. Factor V Leiden discovered in 1994 and named for the city of Leiden, the Netherlands [29]. Prevalence of this mutation varies among different populations and ethnic groups. It is rare in Asian and African populations and is higher in European populations (5-9% healthy subjects). The highest frequency reported in the Eastern Mediterranean region found in Lebanon (14%) [30]. The prevalence of activated protein C resistant in Sudanese patients with DVT found to be 18.8 % [31]. There are other genetic mutations in factor V leading to APCR and venous thromboembloism such as FV Cambridge, Hong Kong and Liverpool mutations but these are extremely rare [28]. In 1997 a specific haplotype of factor V was reported, which may contribute to an APC-resistant phenotype. This so-called HR2 haplotype contains a histidine to arginine Substitution at position 1299 (Hisl299Arg) in the B-domain of factor V [32]. Factor V genotype A5279G is observed in some studies with controversial importance [33].

1.2.1.2. Prothrombin

Prothrombin or coagulation factor ll is a blood protein synthetized in the liver in the presence of vitamin K (vitamin K dependent). It is a thrombin precursor, which induces the formation of fibrin at the end of the coagulation cascade [34]. It is also involved in coagulation control mechanism, binding to thrombomodulin and activating protein C, which also plays a fundamental role in anticoagulant balance [34]. Prothrombin is encoded by a 21 kb long gene localized on chromosome 11, position 11p11-q12 [35]. The prothrombin gene is organized in 14 exons, separated by 13 introns with the 5′ upstream untranslated (UT) region and the 3′ UT region, which may play regulatory roles in gene expression [35].

Among many causes of inherited thrombophilia, the prothrombin G20210A gene mutation remains one of the more recent findings. This mutation was associated with increased venous thrombosis as well as increased levels of circulating prothrombin [36]. It has been shown that carriers of this mutation generate more thrombin than non carriers with similar factor II levels. This fact suggested that thrombin generation is more important for the development of VTE. After factor V Leiden, this mutation is the second most common inherited thrombophilic disorder found in 0.7% and 4% of the general population [36]. It increases the risk for deep venous thrombosis by a factor of 2.7 -3.8 [36]. The 20209 cytosine to thiamine variant of the prothrombin gene has been found

in individuals of African, Caucasian and Jewish-Moroccan origins, associated with venous thrombosis, pulmonary embolism, peripheral vascular disease, deep venous thrombosis, cerebrovascular accedents and obstetrical complications [37]. Initial reports differ as to whether or not this prothrombin variant is associated with increased risk of venous thromboembolism [37]. A novel frameshift mutation in prothrombin gene is found in Japan (Prothrombin Saitama). This variant results from the insertion of T at 7,358-7,359 of exon 8 [37].

1.2.1. 3.Fibrinogen

Plasma fibrinogen is synthesized in the liver. It is a dimeric glycoprotein whose dominant form has a molecular weight of 340,000 [39]. It is found in plasma and in platelet α-granules. Each of the two subunits contains three disulfide-linked polypeptide chains 157 referred to as the Aα (Mr 66,500), Bβ (Mr 52,000), and γ (Mr 46,500) chains. Fibrinopeptides A and B are released from the amino-termini of the Aα and Bβ chains by thrombin cleavage of the Argl6-Glyl7 and Argl4-Glyl5 bonds, respectively [39]. Fibrinogen is converted to fibrin; it forms the structural meshwork that consolidates an initial platelet plug into a solid hemostatic clot. In normal individuals, the plasma half-life of fibrinogen is 3 to 5 days. Only a small proportion of the catabolism results from consumption [39].

The genes for the three chains of fibrinogen are found within a 50-kb length of DNA on chromosome 4 at q23-q32. The genes for all three chains have been sequenced. The genomic sequences show a high degree of homology, suggesting they are derived through duplication of a common ancestral gene. The homology extends to sites upstream of the gene, suggesting common regulatory elements reside in these areas, thus helping to coordinate synthesis of the three chains [39]. Fibrinogen is an acute phase class II protein that plays a vital role in normal haemostasis by promoting platelet aggregation, clot formation and fibrinolysis [13].

1.2.1.3.1. Fibrinogen gene mutations

Increased plasma levels of fibrinogen have been associated with an increased risk for vascular events including myocardial infarction, stroke and venous thromboembolism [11]. Many gene mutations of fibrinogen have been demonstrated. The most common Guanine/Adenine polymorphism has shown in the 455th area of fibrinogen gene. The

presence of A allele leads to increased fibrinogen levels [10]. Another gene mutation of fibrinogen, which is extensively studied, is Single Nucleotide Polymorphism -148C/T (rs1800787) which is located in FGB promoter [40]. Studies have confirmed that SNP -148C/T had been association with high fibrinogen concentration [40]. More recently, the α-fibrinogen Thr312Ala polymorphism has been associated with pulmonary embolism, suggesting a role in thromboembolic complications [41].

1.2.1.4. Plasminogen activator inhibitor type-1 (PAI-1) polymorphism

Plasminogen activator inhibitor type-1 (PAI-1) is a glycoprotein with a molecular weight of approximately 50 kDa [42]. PAI-1 is member of a super family of serine protease inhibitors and is the major plasminogen activator inhibitor in humans. It is synthesized by the vascular endothelium and also exists in platelets [43]. Hormones, cytokines, and growth factors can induce PAI-1 production. PAI-1 is a key element in the inhibition of fibrinolysis by inactivating tissue-type and urokinase-type plasminogen activators. The PAI-1 not only inhibits the fibrinolytic system, but it is also involved in regulation of cell migration, invasion, and adhesion during the inflammatory process. The human PAI-1 gene is located on the long arm of chromosome 7 and includes nine exons and eight introns. Various polymorphisms have been studied within the gene, including a HindIII restriction fragment length polymorphism, a cytosine–adenine (CA) dinucleotide repeat and a single nucleotide insertion/deletion (4G/5G) polymorphism 675 bp upstream of the transcriptional start site in the promoter [44].

1.2.1. 5. Protein C

Protein C, the key component of the PC anticoagulant system, is an important vitamin K-dependent protein that regulates the physiological coagulation cascade by inactivating factors Va and VIIIa upon activation by thrombin. The mature PC molecule is a single-chain, 62-kDa glycoprotein that is synthesized by hepatocytes as a 461-amino acid precursor from which a 42-amino acid signal peptide is cleaved. Protein C is composed of a C carboxy-glutamic acid residue (Gla) domain, two epidermal growth factor (EGF)-like domains, a short activation peptide, and a serine protease domain. Thrombin cleaves PC at Arg169, removes the activation peptide and generates activated protein C (APC). Activated PC cleaves critical sites in the activated procoagulant factor V (FV) and FVIII,

and inactivates the two factors. This process is augmented by protein S (PS), FV and lipid cofactors of lipoproteins and phospholipids [45]. In addition to its anticoagulant properties, APC has anti-inflammatory and cytoprotective functions, which are exerted when APC activating protease activated receptor-1 (PAR-1). The human protein C gene (PROC) is located on chromosome 2q13-q14 and comprises nine exons spanning 11 kb [46].

Protein C (PC) deficiency, first described in a family with recurrent thrombosis by Griffin et al. in 1981 , is an autosomal dominant inherited disorder caused by mutations of the gene (PROC, OMIM #176860) located on chromosome 2q13–14, and its clinical expression is widely heterogeneous. Thus far, 151 different mutations have been described . In the neonatal period, homozygous subjects often express severe clinical manifestations, such as purpura fulminans or disseminated intravascular coagulation with undetectable PC level. Homozygous or compound heterozygous kindreds, with very low PC levels associated with severe clinical manifestations, have recurrent thrombotic episodes during the first decade of life [47].

1.2.1. 6. Protein S

Protein S (ProS), a vitamin K-dependent glycoprotein, is a crucial naturally occurring plasma anticoagulant involved in the regulation of blood coagulation. The human ProS precursor contains 676 amino acid residues. Mature ProS circulates in the plasma as a single-chain, 635-amino acid protein with a molecular weight of 75 kDa and comprises a g-carboxyglutamic acid domain (Gla), a thrombin-sensitive region (TSR), four epidermal growth factor (EGF)-like domains (EGF1-4), and a large domain homologs to the sex hormone-binding globulins (SHBG). The Gla domain is pivotal for binding to negatively charged phospholipid membranes in a Ca 21-dependent manner. Factor Xa inactivates ProS by proteolytic cleavage at the TSR region [48].

Under normal conditions, more than 60% of protein S is bound to the C4b binding protein, and unbound protein S is available to form a complex with activated protein C in the presence of phospholipids and calcium ions [49].

Increased risk of venous thromboembolism is associated with hereditary deficiencies of protein S and protein C [50]. The frequency of deficiencies of protein C and protein S in VTE patients of Western ethnicity was reported to be 1.4–8.6 and 1.4–7.5%, respectively [51]. Compared with Caucasians, deficiencies of protein C and protein S in Asians were

higher in both the general population and in VTE patients. The most prevalent deficiencies in Asian VTE patients were protein S deficiency, followed by protein C deficiency [51].

1.2.1.7. Methylenetetrahydrofolatereductase polymorphism

Homocysteine is a chemical compound with the formula HSCH2CH2CH (NH2) CO2H and is a methylation of cells within the process of intermediate products. The change of plasma Hcy concentration can directly affect the genomic DNA methylation, which tends to repress gene expression while demethylation or hypomethylation activates the gene expression [52]. As a sulphur amino acid in the human body, Hcy is an intermediate product in the metabolism of methionine. It is suggested that high plasma levels of total homocysteine (tHcy) result from the interaction between genetic and acquired determinants [53]. Methylenetetrahydrofolatereductase is the key enzyme in the remethylation of homocysteine to methionine. In this reaction, methylenetetrahydrofolatereductase reduces 5- methylentetrahydrofolate to methyl donor methyltetrahydrofolate. Methylenetetrahydrofolatereductase deficiency is an autosomal recessive disease characterized by high levels of homocysteine and low or normal levels of methionine.

Hyperhomocysteinemia is a consequence of single nucleotide polymorphisms (SNPs) in MTHFR 677 C>T that can cause homocysteine levels in the blood to increase, usually exceeding 15 μmol/L. MTHFR 677 C>T is the result of a missense mutation occurring at position 222, where alanine is substituted with valine [54]. The methylenetetrahydrofolatereductase C677T homozygote mutation exhibits a prevalence of 10-20% in some societies [57]. The plasma Hcy level of the T/T genotype was significantly higher than that of the C/T or C/C genotype as reported by Ni MAO et al, 2012(31). Hyperhomocysteinemia is not only associated with the MTHFR C677T mutation, it is found that there was obvious negative correlation between the plasma levels of Hcy and the serum levels of folate [31, 50]. The accumulation of homocysteine has been shown to be associated with carotid plaque thickness and carotid stenosis, which have vital roles in clot formation and can contribute to cardiovascular disease as well as stroke [46]. Hyper-Hcy is well known to cause venous and arterial thrombo-embolism.

Epidemiological studies also have suggested that even mild hyper-Hcy is associated with occlusive arterial vascular disease and venous thromboembolism [53].

1.2.1.8. FactorXlll

Coagulation factor XIII (FXIII) is a transglutaminase, which plays a major role in the final stage of blood coagulation. FXIII circulates as a heterotetramer consisting of two active A subunits (FXIII-A) and two carrier B subunits (FXIII-B). Thrombin cleaves a 37-amino acid peptide from A-subunit and activates the molecule. FXIII catalyses cross-linking between fibrin molecules, increases the mechanical strength of the clot, and increases the resistance to plasmin. It also catalyses cross-linking reactions between fibrin molecules and other adhesive proteins, and eventually anchors the clot to the injured vessel wall [12].

The A subunit contains the active site of the enzyme, and is synthesized by hepatocytes, monocytes, and megakaryocytes [12]. The B-subunit serves as a carrier for the catalytic A-subunit in plasma, and is synthesized by the liver [12], The main source of FXIII A-subunit in plasma was found to be platelets but not monocytes as reported previously in patients with autologous stem cell transplantation following myeloablation [13, 56]

The FXIII-A gene is highly polymorphic and located on chromosome 6 p24-25. In addition to mutations leading to FXIII deficiency, other polymorphic sites have been described at Val34Leu, Pro564Leu, Val650Ile, Glu65 lGln, and Tyr204Phe. FXIII Val34Leu polymorphism with a valine to leucin change that is close to the thrombin activation site of the molecule has been reported to be protective against both arterial and venous thrombosis [12].

1.2.2. Predisposing factors of venous thromboembolism

1.2.2.1. Pregnancy

Pregnancy is a normal physiological state that may predispose to thrombosis, a hypercoagulability (thrombophilia), determined by changes in the body, arising from the special hormonal constellation [57]. Normal pregnancy is characterised by a significant increase in procoagulant factors V, VII, VIII, IX, X, XII, fibrinogen and von Willebrand factor, which is maximal around term. Acquired activated protein C (APC) resistance has previously been reported .An increase in C4B-binding protein has previously been reported during pregnancy. The consecutive fall in free protein S levels in pregnancy may

contribute to a state of hypercoagulability [58]. The risk of venous thromboemblolism increases throughout pregnancy with an average incidence rate of 1 per 1000 pregnancies [49, 59]. In a systematic review of studies covering a search period of 1966 to 2009, Chan and colleagues found that the distribution of deep vein thrombosis was different in pregnant women than in other patients. They confirmed earlier findings that deep vein thrombosis mainly affects the left leg and is usually isolated to the proximal iliac and/or femoral veins [60]. This is because of increased venous stasis in the left leg due to the compression of the left iliac vein by the right iliac artery, in addition to compression of the inferior vena cava by the gravid uterus [57].Although clinical assessment of deep venous thrombosis (DVT), based on risk factors and physical signs, cannot be used alone to safely exclude or diagnose DVT , clinical assessment of DVT using a combination of risk factors and physical signs plays an important role in current diagnostic strategies of DVT in the general population [61]. During pregnancy, an accurate diagnosis is required in case of suspected deep vein thrombosis (DVT). Indeed, false positive tests lead to inappropriate anticoagulant treatment that increases the risk of bleeding and requires daily heparin injections during the entire pregnancy. Conversely, false negative tests might lead to a life-threatening thromboembolic event [62].

1.2.2.2. Antiphospholipid

Antiphospholipid antibodies (aPL) are a wide group of autoantibodies detected in serum or plasma by diverse laboratory tests employing phospholipids. The association of these antibodies with thrombotic events (deep venous thrombosis(DVT), pulmonary embolism (PE), myocardial infarction (MI), stroke or transient ischaemic attack (TIA) and pregnancy complications is well established. The combination of aPL with either venous or arterial thrombosis or obstetric complications is referred to as antiphospholipid syndrome (APS) [63].Aniphospholipid Syndrome also known as sticky blood or Hughes' syndrome, is an autoimmune disease that can cause abnormal blood clotting in any blood vessels- both arteries and veins. As a result it can cause many different problems [64]. APS may be isolated (primary) or related to auto-immune disorders such as systemic lupus erythematosus (SLE) [65]. The mechanisms of thrombosis caused by aPL in cerebral thrombosis (CT), venous thromboembolism (VTE), and obstetric morbidity are poorly understood. However, inhibition of natural anticoagulants, activation of platelets and

endothelial cells , blocking of the fibrinolytic system , and triggering of the complement cascade have been speculated. aPL from patients with APS preferentially targets the negatively charged phospholipids (PL) and/or their complex with plasma proteins including b2-glycoprotein I (b2GPI) [66]. The results of previous data showed that double or triple positivity of aPL antibodies (LA, aCL, ab2GPI) carries a much higher risk for future thrombotic events(either venous or arterial) in asymptomatic aPL carriers than any single positive test [63].

1.2.2.3. Immobility

Immobility is associated with reduced venous blood flow, particularly in the pockets of the venous valves, leading to inflammation and hypercoagulability. In young and middle-aged individuals, immobility, such as that due to hospitalization or minor injuries, is an established risk factor for thrombosis with relative risk estimates ranging from 3 to 11 . However, it is unknown to what extent immobilization increases the risk of venous thrombosis in older individuals [63]. According to the literary data, DVT frequency in the risky hospitalized patients without prophylactic therapy is 10–40%, where, 70–80% of such thromboses are clinically neither silent -nor asymptomatic [68].

1.2.2.4. Trauma

Among hospitalized adults, trauma patients have the highest risk of deep venous thrombosis (DVT), with reported incidences of more than 50% without prophylaxis [69].Trauma and general surgery patients have a multitude of factors contributing to VTE, including venous stasis, endothelial injury, impaired fibrinolysis, and decreased levels of serum anticoagulants. Despite standard prophylaxis, the incidence of pulmonary embolus after trauma has been reported to be as high as 2% to 22 % [70]. Even studies published from the West show variation in VTE incidence. The largest retrospective published study to date of VTE from North America has shown that the overall VTE incidence in general trauma patients may be lower than expected, finding an incidence of only 0.36 %for VTE and 0.13 % for PE [71].

1.2.2.5. Contraceptive pills

The pill is the most commonly used contraceptive method and approximately 50–80% of women use it at some stage during their reproductive lives. There is now a large range of products available with over 30 different registered brands [72]. While many of these pills contain similar hormones and doses, there are multiple formulations for the prescriber to consider. These pills contain an oestrogen component (ethinyloestradiol, mestranol, oestradiol or its pro-drug oestradiolvalerate) and a progestogen (levonorgestrel, norethisterone, gestodene, desogestrel, drospirenone, nomegestrol, dienogest or cyproterone) [72]. Oral combined contraceptive pill (OCCP) is an effective form of birth control and is used to treat a number of other conditions. Like any other drug, OCCPs are not free from risks. Women taking OCCP have higher risk of developing deep vein thrombosis (DVT), usually in the legs, and this may lead to pulmonary embolism, a serious complication. For every 100,000 women aged 15–44 years not taking pill, approximately 5–10 are likely to develop blood clot in 1 year and this risk increases 3–4 times in those using second generation OCCP and 6–8 times in those using third generation OCCP [73].

1.2.2.6. Diabetes

Diabetes mellitus is a very frequent disease with an estimated prevalence of 6% among the population worldwide [74]. There is increasing evidence that diabetes mellitus is associated with several defects of coagulation and fibrinolysis that lead to a procoagulant, thrombogenic predisposition [75]. Diabetes mellitus is commonly associated with both macrovascular and microvascular complications. Macrovascular events are likely due to thrombotic events in the large arteries, whereas microvascular complications result from long-standing functional and metabolic derangement in small vessels. Although the mechanisms of these complications are complex and not yet completely clarified, a prothrombotic state characterized by activation of the coagulation system has been reported as a plausible cause of vascular complications. Advanced glycation end products can induce an intracellular oxidative response, which results in tissue factor (TF) expression of endothelial cells. High levels of thrombin markers, such as prothrombin fragment 1 + 2 and thrombin–antithrombin complexes have been detected in patients with diabetes, in comparison with healthy controls [76].

1.2.2.7. Obesity

The prevalence of obesity [body mass index (BMI) > 30 kg/ m2] has increased substantially in recent years and forecasts suggested that if current trends continue, up to 58% of the world's adult population will be overweight or obese by 2030. Obesity is an independent risk factor for venous thromboembolism and ischaemic heart disease and adversely impacts on overall health with healthcare utilization, including inpatient stay, reported to increase in parallel with BMI [(77)]. Obesity is associated to a haemostatic derangement characterised by inflammatory state, early endothelial dysfunction and increased thrombin generation [(78)]. It was shown that obesity is associated with a hypercoagulable state either with increased levels of clotting factors or inhibition of the fibrinolytic pathways [(79)].

1.2.2.8. Cancer

The association between cancer and venous thromboembolism (VTE) is well-established and strong [(80)]. Active cancer with and without chemotherapy increases VTE risk by 5- to 6-fold [(80)]. Prevalence of DVT and PE among patients with upper GI cancer was estimated to be 5.2 and 7.7 per cent respectively [(81)]. Venous thromboembolism in lung cancer patients is associated with significant morbidity and mortality. Lung cancer patients have high rates of VTE recurrence in the first few months after diagnosis [(82)]. It is reported that VTE occurred in almost 15% of lung cancer patients [(82)]. Other studies showed an association between ovarian cancer and VTE, the prevalence was 14.5 % [(83)]. Active cancer is associated with intra-abdominal and bilateral leg DVT [(80)].Thromboembolism (TE) is a well-recognized complication of acute lymphoblastic leukemia (ALL) and its therapy, with a significant impact on the overall outcome of ALL and subsequent quality of life. Thromboembolism in children is associated with 1.5–2 % mortality and significant morbidity, including recurrence, loss of venous access, lack of thrombus resolution and the development of postthrombotic syndrome. Estimates of incidence of TE in children with ALL, gathered from prospective studies, range from 3 % to 36.7 % compared to about 1 per 100,000 children in the general pediatric population [(84)]. Philadelphia-negative myeloproliferative neoplasms, polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF), are considered to be

acquired thrombophilic states [82].They are associated with the JAK2 V617F somatic mutation, and carry an increased risk of thrombosis [85].

1.2.3. Diagnosis of deep vein thrombosis

Several strategies have been developed to improve diagnostic accuracy and minimize health consequences of misdiagnosis and overtreatment. The diagnostic strategies for DVT usually consist of clinical pretest probability assessment, using structured scoring systems, followed by sequential testing using the D-dimer assay and imaging studies. The Wells score is the most studied structured scoring systems and categorizes patients as having low (5.0%; 95% CI, 4.0-8.0%), moderate (17%; 95% CI, 13-23%), or high probability of having DVT (53%; 95% CI, 44-61%). Compression ultrasound (CUS) of the proximal veins is the commonly used imaging test. Other tests, such as contrast venography, which is still considered the reference standard for DVT diagnosis, computed tomography, and magnetic resonance imaging may be occasionally used [86].

1.2.3.1. Detection of gene mutations

The discovery of genetic risk factors for thrombosis came after the identification of families in whom the thrombophilia segregated with an abnormal result in a plasma test (protein C, protein S**,** antithrombin, and APC resistance). However, despite the ever growing insight into the processes of coagulation and fibrinolysis, the underlying cause of many inherited thrombotic events remains unsolved [87]. Several different molecular genetics techniques that use restriction enzymes, allele-specific amplification, hybridization probes at a single detection temperature or the Light Cycler system have been described [88].

With Polymerase Chain Reaction (PCR), gene sections that are limited supply can be targeted and replicated. This can only be done if parts of the sequence in question are known. These sequences can be used to produce oligonucleotides, usually 20-25 bases in length, known as primers. They mark the starting points of DNA synthesis once DNA polymerase and deoxynucleoside phosphates have been added [89].

RT-PCR is a method that allows the quantification of genetic amplification products with the follow-up of PCR reaction in all phases and cycles. Accordingly, it dispenses with the use of conventional electrophoresis, optimizing the process [90]. The advantage of RT-

PCR relies on the fact that it offers a higher sensibility when compared with conventional PCR, allowing the detection of low concentrations of genetic material in the sample [90].

Minisequencing is one of the polymerase chain reaction (PCR)-based methods which is used for detection of a single nucleotide polymorphisms or point mutations. This method relies upon the incorporation of a single fluorescently labeled dideoxynucleotide (ddNTP) at the 3′ end of a special primer which is complementary to the sequence and is located one nucleotide before the examined polymorphic site. The incorporation of one out of four ddNTPs labeled by different color dyes depends on the genotype. After reaction, the products of the reaction are separated by capillary electrophoresis. The color of the single signal (obtained for homozygote), or two signals (obtained for heterozygote), allows the identification of the incorporated ddNTP and the complementary deoxynucleotide in DNA sequence [91].

1.2.3.2. D-dimer

D-dimers are fibrin degradation products formed as a result of fibrin clot dissolution by plasmin. They participate in the entire homeostasis mechanism including coagulation and fibrinolysis processes. Under physiological conditions, both processes should take place simultaneously, maintaining some balance [92]. D-dimer does not represent a simple homogeneous molecule, and most problems in measurement emerge from its biochemical heterogeneity [93].The use of D-dimer levels in the investigation and management pathway of venous thromboembolism is well established. This marker has a high sensitivity and specificity in excluding thromboembolism when a well-defined assay is used in the appropriate clinical setting [94].

D-dimers are detected by immunoassays using monoclonal antibodies specific for the cross-linked D-dimer domain in fibrinogen. Commercially available assays include latex agglutination, immunoturbidimetry, and enzyme-linked immunosorbent assay (ELISA). Efforts made to standardize D-dimer results have not been successful thus far, because the D-dimer analyteis not uniform across the different assays [95]. Many factors may affect the D- dimer results. Firstly its concentration is influenced by several demographical factors. Secondly, the method used (quantitative assays are preferred over semiquantitative or qualitative assays). Finally, the unit of measurement is an important issue that often generates confusion and jeopardizes the harmonization of test results

across laboratories. Results of D-dimer can be expressed as fibrinogen equivalents (FEU) or D-dimer units (DDU), according to the type of calibrator used. The mass of one unit of FEU is approximately twice that of one unit of DDU (1 µg/L DDU basically equals 2 µg/L FEU) [93]. The cutoff point for D-dimer is 500 µg/L, but this value should be adjusted in patients 50 years or more [96].

1.2.3.3. Thrombin generation

The serine protease thrombin is the most potent platelet agonist [97]. Thrombin has multiple functions in blood coagulation and its regulation is central to maintaining the balance between hemorrhage and thrombosis [98]. Physiologically, thrombin generation is triggered by the interaction between cellular tissue factor and circulating factor VII resulting in the active serine protease factor VIIa. Subsequently, the tissue factor:factorVIIa complex generates factor Xa, which in conjunction with trace amounts of its cofactor factor Va, converts prothrombin into thrombin. The final step in coagulation, conversion of fibrinogen into fibrin, is catalyzed by thrombin [99].

Recently, there has been increasing evidence to suggest that measurement of thrombin generation provides useful information about the coagulation status [100]. The thrombin generation (TG) assay is increasingly being recognized as a versatile diagnostic tool in the field of thrombosis and hemostasis. It is well accepted that the transient TG profile in clotting plasma is a better determinant of the overall function of the hemostatic system than are clotting time– based assays, e.g., prothrombin time (PT) and activated partial thromboplastin time [101]. In the thrombin generation assay with an automated calibrated thrombogram, thrombin generation curves can be operationally characterized to display initiation, propagation, and termination phases. After stimulation with TF, the consequent induction of endogenous thrombin potential (ETP) is measured in plasma [100].

1.2.3.4. Protein C

Protein C, the key component of the PC anticoagulant system, is an important vitamin K-dependent protein that regulates the physiological coagulation cascade by inactivating factors Va and VIIIa upon activation by thrombin [102]. Two different types of assays are available for the diagnosis and classification of PC deficiency, functional tests and antigen assays. For screening, a functional test should be performed, and if the results are abnormal, the antigen assay can distinguish between types I and type II deficiencies, with

concentrations of antigen being normal in the latter [103]. Healthy adults show a wide range of plasma protein C activity approximately between 65 and 135 IU⁄dL. As reported, the definition of 'mild' PC deficiency indicates activity levels higher than 20 IU ⁄ dL, 'moderately severe' protein C deficiency activity levels in the range of1–20 IU ⁄ dL, and 'severe' deficiency levels lower than 1 IU⁄dL (5). Thrombotic events may be observed in adolescents and in adults with 'moderately severe' PC deficiency [101].

1.2.3.5. Protein S

Protein S acts as a nonenzymatic cofactor for APC in the inactivation of the procoagulant factor Va and factor VIIIa on phospholipid membranes, as well as in the inhibition of thrombin generation [102].Under normal conditions, more than 60%of protein S is bound to the C4b binding protein, and unbound protein S is available to form a complex with activated protein C in the presence of phospholipids and calcium ions [103]. Measurement of protein S performed using a latex ligand immunoassay for the determination of free antigen and using clotting assays that detect protein S–mediated enhancement of the anticoagulant function of activated protein C [104]. In addition to congenital deficiencies, there are numerous physiologic and pathologic conditions that lead to decreased protein S levels. Inflammation leads to increased C4b binding protein levels and concomitantly decreases the levels of unbound and functional protein S [50].plasma concentration of protein S is 20–25 mg/L and PS circulates with +a half-life of 42 h [105].

1.2.3.6. Fibrin monomer complex

Soluble fibrin monomer appears in the bloodstream during the extremely early stage of blood coagulation. It generally forms a complex with fibrinogen after releasing fibrinopeptide A and/or fibrinopeptide B, which is termed soluble fibrin (SF) or soluble fibrin monomer complex (FMC). These complexes are found at a high concentration in hypercoagulable state blood. When a cross-linking reaction is induced by activated factor XIII (XIIIa), FMC or SF is converted to insoluble fibrin and forms thrombus. Therefore, FMC is well known as a good indicator of thrombogenic conditions, such as congenital thrombotic tendency or disseminated intravascular coagulation [106] FMC testing has not yet been widely used for the detection of coagulation status, possibly due to its nonquantitative features and a complicated measurement procedure. New methods to qualify FMC have recently been developed. Latex immunoassay (LIA) uses monoclonal

antibody to FMC. Several clinical studies have used LIA to predict or detect thrombotic status, including DVT, in patients with various diseases, and the FMC concentration appears useful in discriminating thrombotic status from normal coagulation status in orthopaedic patients. Nevertheless, no studies have yet examined the relationship between FMC concentration and pregnancy [107].

1.2.4. Signs and complications of DVT

1.2.4.1. Signs

Clinical signs and symptoms of DVT are similar to many other conditions and often it is clinically silent. Therefore accurate diagnosis requires special strategies. The dorsiflexion sign, first mentioned as a clinical sign of DVT in 1941 by Dr. John Homan, was described as "discomfort behind the knee on forced dorsiflexion of the foot". Dr. Homan also referred to tenderness and swelling of the affected extremity, tachycardia, pain or soreness of the calf muscles upon dorsiflexion of the foot and slight rise in temperature as signs and symptoms indicative of DVT [108]. Although a number of reports have questioned the clinical utility of Homans' sign, it is still widely used in clinical practice. This may stem from its historical role in clinical examination prior to the availability of the more reliable diagnostic studies, as well as from the ease of performing the Homans' sign test [109]. Thus, this test should not be the only criterion for diagnosing DVT [110]. A simplified model is designed to include nine clinical characteristics, each worth one point. These characteristics are active cancer, paralysis or casting of an extremity, bedridden more than 3 days or major surgery with general anesthesia during the previous 3 months, localized tenderness along the deep venous system, swelling of the entire leg, calf swelling of greater than 3 cm larger than asymptomatic side, pitting edema confined to the symptomatic leg, dilated superficial veins of the affected leg, and previously documented DVT [111]. A score of 2 or more places the patient in a high-risk category and a score of less than 2 indicates DVT is unlikely [111]. Some authors suggested that patients with even one of the predisposing factors should be considered at high risk for developing DVT, and should be assessed at least twice daily for clinical signs [115]. Patients who score 2 or higher should be assessed further for the presence of DVT through D-dimer assay blood testing and ultrasound [112].

1.2.4.2. Complications

DVT is a commonly occurring condition with potentially serious complications. The main hazard of DVT is acute stage thrombus shedding, which can cause a pulmonary embolism (PE) and acute respiratory distress syndrome when the blood flow blocks the pulmonary artery [113]. Pulmonary embolus (PE) refers to obstruction of the pulmonary artery or one of its branches by material (e.g., thrombus, tumor, air, or fat) that originated elsewhere in the body. Despite the high-incidence of pulmonary embolism, its diagnosis continues to be difficult primarily because of the nonspecific nature of the presenting signs and symptoms. The most common sources of pulmonary embolism are the pelvic veins or deep veins of the thigh [114]. Pulmonary embolism (PE) represents the third most common cause of cardiovascular death after ischemic heart disease and stroke [108]. If a PE is left untreated, mortality can increase to as high as 65% within the first hour and 92.2% in the first 2.5 hours . When appropriate treatment is instituted in a timely fashion, however, death occurs in less than 10% of patients [110]. A diagnosis of PE can be a major challenge for clinicians since its signs and symptoms are nonspecific [115]. With the increasing use of the computed tomography (CT) angiography as the main diagnostic method in pulmonary thromboembolism, new approaches for categorizing the severity of pulmonary embolism have been conducted mainly based on thrombus burden and its impact on the right ventricle [116]. Chronic thromboembolic pulmonary hypertension (CTEPH) is a rare complication of acute pulmonary embolism due to unresolved emboli initiating remodeling of the non-obstructed pulmonary arteries leading to progressive increase in pulmonary vascular resistance (PVR). The incidence of CTEPH is not known, but recent studies suggest that 1–4.6% of patients develop the condition within 2 years after an episode of acute pulmonary embolism [117]. Acute respiratory distress syndrome (ARDS) is a clinical syndrome characterized by acute onset respiratory failure resulting from various direct or indirect injuries to pulmonary parenchyma or vasculature [118].

Normal pregnancy is related with an acquired hypercoagulable state due to increased levels of coagulation factors, decreased levels of anticoagulants and decreased fibrinolytic activity. This hypercoagulability may be exacerbated in women with heritable predisposition to thrombosis, known as thrombophilia, and may contribute to various pregnancy complications such as venous thromboembolism (VTE), deep venous

thrombosis (DVT), first trimester abortion (FTA), mid-trimester abortion (MTA), intrauterine fetal death (IUFD), preeclampsia (PE), placental abruption (PA), and fetal growth restriction (FGR). The most common types of inherited thrombophilias are the following: factor V Leiden (FVL) mutation, prothrombin G20210A (PT) mutation, deficiency of protein C (PC), deficiency of protein S (PS) and the most thrombogenic, antithrombin (AT) deficiency [119].

1.2.5. Prevention

Venous thromboembolism (VTE), including deep-vein thrombosis (DVT) and pulmonary embolism (PE), is associated with fundamental morbidity and mortality. Detailed estimates of the incidence of VTE events are hard to obtain because VTE is difficult to diagnose. This is because of various factors; VTE is often clinically silent and, in many cases, the first sign of the disease is a sudden fatal PE [120]. Post-thrombotic syndrome (PTS) is a chronic condition characterized by symptoms and signs that develop after deep venous thrombosis (DVT). Post-thrombotic sequelae range from minor skin changes to established ulceration. PTS is the most common long-term complication of venous thrombo-embolism affecting 20–40% of patients after DVT of the lower limb. PTS can be debilitating to patients, and have a significant impact on their quality of life. Most hospitalized patients have one or more risk factors for VTE. These risk factors are generally cumulative. For example, patients with fractures of the hip are at particularly high risk for VTE because of their usual advanced age, the presence of a proximal lower extremity injury as well as its operative repair, and the frequent marked reduction in mobility for weeks after surgery. If cancer is also present, the risk is even greater. Without prophylaxis, the incidence of objectively confirmed, hospital-acquired DVT is approximately 10 to 40% among medical or general surgical patients and 40 to 60% following major orthopedic surgery. One quarter to one third of these thrombi involve the proximal deep veins, and these thrombi are much more likely to produce symptoms and to result in PE [121]. Therefore, prevention and prophylaxis of VTE is of great importance in immobilized patients or post operative patients. Prophylaxis of VTE should be implemented according to individual's situation.

1.2.5.1. Exercise

DVT and its chronic complication, PTS, affect adults of all groups; many of them are active and do physical exercise. Exercise training is an effective treatment for the improvement of post-thrombotic syndrome.during exercise, muscle contraction increases the pressure outside of the veins drives blood back to the heart(calf muscle pump), reducing the hydrostatic pressure gradient required for edema formation [122].

1.2.5.2. Compression therapy

1.2.5.2.1. Intermittent pneumatic compression

IPC is an effective mechanical method of DVT prophylaxis, exerting an antithrombotic effect that appears to be a result of reduced plasminogen activator inhibitor-1 levels and increased tissue plasminogen activator activity. These actions stimulate fibrinolytic activity and increase venous blood flow velocity, thereby reducing stasis and altering hypercoagulability. However, the efficacy of this treatment is determined in part by patient compliance with the treatment protocol for instance, it is suitable method if the patient suffers bleeding after antithrombotic therapy. Nevertheless, the use of ICP is limited in cases with certain diseases such as pulmonary edema, non-congestive heart failure, and trauma or fracture of the leg [123].

1.2.5.2.2. Compression stocking

Compression therapy, which may include use of bandages or elastic compression, should be started soon after initiation of anticoagulant therapy. Compression is recommended for at least 2 years, and longer if the patient has continuous symptoms of PTS and stockings are helpful. Most patients adhere to compression therapy; however, patient conditions may hinder adherence, such as stocking application, discomfort, allergic hypersensitivity to the skin, and cost. The risk of major harm from compression therapy is low and some patients report improvement of symptoms with their use; therefore, compression therapy is reasonable to trial in Post-Deep Vein Thrombosis [124].

1.2.5.3. Pharmaceutical therapy

1.2.5.3.1. Aspirin

Acetylsalicylic acid (aspirin) is the most widely used inhibitor of platelet aggregation. It has been shown in recent years to be effective in the primary and secondary prevention of arterial occlusion. Aspirin induces a long-lasting functional defect in the platelet, by permanently inactivating the enzyme prostaglandin G/H synthase (now cyclooxygenase or COX), which catalyses the first step in the synthesis of prostaglandins. Thereby, thromboxane (TX) A2 cannot be formed in platelets from its substrate arachidonic acid. Thromboxane A2 is a potent vasoconstrictor and platelet aggregating agent [(125)]. The minimum dose required to produce platelet inhibition remains controversial. Single oral doses of as low as 5 mg aspirin have been shown to cause inhibition of platelet COX activity in both normal subjects and in patients with atherosclerotic vascular disease [(129)]. Aspirin and other anti-platelet drugs are highly effective at reducing major vascular events in patients who are at risk for or who have established atherosclerotic disease. Evidence, suggests that antiplatelet agents also provide some protection against VTE in hospitalized patients who are at risk. However, it is not recommend the use of aspirin alone as VTE prophylaxis for several reasons. First, much of the evidence citing a benefit for the use of antiplatelet drugs against VTE is based on methodologically limited studies [(124)]. Second, a number of trials found no significant benefit from aspirin therapy, or found that aspirin was inferior to other prophylactic modalities. Finally, aspirin use is associated with a small but significant increased risk of major bleeding, especially if combined with other antithrombotic agents [(121)].

1.2.5.3.2. Heparin

Traditionally heparin has been used once DVT or PE is diagnosed or highly suspected due to its rapid anticoagulation activity. A vitamin K antagonist (VKA) such as warfarin is usually started at the same time as heparin, or slightly later, but takes several days to produce an anticoagulant effect, hence the need for initial heparin in patients who are to receive warfarin [(127)] . Low molecular weight heparin (LMWH) is preferred over unfractionated heparin (UFH). It is the preferred treatment of DVT in cancer patients and in pregnancy because it does not cross the placenta. LMWH is given by subcutaneous injection and, as it has a longer half-life than standard heparin, it can be given once a day

in prophylaxis, or once or twice daily for treatment [128]. It is recommended that when using heparin, the risk of heparin-induced thrombo-cytopenia (HIT) be taken into account. When using unfractionated heparin (UFH), platelet count should be regularly monitored [127]. LMWH is superimposed with oral warfarin for the first few days, decreasing the dose gradually. Coagulation parameters should be checked after three days, and the oral warfarin dosage increase or decrease in order to maintain an international normalized ratio (INR) within 2.0–2.5. When the INR reaches 2.0, LMWH will be discontinued [129].

1.2.5.3.3. Warfarin

Coumadin (4-hydroxycoumarin, warfarin sodium) is a member of the coumarin drug family and is the formulation of coumarin most often used in North America [131]. Warfarin is an oral anticoagulant recommended by the American College of Chest Physicians for a variety of indications, including the treatment of venous thromboembolism and prevention of embolism in patients with prosthetic heart valves or atrial fibrillation [132]. Coumadins produce their anticoagulant effect by interfering with the cyclic interconversion of vitamin K and its epoxide (vitamin K epoxide), thereby modulating the γ-carboxylation of glutamate residues (Gla) on the N-terminal regions of vitamin K-dependent proteins. The vitamin K coagulation factors II, VII, IX, and X require γ-carboxylation for their procoagulant activity, and treatment with coumarins results in the hepatic production of partially carboxylated and decarboxylated proteins with reduced coagulant activity [133]. Coagulation monitoring has formed the cornerstone to the effective management of patients receiving warfarin. Monitoring of the international normalized ratio (INR) levels is required to compensate for the complex pharmacokinetics of the coumadins, especially the high inter- and intrapatient variability and multiple food and drug interactions that leads to the need for dose adjustment [134]. The physician adjusts the Coumadin dosage to achieve the desired INR of 2 to 3, or 2.5 to 3.5 if the patient has a mechanical heart valve. INRs greater than 4 are associated with increased risk of hemorrhage and require immediate communication with the clinician who is managing the patient's case [131].

1.2.5.3.4. Rivaroxaban

Rivaroxaban is an active factor Xa inhintor, factor Xa that is bound by factor IXa, and clot-bound factor Xa [131, 115]. As a consequence of factor Xa inhibition, rivaroxaban dose-dependently inhibited thrombin generation via both the intrinsic (collagen-induced) and extrinsic (tissue factor induced) coagulation pathways. Unlike warfarin, which is associated with dosage and monitoring and dose adjustment difficulties , rivaroxaban is administered at a fixed oral dose and generally does not require routine monitoring of coagulation parameters [135].

1.2.5.3.5. Apixaban

Apixaban is a selective direct factor Xa inhibitor. It is believed that Apixaban terminates the burst of thrombin generation and result in inhibition of thrombus formation with a favorable safety profile. Apixaban has been studied for the treatment and prevention of venous thromboembolic disease, in addition to stroke prevention among patients with nonvalvular atrial fibrillation, and additional evidence suggests that among the direct oral anticoagulants (DOACs), the risk of major bleeding with apixaban is low [136].

Rationale

Venous thromboembolism (VTE) comprises deep vein thrombosis (DVT) and pulmonary embolism (PE). VTE constitutes the third most common cardiovascular disease, after acute coronary syndrome and stroke. DVT and PE affect individuals over a wide age range, from teenagers to the elderly. This illness strikes all socioeconomic groups in developed Western countries. The mortality rate in the United States alone is between 100,000 and 180,000 deaths annually. In addition, the economic burden is high. The most effective and economical approach to decreasing the burden of VTE is to prevent the development of DVT and PE in patients at high risk. We aimed to study common prothrombatic gene mutations and their role as risk factors for VTE. Moreover, the study aimed to find out the prevalence of each mutation and its association with the occurrence of DVT. In addition, we aimed to estimate concentration of proteins that related to the regulation of hemostasis. Finally, we aimed to provide enough data on risk factors of VTE, their prevalence, and laboratory diagnosis to improve strategies for the prevention of the condition.

Objectives

General objective:

To detect prothrombotic gene variants in patients with deep venous thrombosis.

Specific objectives:

1- To detect factor V Leiden G1691A mutation.

2- To detect prothrombin G20210A mutation.

3- To detect Methylenetetrahydrofolatereductase (MTHFR) C677T mutation.

4- To detect Beta fibrinogen 455G/A mutation.

5- To detect Plasminogen activator inhibitor type-1 4G/5G 675 mutation.

6- To detect Factor XIII val 34 leu mutation.

7- To measure protein C, protein S, plasma fibrinogen and D-dimer .

8- To perform coagulation profile: PT, APTT and platelets count.

9- To calculate prevalence of each parameter in the study group and compare it with the control group.

Chapter two
Materials and methods

2.1. Study design

This is a case control study. It was conducted in Khartoum state, Sudan, in order to determine the prevalence of genetic mutations that predispose thrombophilia among deep venous thrombosis patients. A total of one hundred and fifty individuals were included in this study, one hundred of them were DVT patients and the remaining fifty were healthy volunteers assigned as control group. The study group (DVT patients) consists 30 men and 70 women; median age 48.14 years; ranging from 19 to78 years. Patients were recruited to the study during their visit to outpatient clinic at Ommdurman teaching hospital when they came for regular follow up. Seventy eight were under heparin therapy while twenty two were taking warfarin. The control group (healthy volunteers) included 29 men and 21 women; median age 32.4 years; ranging from 22 to 47 years). Both case and control group were unrelated and randomly selected.

2.2. Inclusion criteria

Confirmed DVT patients were included. Fifty normal individuals who did not experience any episodes of deep venous thrombosis nor had a history of thrombophilia were included as control group.

2.3. Samples

Blood samples were collected under sterile condition and the vein puncture was well dressed. Five milliliter of blood was drawn from each participant. Two milliliter of whole blood poured in ethylenediamine tetra acetic acid (EDTA) container which used for the molecular techniques. One point eight milliliter was collected in sodium citrate tubes for clot based methods, and the remaining blood placed in plain containers for proteins estimation. Platelet poor plasma prepared by centrifugation of citrated tubes at 3500 RPM for fifteen minutes and transferred to eppindorf tubes. Serum prepared by centrifugation of blood collected in plain containers at 2500 RPM for ten minutes and transferred to eppindorf tubes as well. Plasma, serum and EDTA blood were stored at minus seventy degrees Celsius tell the time of examinations.

2.4. Ethical consideration

The volunteers were informed by the aim of this study and they were aware with the consequences of the research and their written consent was taken.

2.5. Data analysis and presentation

Data was analyzed with Chi-square test, T independent test, and percentage using SPSS software program version 16.0 and presented in form of tables and graphs.

2.6. Materials and methods

2.6.1. Molecular biology

2.6.1.1. DNA extraction

2.6.1.1.1. Red blood cells lyses buffer

This solution is composed of: chlorine (NH4CL), potassium bicarbonate (KHCO3) and EDTA 5%, all ingredients dissolved in one liter distilled water and kept at room temperature.

2.6.1.1. 2. White cells lysis buffer

WBC lysis buffer prepared by dissolving four chemicals in 500 distilled water. These chemicals include: Tris-Hcl, EDTA, Nacl and SDS.

2.6.1.1. 3. Guanidine hydrochloride

Strong chaotropic agent used for the denaturation and subsequent refolding of proteins. Prepared by weighing 57.2 grams powder and dissolved in 100 ml distilled water.

2.6.1.1. 4. Protienase K

Proteinase K is commonly used in molecular biology to digest protein and remove contamination from preparations of nucleic acids. Addition of Proteinase K to nucleic acid preparations rapidly inactivates nucleases that might otherwise degrade the DNA or RNA during purification [(137)].

2.6.1.1. 5. Chloroform -20

Chloroform ensures phase separation of the two liquids because chloroform is miscible with phenol and it has a higher density (1.47 g/cm3) than phenol; it forces a sharper separation of the organic and aqueous phases thereby assisting in the removal of the aqueous phase with minimal cross contamination from the organic phase [(138)].

2.6.1.1. 6.Ethanol

DNA is insoluble in alcohols (Ethanol and Isopropanol), therefore 100% alcohol used for the precipitation so we get good amount of DNA. Washing with 70% alcohol is to remove the excess of salts that might have come along with the extraction buffers (i.e. the excess of salts dissolve in the 30% of water).

2.6.1.1.7. DNA extraction protocol

The genomic DNA was isolated from peripheral blood leucocytes using Guanidine chloroform Extraction Method. The protocol was performed in three days. First day started with washing whole blood in RBC lysis buffer after thawing at room temperature. Blood placed in sterile falcon tube, lysis buffer added up to the 10 ml mark, tubes centrifuged at 6000 rpm for 15 minutes. After centrifugation supernatant was discarded. These steps were repeated several times until the supernatant became clear. After that 2ml of WBC lysis buffer, 1ml guanidine, 300 µl ammonium acetate and 25 µl proteinase K were added to the bellet. Last step of day one was the incubation of tubes at 37 ^{0}C for overnight.

On the second day tubes were brought to the room temperature. Two ml of chilled chloroform added to each tube. After that, tubes were agitated in a vortex and centrifuged for 5 minutes at 2500 rpm. Three layers were obtained. The upper layer transferred to a new falcon tube containing 10 ml chilled ethanol previously kept at -20. Then all tubes shaken very well and the second day finalized by placing tubes at -20 for overnight.

On day three, tubes were centrifuged and the ethanol discarded, tubes inverted on dry gauze for two hours. When they are dry tubes washed in 70 % ethanol, inverted on gauze until they were dry. After that 100 µl of doubled distilled water added and the mixture was transferred to appindorf tube. Finally, the product kept at -20 [(138)].

2.6.1.2. Mutations detection

2.6.1.2.1. Factor V Leiden

2.6.1.2.1.1. Primers design and preparation

The primers used were designed especially for this study using the NCBI and prime 3 soft ware. The specificity was checked in the NCBI. Primers are shown in table (2.1.). Primers purchased in a lyophilized form. They dissolved in distilled water as described by the manufacturer. From the stock primers 10µl added to 90 µl DW to prepare a working solution.

2.6.1.2.1.2. PCR protocol

Polymerase chain reaction was performed in a 20-µL reaction mixture containing 3µl DNA template, 1 µl from each primer and 15 µl DW in addition to master mix contents. The PCR reaction conditions were as follow: the reaction was begun with initial denaturation at 93°C for 7 minutes followed by 35 cycles of denaturation at 94°C for 30

seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension at 72°C for 10 minutes.

2.6.1.2.1.3. Detection of the PCR product

The product was detected in agarose gel electrophoresis. The gel concentration was 2 % to which 2 µl ethedium bromide was added. The 388 bp product was digested by MNL 1 restriction enzyme. Wild type of the gene had two restriction sites yielding three fragments: 246, 105 and 37 bp. The mutant allele had one restriction site that resulted in two fragments; 246 and 142 bp [(140)].

2.6.1.2.2. Prothrombin G20210A

2.6.1.2.2.1. Primers design and preparation

Primers used were described by Farah Parveen *et al*. [(10)]. They were prepared as mentioned in factor V Leiden. Primers are shown in table (2.1.).

2.6.1.2.2.2. PCR protocol

Detection of prothrombin gene polymorphism was done according to method described by Farah Parveen *et al*. Presence of 20210GA allele was screened by PCR followed by Hind III enzyme digestion. The PCR conditions were as follow: initial denaturation at 95 ^{0}C for 5 minutes, 35 cycles of 94 ^{0}C for 30 seconds, 59.3 ^{0}C for 45 seconds, and 72 ^{0}C for 1 minute followed by final extension of 72 ^{0}C for 5 minutes.

2.6.1.2.2.3. Detection of PCR products

PCR product checked on 2.0 % agarose gel at 120 V for 60 minutes stained with 0.5 lg/mL ethidium bromide in Tris. The amplified fragment of 345 bp was digested by 5 U/sample of Hind III enzyme at 37 ^{0}C for 18 hours. The 20210A allele generated a restriction site in the amplified fragment and was digested into two fragments of 322 and 23 bp, respectively. Wild-type allele (20210G) lacked the restriction site and therefore remained undigested 345 bp [(10)].

2.6.1.2.3. Beta-fibrinogen 455 G/A gene polymorphism

2.6.1.2.3.1. Primers design and preparation

Primers were used previously by Jeddi-Tehrani M and

Torabi R, 2011[(140)]. They were prepared as described above. Table (2.1.) shows primer design.

2.6.1.2.3.2. PCR protocol

The beta fibrinogen G455A polymorphism was detected by conventional PCR. The protocol was started with initial denaturation at 94 ^{0}C for 5 minutes. Then the amplification was performed with 36 cycles (denaturation at 95 ^{0}C for 15 seconds, annealing at 53 ^{0}C for 45 seconds, extension at 72 ^{0}C for 30 seconds). After that a final step of extension for 5 minutes at 72 ^{0}C was done [139].

2.6.1.2.3.3. Detection of PCR product

The PCR product was applied on 1.5 % agarose gel electrophoresis to which ethedium bromide was added. The 495 bp band was digested with Hea III restriction enzyme. A 5 µl from the product was incubated with 5units Hae III restriction enzyme at 37^{0}C for 18 hours. After that the enzyme reaction was inhibited by raising the temperature to 65 ^{0}C. Finally, the mixture applied on 3% agarose. The mutant type of the gene had no restriction site; therefore it remained undigested while the wild gene had two restriction sites resulting into three fragments, 383 bp, 86 bp and 26 bp.

2.6.1.2.4. Methylenetetrahydrofolatereductase (MTHFR) C677T

2.6.1.2.4.1. Primers design and preparation

Primers were designed using of primer 3 software program after getting the gene sequence from the NCBI. Working solution of each primer was prepared as mentioned earlier. Primers are shown in table (2.1.).

2.6.1.2.4.2. PCR protocol

The MTHFR C677T mutation was analyzed by PCR based restriction fragment length polymorphism (RFLP) methods. PCR protocol was consisted of an initial melting step of 5 minutes at 94◦C; followed by 35 cycles of 30 seconds at 94◦C, 30 seconds at 61◦C, and 30 seconds at 72◦C; and a final elongation step of 5 minutes at 72◦C(Inanir A *et. al.*)

2.6.1.2.4.3. Detection of product

After amplification, the 198 bp PCR product was digested with Hinf I in a 15 μl reaction solution containing 10 μl of PCR product, 1.5 μl of 10X buffer, and two units of Hinf I at 37◦C [140].

2.6.1.2.5. Plasminogen activator inhibitor type-1 (PAI-1)

2.6.1.2.5.1. Primers design and preparation

Genotypic screening protocols for 4G/5G were done through allele-specific PCR. Amplification of genomic DNA was done using the allele-specific primers, insertion

5G allele: 5′-GTC TGG ACA CGT GGG GG-3′, deletion 4G allele: 5′-GTC TGG ACA CGT GGG GA-3′ each in a separate PCR reaction together with the common downstream primer 5′-TGC AGC CAG CCA CGT GAT TGT CTA G-3′ and a control upstream primer 5′-AAG CTT TTA CCA TGG TAA CCC CTG GT-3′. The control upstream primer is used to verify the occurrence of DNA amplification in the absence of the allele on the genomic DNA [(141)].

2.6.1.2.5.2. PCR protocol

The PCR protocol included initial denaturation step for 5 minutes at 94 ^{0}C followed by a 40 cycles: denaturation at 95 ^{0}C for 45 seconds, annealing at 59 0C for 45 seconds, extension at 72 ^{0}C one for minute, and a final extension at 72 ^{0}C for 5 minutes.The product was detected in 1.5 % agarose gel electrophoresis.

2.6.1.2.5.3. Detection of PCR product

The normal gene showed a 260 bp band and the mutant gene yielded a 140 bp for both 5G and 4G polymorph.

2.6.1.2.6. Factor XIII (FXIII) val 34 leu mutation

2.6.1.2.6.1. Primer design and preparation

Primers were previously used by Diz-Kucukkaya R *et. al*. (2004)[(143)]. Preparation of primers was mentioned earlier in this part of the chapter.

2.6.1.2.6.2. PCR protocol

DNA was amplified by polymerase chain reaction (PCR) using 3 µl genomic DNA, 0.5 pmol of each primer. The PCR protocol included the following sequences: initial denaturation at 95 °C for five minutes, then 40 cycles of 95°C for one minute, 55°C for one minute, 72°C for one minute, and a final 10 minutes extension at 72°C [(142)].

2.6.1.2.6.3. Product detection

Tbe PCR products were digested witb *Hinf* I enzyme, and digested products were separated on 2.5% agarose gels. Bands of 94 and 114 base pairs corresponded to alleles Vand L, respectively.

Table 2.1 Primers design and the location of the polymorphism

Polymorphism	Primers
Factor V Leiden G1691A	F5′TGGTTCCAGCGAAAGCTTATT3′(prime 3) R5′TGCCCCATTATTTAGCCAGGA3′
Prothrombin G20210A	F5′TCTAGAAACAGTTGCCTGGC3′(Farah P. *et. al*) R5′GTAGTATTACTGGCTCTTCCTGAG3′
Fibrinogen G455A	F5′GCTTGTGGGAAATGAAGGAA3′(Jeddi *et.al*.) R5′GGCAACCACTAAAATCGTGA3′
MTHFR C667CT	F5′AGGAGGGTGCGGTGAGAGTG3′ (NCBI) R5′TGAAGGAGAAGGTGTCTGCGGGA3′
Factor XIII Val34Leu	F5′ACTTTCAGGACCGCCTTTGGAGGC3′ R5′GTTGACGCCCCGGGCACCG3′(Diz *et.al*)

2.6.2. Estimation of proteins

2.6.2.1. Fibrinogen estimation

2.6.2.1.1. Principle of the test

Determination of fibrinogen based on a modified Clauss method. Citrated plasma is diluted, mixed with an excess of thrombin (80 I U/ ml) and the coagulation time was determined. The concentration of fibrinogen is then got from a standard curve previously prepared using a fibrinogen standard.

2.6.2.1.2. Preparation of the samples

Patient's plasma and controls was diluted 1 in 10 in Owrens buffer.

2.6.2.1.3. Fibrinogen standard calibration curve

Serial dilutions of fibrinogen standard were done by diluting the standard with Owren's buffer. Then clotting time of each dilution was done. After that clotting times were blotted against their concentrations on a logarithm paper. (Fig. 2.1)

2.6.2.1.4. Test procedure

Clotting time of test and controls were measured in Hosptex coagulometer using thrombin reagent from technoclone GmbH, Viena, Austria. Concentration of fibrinogen was gotten from the standard calibration curve. Results were expressed in mg/dl.

2.6.2.2. D-dimer

2.6.2.2.1. Reagents and instrument

Mispa-i2 instrument and its reagent were used in the determination of D-dimer. They were manufactured by AGAPPE DIAGNOSTICS LTD. Kerala. India.

2.6.2.2.2. Principle of the test

The D-dimer assay is a nephelometric assay that utilizes antibody coated latex particles. In the presence of D-dimer, the particles aggregate and light scattering increases. The increase in scattering was proportional to the amount of D-dimer in the sample.

2.6.2.2.3. Test procedure

The test procedure and the calibration data were provided in a smart card along with the kit. The card was inserted in the instrument and instructions were followed. Reagent 1 and sample placed in the cuvette, 180 µl and 6 µl, respectively. After incubation 60 µl from reagent 2 added to the cuvette using attached sensor pipette. The result showed in the display and printed out. The results expressed in µg/ml. The cut-off point considered as 0.5 µg/ml.

2.6.2.3. Protein C

2.6.2.3.1. Principle of assay

The assay was a sandwich ELISA using microplates coated with a capture antibody specific for human protein C. Samples were diluted 1:51 and incubated in the wells allowing protein C present in the sample to bind to the antibody. The unbound fraction removed by washing. Afterwards anti-human protein C detection antibody conjugated to horseradish peroxidase (conjugate) is incubated and reacts with the antigen-antibody complex on the microwell surface. Following incubation, unbound conjugate is washed off. Addition of TMB-substrate generates an enzymatic colorimetric (blue) reaction which is stopped by diluted acid (color changed to yellow). The rate of color formation from the chromogen is measured in optical density units with a spectrophotometer at 450 nm. Using a curve prepared from the reference plasma provided with the kit, protein C antigen relative percent concentration in plasma is determined.

2.6.2.3.2. Preparations before starting

Sample buffer diluted 1:5 with distilled water. The concentrated wash buffer diluted 1:50 with distilled water. Reference plasma reconstituted by adding 0.4 ml distilled water,

shaken well and allowed to stand for 10 minutes at room temperature. Control N and control D reconstituted in 0.2 distilled water.

2.6.2.4. Protein S

2.6.2.4.1. Principle of the test

The AESKULISA is a sandwich ELISA using microplates coated with a capture antibody specific for human protein S. Patients plasma diluted 1:51 and incubated in the wells allowing protein S present in the sample to bind to the antibody. The unbound fraction removed by washing. Afterwards anti-human protein S detection antibody conjugated to horseradish peroxidase (conjugate), is incubated and reacts with the antigen-antibody complex on the microwell surface. Following incubation, unbound conjugate is washed off. Addition of TMB-substrate generates an enzymetic colorimetric (blue) reaction which is stopped by diluted acid (color changed to yellow). The rate of color formation from the chromogen is measured in optical density units with a spectrophotometer at 450 nm. Using a curve prepared from the reference plasma provided with the kit, protein C antigen relative percent concentration in plasma is determined.

2.6.3. Clot based methods

2.6.3.1. Prothrombin time (PT)

2.6.3.1.1. PT reagent

Reagents used were manufactured by biomed diagnostics company, Germany. It was consisted of thrompoblastin and calcium. The thromboplastin used is recombinant product which has International Sensitivity Index (ISI) closed to one (1.04). Calcium ions obtained from calcium chloride (CaCl2) 0.025 mol/L.

2.6.3.1.2. Principle of the test

The PT test measures the clotting time of plasma in the presence of an optimal concentration of thromboblastin and indicates the overall efficiency of extrinsic pathway of the coagulation system.

2.6.3.1.3. Procedure of the test

First of all, all reagents and samples brought to the room temperature, 200 µl of the reagent mixture delivered to glass tube. After that samples and reagents placed in a water bath for 1 to 3 minutes to warm up. Then 100 µl of plasma added the mixture and the stop watch simultaneously started. Finally, clot formation was observed and time is

recorded for each sample. The results were expressed as the mean of duplicated readings in seconds [143,144].

2.6.3.2. Activated Partial Thromboplastin Time (APTT)

The APTT (also called the partial thromboplastin time, or PTT) is performed to monitor the effects of unfractionated heparin therapy and to detect lupus antibodies (LA) and specific anticoagulation factor antibodies such as anti-factor VIII antibody. The APTT is also prolonged in all congenital and acquired procoagulant deficiencies, except for deficiencies of factor VII or XIII.

2.6.3.2.1. Reagents

The APTT reagent contains phospholipid and a negatively charged particulate activator such as silica, kaolin, ellagic acid, or celite in suspension. These activators provide a negative charge surface for the activation of contact factors. Calcium chloride is used as a source for calcium ions. Reagents used were manufactured by biomed diagnostics company, Germany.

2.6.3.2.2. Procedure

To initiate contact activation, 100 µL of warmed (37°C) reagent consisting of phospholipid and particulate activator was mixed with an equal volume of warmed PPP. The mixture was incubated for the exact manufacturer-specified time (3 minutes). After that, 100 µL of warmed 0.025 M calcium chloride is forcibly added to the mixture, and a timer was started. When a fibrin clot formed, the timer stopped, and the interval was recorded. The test was done in duplicate, and the two results were matched within a difference less than 10%, then the average was reported in seconds [143,144].

2.6.4. Platelets count

Platelets count performed using Sysmex cell counter. The principle was based on electrical impedance method; in which whole blood is passed between two electrodes through an aperture so narrow that only one cell can pass through at a time. The impedance changes as a cell passes through. The change in impedance is proportional to cell volume, resulting in a cell count and measure of volume [143].

Chapter three
Results

3.1. Demographic data

This study conducted in Khartoum state in the period from December 2014 to May 2018 to detect gene mutations associated with DVT. One hundred and fifty individuals were included in this study. The study group (DVT patients) consists 30 men and 70 women; median age 48.14 years; ranging from 19 to78 years. The control group (healthy volunteers) included 29 men and 21 women; median age 32.4 years; ranging from 22 to47 years.

Among patient group 25 % (25/100) were post surgery, 6 % (6/100) smokers and 14 %(14/100) were obese. Pregnant women represented 22.5 % (16/71) out of the female patients.

Both patient group and control group were investigated for six gene mutations that considered being predisposing factors for thrombophilia. Four plasma proteins that related to hemostatic process and coagulation profile were also measured for all of the participants.

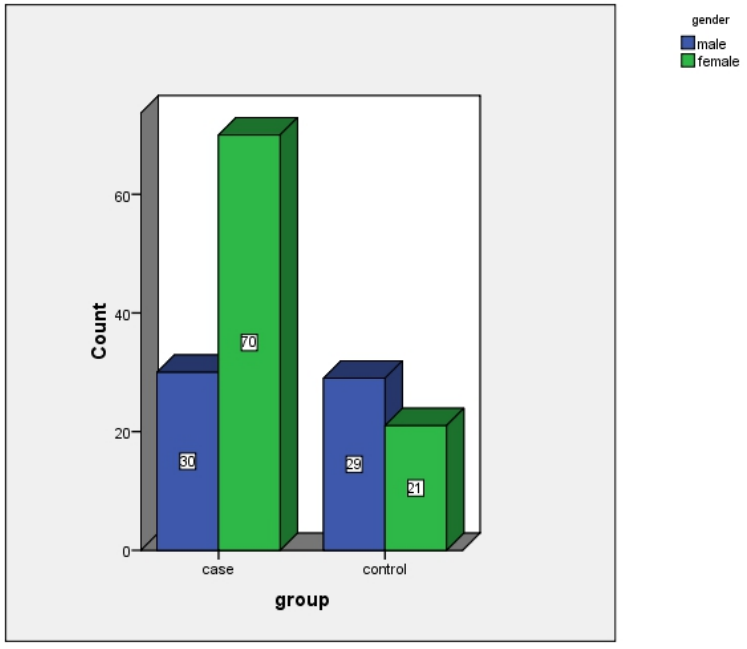

Figure 3.1 Distribution of gender.

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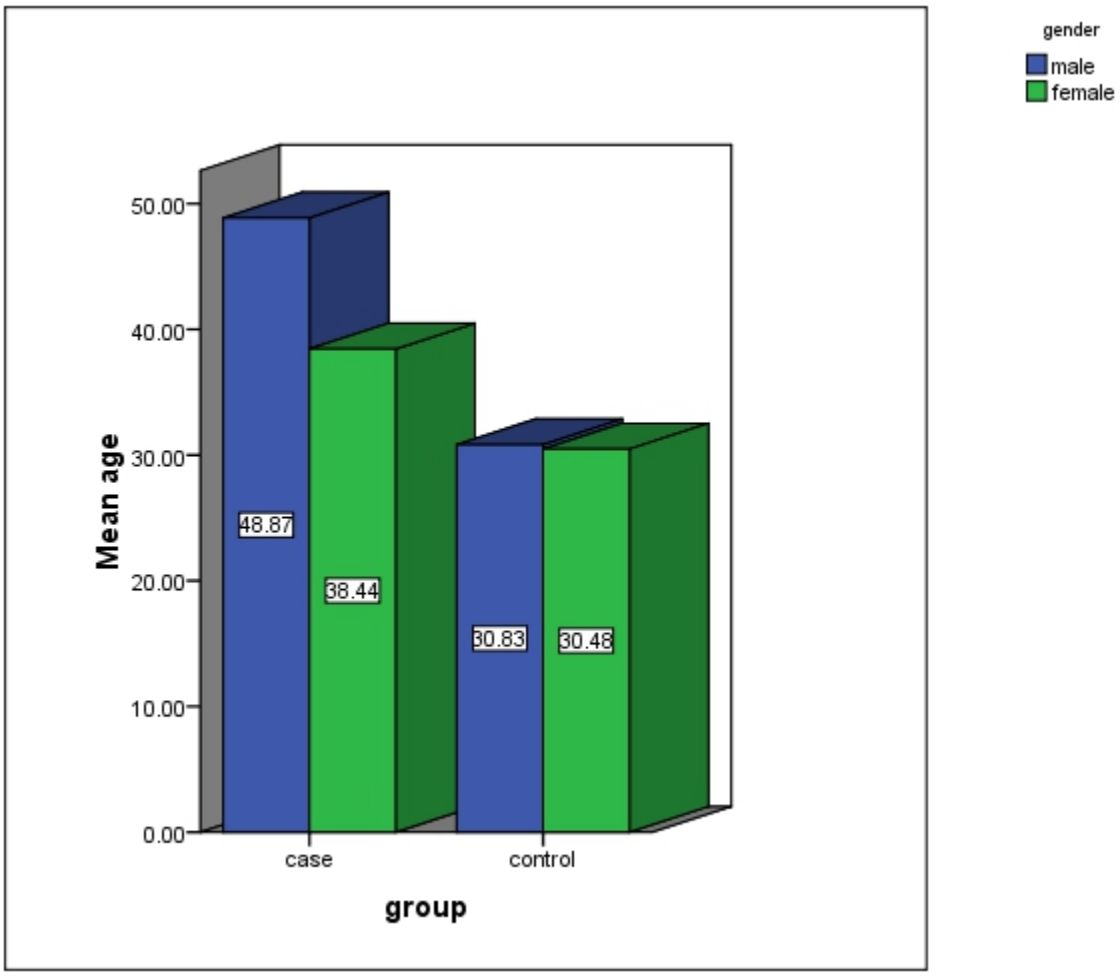

Figure 3.2 Mean age of patients and control group.

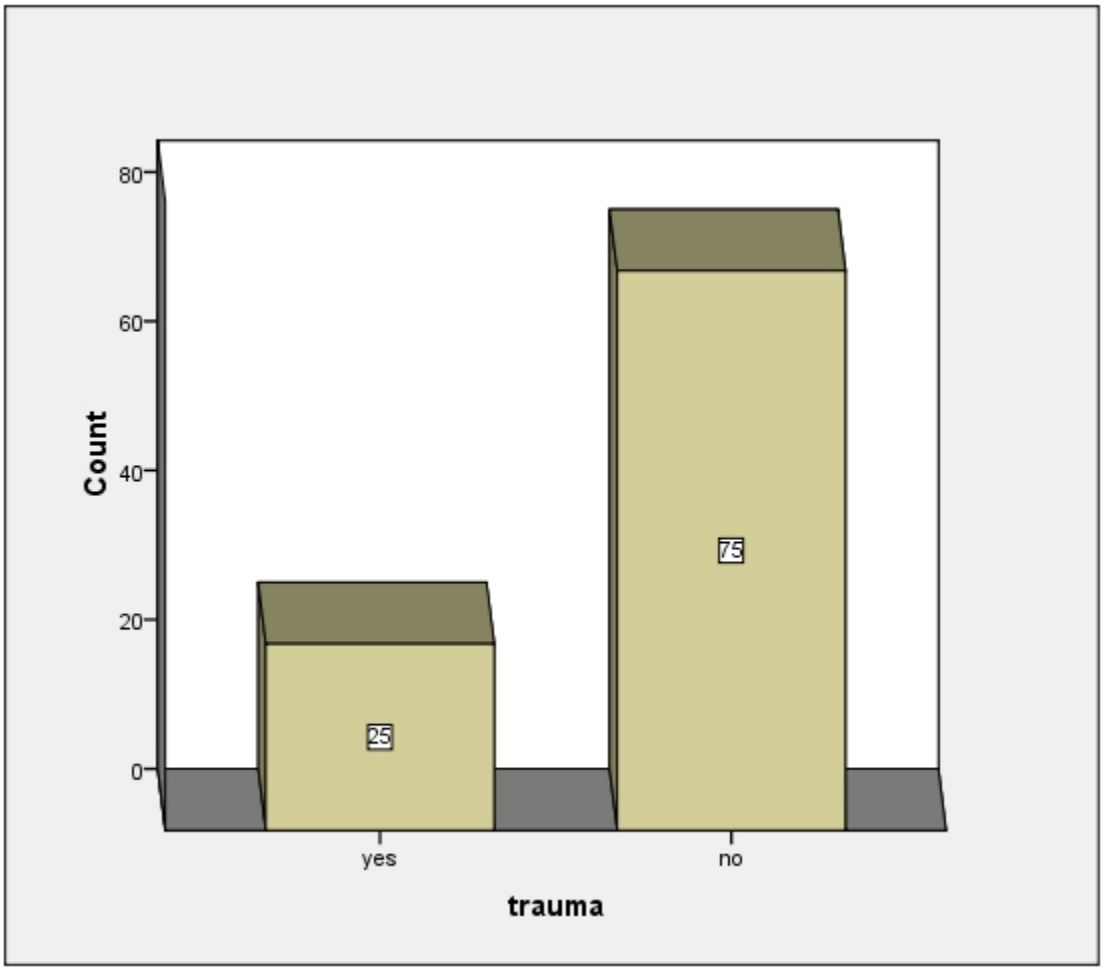

Figure 3.3 Distribution of trauma in patients group.

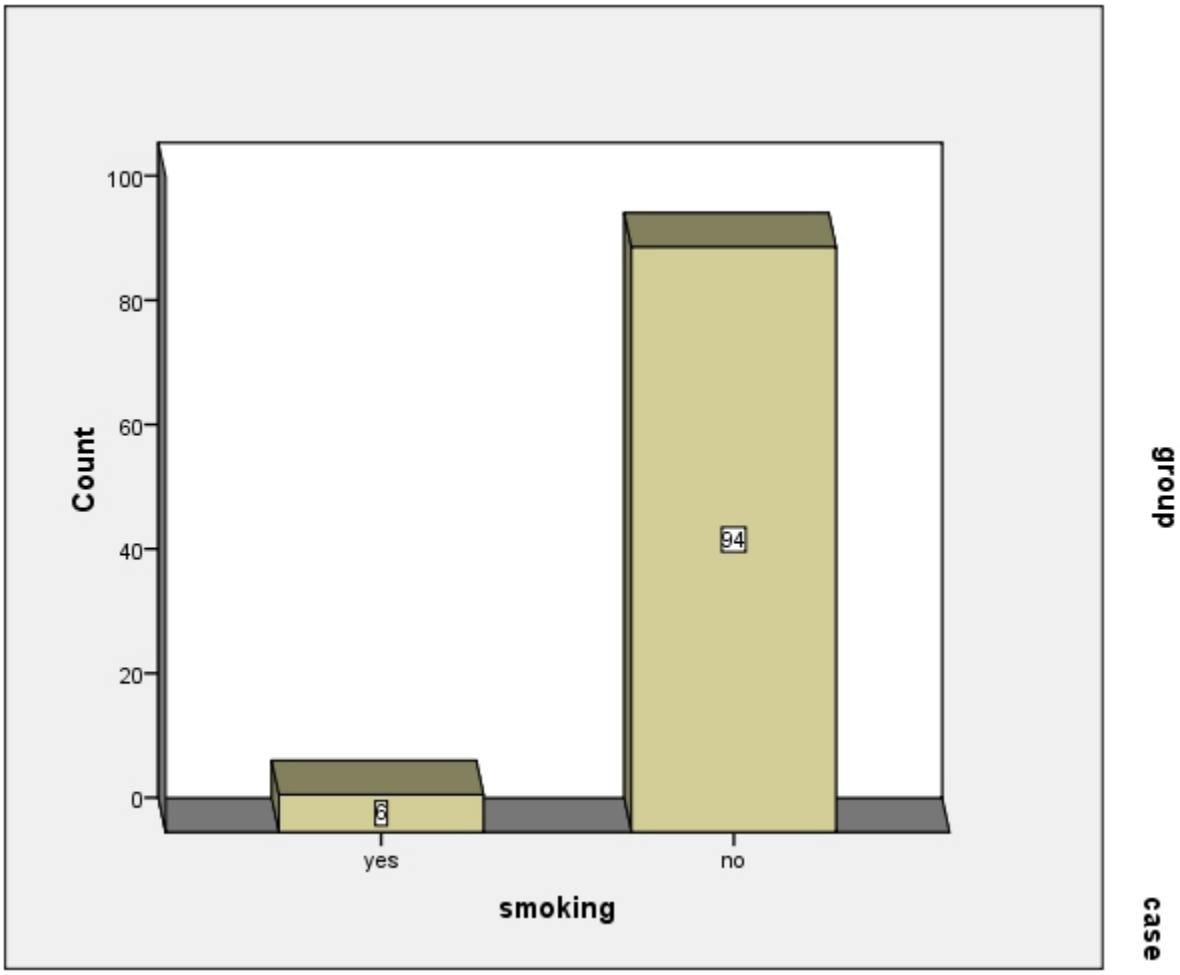

Figure 3.4 Distribution of smokers in patients group.

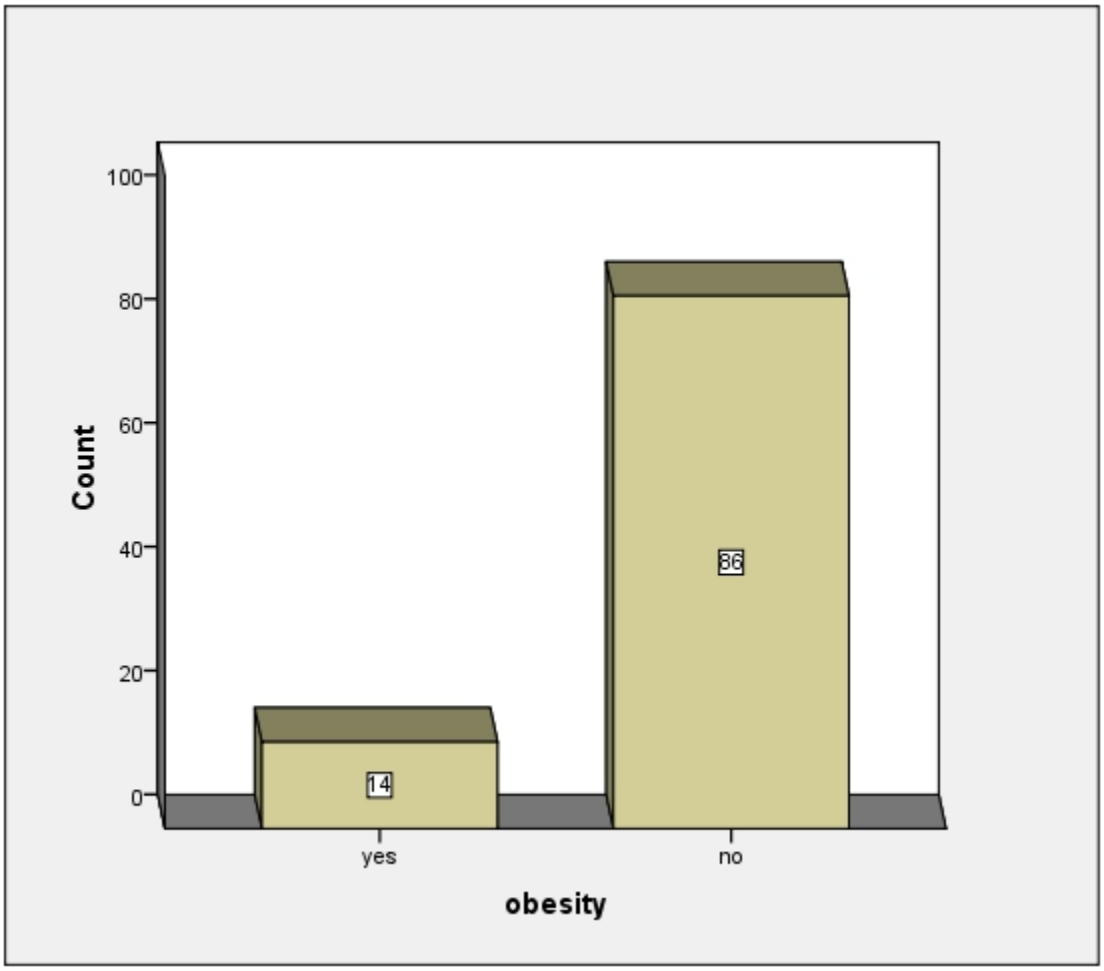

Figure 3.5 Distribution of obesity in patients group.

3.2. Genetic analysis

The plasminogen activator inhibitor-1 deletion/insertion polymorphism (4G/5G) was detected in 9% (9/100) patients with DVT. The prevalence of mutant type (4G/4G) was (7%), the (5G/5G) type was (2%) and the normal type (4G/5G) was (91%). The mutation was detected in 4 healthy individuals (Table 3.2). The difference between patient group and control group is statistically significant (*P*= 0.012) table 3.1

Table 3.1. Frequency of plasminogen activator inhibitor-1 mutation in patient and study group

PAI-1 mutation	Group		Total	P-value
	Case	Control		
4G/4G 5G/5G 4G/5G	7 2 91	4 3 16	11 5 107	0.012
Total	100	23	123	

Factor XIII mutation val 34 leu was detected 8% (8/100) patients with DVT. The prevalence of the mutant allele (LL) was (8%) the heterozygous gene was not detected in the patient group. The wild type allele (V V) was detected in 92(92%) of the patients. Neither mutant allele nor mixed allele was detected in the normal control group (table3.1). At diagnosis, the association between the mutation and the disease was statically significant (*P*=0.04).

The *β*–fibrinogen 455A/A polymorphism was detected 4% (4/100) patients with DVT. The mutant type (AA) was detected in 1(1%) patient. The prevalence of mixed allele (GA) was 4%. The normal type (GG) was detected in 96 (96%) of the patients. The mutation was detected in 6 (12%) of the control group. The mutation was detected in 6

normal individuals (Table 3.1). The association between this mutation and DVT was statically insignificant (*P*=0.17).

The MTHFR mutation C677T was detected in 4% (4/100) of the patients. The prevalence of the mixed allele (CT) was 4%. The mutant allele (TT) was not detected in the patient group. The wild type(C C) was detected in 96 % (96/100) of the patients with DVT. The MTHFR mutation was not detected in the normal control group (Table 3.1). At the time of diagnosis, results showed no significant difference between patients and normal control group (P=0.152).

Prothrombin G20210A mutation was detected in 4% (4/100) of DVT patients. The prevalence of the homozygous type (GG) was 4% the heterozygous type was not detect. Both the homozygous and heterozygous were not detected in the control group (Table 3.1). The relation between DVT and this mutation was statically insignificant (*P*= 0.152).

Factor V Leiden was detected neither in patient group nor in control group. At the time of diagnosis, the association between DVT and factor V Leiden was not found (Table3.2).

Table. 3.2. Frequency and percentages of mutations in patient and control group

		Study group		Control group		P-value
		frequency	Percentage	frequency	percentage	
MTHFR mutation C677T	*Mutant allele*	0	0%	0	0	0.152
	Mixed allele	4	4%	0	0	
	Wild type	100	100%	50	100	
factor XIII mutation val 34 leu	*Mutant allele*	8	8%	0	0	0.04
	Mixed allele	0	0%	0	0	
	Wild type	92	92%	50	100	
fibrinogen mutation G 455A	*Mutant allele*	1	1	1	2	0.17
	Mixed allele	3	3	5	10	
	Wild type	96	96	44	88	
prothrombin mutation G20210A	*Mutant allele*	4	4	0	0	0.152
	Mixed allele	0	0	0	0	
	Wild type	96	96	50	100	
Factor V leiden	*Mutant allele*	0	0%	0	0	
	Mixed allele	0	0%	0	0	
	Wild type	100	100%	50	100	

3.3. Plasma proteins

D-dimer results showed a significant difference between study group and control group (Table3.3.). Mean of D-dimer was 0.393 microgram/ml and 0.186 microgram/ml for patient group and control group, respectively (*P*=0.014).

Plasma fibrinogen was high in patients with DVT (mean 474.11 mg/dl) when compared to normal control group (mean369.3 mg/dl)(Table 30.3). The difference was statistically insignificant (p-value=0.109).

Protein C showed lower **results** in patient group when compared with the control group. The mean in patient group was 93.19 mg/dl and in control group was 97.06 mg/dl. The difference was insignificant (*P*= 0.263).

The mean of protein S in patient group was 75.28 mg/dl while it was 79.27 mg/dl in control group. The difference between patient and control group was statistically insignificant (*P*= 0.534) table3.3.

Table.3.3. Mean of protein S, protein C, plasma fibrinogen and D-dimer

	Group	N	Mean	P-value
protein S mg/dl	Case	100	75.28	0.534
	Control	50	79.27	
protein C mg/dl	Case	100	93.19	0.263
	Control	50	97.06	
fibrinogen mg/dl	Case	100	474.11	0.109
	Control	50	369.30	
d-dimer microgram/ml	Case	100	.392	0.014
	Control	50	.186	

3.4. Coagulation profile

The mean of APTT in patient group was 36.79 seconds and 31.64 seconds in control group (Table 3.4). The association between APTT and DVT was statistically significant (P-value 0.000).

The difference of prothrombin time between patients with DVT and normal group time was statistically significant (*P*=value 0.001). The mean of PT was 13.58 seconds in patient group and 12.78 seconds in control group (table3.4).

Platelets count was low in the patient group. The mean platelets count was 256000/cumm in patient group and it was 331400/cumm in the control group. The association between the disease and platelets coun was statistically significant (*P*=value 0.000).

Table.3.4. Mean of platelets count, PT and APTT

	Group	N	Mean	P-value
Platelet count /L	Case	100	$256x10^9$	.000
	Control	50	$331.4x10^9$	
PT in seconds	Case	100	13.58	.001
	Control	50	12.61	
APTT in seconds	Case	100	36.78	.000
	Control	50	31.64	

Chapter four
Discussion, conclusions and recommendations

4.1. Discussion

Present study is a cross sectional study designed mainly to investigate mutations associated with deep venous thrombosis. A total of one hundred and fifty individuals were included in this study, one hundred of them were DVT patients and the remaining fifty were healthy volunteers assigned as control group. The study group (DVT patients) consisted of 30 men and 70 women; median age 48.14 years; ranging from 19 to78 years. The control group (healthy volunteers) included 29 men and 21 women; median age 32.4 years; ranging from 22 to 47 years).

In this study six mutations were detected in deep venous thrombosis patients and in control group. Moreover, four coagulation and hemostatic regulatory proteins were measured, in addition to coagulation profile and platelets count. The results obtained in the majority of studies describing genetic polymorphisms in DVT are conflicting. Many of the reported associations have not been reproduced in previous studies. This is partly due to the fact that the associations may vary in different ethnic populations and they may be biased because of limited area we covered. This may be true in the present study because of the ethnic diversity in Sudan.

This study have considered at the risk posed by 4G/5G genotype of PAI-1 gene. To our knowledge, there are no reports on the prevalence of this polymorphism in Sudanese patients with DVT. Recent study found 7, 2, and 91 for 4G/4G, 5G/5G, and 4G/5G, respectively. The difference between patient group and control group is statistically significant (P= 0.012). These results were sustained by many authors. In Asian Indian population Akhter *et.al.* , 2010 found a high prevalence of the 4G/G4 and 4G/5G alleles (P-value <0.05) [146]. Similar results were also reported in Spain by Seguõ *et.al*., 2000. They proved the association between the prevalence of 4G/5G genotype and DVT (P-value<0.05 [147]. Another conclusion that goes with recent study is described by Vesa *et. al.* 2016). They found a significant difference between patient and the study group (P-=0.023) [148].

In this study factor V Leiden mutation G1691A didn't found in any participant of the study group or control group, neither the mutant allele (A/A) nor the mixed allele (G/A).

Only the wild type (G/G) detected. The results were confirmed using a positive control sample for that mutation. Depending on these findings, it's obvious that factor V Leiden was not a predisposing factor for DVT in this study. Results of current were in accordance to results obtained by Yousif *et.al*. 2017. They didn't find any mutation in their study [149]. Results of recent study were also similar to results stated in Saudi Arabia by Algari *et.al.* 2016 who didn't find any of their participants having factor V Leiden mutation [150]. Current study is completely agreed with a gene test review done by Kujovich *et.a.l,* 2011 which showed a complete absence of the gene in Africans [151]. Similar results reported by Hira *et. al*, 2003 in South Africa. They didn't find any mutant allele in cases or control of their study [152]. Recent findings are also backed by Ardestani et.al.2013, who found a few number of mutant alleles in a case control study conducted in Iranian women with recurrent pregnancy loss. Their findings were insignificant when they compared the study group with the control group (*P*. value >0.05) [153]. Other findings that agreed with results obtained in this study, detected in Sudanese pregnant women by Awad al Kareem *et. al*., 2017. Their results revealed that 4.3 % of the cases were heterozygous for factor V Leiden [154]. Markedly different findings were found in Lebanon. The study conducted by Kreidy *et.al*. 2012 who found factor V Leiden in 56.9% of their tested DVT patients [155]. Results of this study were also disagreed with Meyer *et. al*., 2001, in France. They determined factor V Leiden mutation in patients with DVT. Their study showed a significant correlation between the mutation and the disease (*P* =0.007) [156]. High prevalence of factor V Leiden (38.3%) also found in Turkish population in a study conducted by Dolek *et. al*., 2007 [157]. The explanation for the absence of association between factor V Leiden and DVT in our study, as believed, is due to the African ancestry of most of Sudanese population. In literature most of studies reported the absence of this mutation in Africans which in turn support our hypothesis [151,152].

This study showed that the FGB G455A polymorphism present in both study and control group. In the study group one (1%) subject had mutant allele (A/A), three (3%) had mixed allele (G/A), and ninety six (96%) were wild type (G/G). In control group one mutant allele (2%) was found, five had mixed allele (10%), and forty four were wild type (88%). The correlation between this mutation and the disease was statically insignificant

(*P* = 0.17). Previous studies showed different results in different areas of the world. Results of this study were similar to a study done in Italy by Marchetti G. *et. al*.2003. Their results showed no difference between study group and control group (*P* =0.110) [(158)].

In India, Kumari *et. al*., 2014 got a result that, to some extent, in accordance to results found in this study. They found the mutant allele in 6.68% of the control group while it was 0.0% in the study group (*P* =0.09) [(159)]. In Sudan Ahmed *et al*., 2016 found that 62.3% of their cases were mutant, 14% mixed allele, and 32.7 were wild. These results were completely different from the results reported in this study [(160)]. No any study found in Sudan to support findings of recent study. A study carried out in North Western Russia by Harrington *et. al*., 2013 showed results that disagree with current study findings. They found 65.5%, 32.8% and 1.7% for the wild type, mixed allele, and mutant allele, respectively [(161)].

The FGB 455 G/A polymorphism had no effect on the fibrinogen in plasma level in our study (*P*= 0.731) although the mean concentration of plasma protein is higher in the patient group than in control group. The insignificant findings of this polymorphism observed in our study can be due to the fact that no appreciable association between this mutation and the disease (DVT).

In recent study we found four individuals (4%) of our study group had mutant allele (A/A) for the prothrombin mutation G20210A. The remaining 96 (96%) had the wild type (G/G). We didn't find the mixed allele (G/A). The mutant allele and the mixed allele were not found in the control group. The difference between study group and control group was insignificant (*P* = 0.197) indicating that no association between this mutation and DVT. These findings are supported by a study conducted in Sudan by Yousif *et al*., 2012 who didn't detect the mutation in both study and control group [(149)]. The absence of this mutation was also supported by similar findings in a study carried out in Saudi Arabia, Algari *et al.*, 2017; they found no association between this mutation and DVT patients (*P*-value>0.05) [(153)]. In France Meyer *et.al*, 2001 found results that were consistent with results of this study. The mutant allele found in 8 % of their study group (DVT patients) but the association was insignificant (*P* =0.2) [(162)]. This study didn't agree with a study done in turkey by Dölek *et. al.*, 2007 [(157)].

They found 8.6 % of the study group having the mutant allele while the mutant allele represented 1.8 % of the control group. They concluded that there was association between factor II mutation and DVT. In Italy Martinelli *et. al*.1999 found the mutant allele in 9.4 % of patients and 2.5 % in the control group. They presumed that the mutation is significant if it is combined with acquired factor [(163)].

Current study showed that the MTHFR mutation C677T found in the following pattern: mixed allele (C T) in four patients (4%), ninety six patients (96%) had wild type (C C), and no mutant type (T T) in the patient group. Study found neither mutant type nor mixed allele among the control group. The findings showed no any discrepancy between study group and control group (*P*=0.152) which means there is no relation between this mutation and the disease (DVT).

In Sudan, Elhassan *et al*., 2015 found the mixed allele in 12% of their study group and 8% in the control group. They didn't find the mutant allele in any participant. Their finding was statically insignificant (P =0.508) [(164)]. Another study conducted in Iran by Ghaznavi *et. al*., 2015 supported these results. They found 7.5% mutant allele in the study group and 4.5 % in the control group. The mixed allele was 41.8% and 34.3% for study group and control group, respectively. The difference was statistically insignificant (*P*-value>0.05) [(165)]. Findings that disagreed with the results of current study obtained by Hosseini *et. al* 2015 from Iran. They found 9.3% and 0.8% of mutant allele in the study group and control group, respectively. The mixed allele was 1.6 % and 1.2% for the study group and control group, respectively. They found a significant correlation between the mutation and the disease (*P*-value<.001) [(166)].

The present study found that factor XIII val34leu mutation has no impact on DVT. Results showed eight patients (8%) had mutant allele (L L). Ninety two (92%) had wild type allele (V V). The entire control group had the wild type allele (V V). The difference between study group and control group is significant (*P* =0.04). Recognition in this study is the fundamental one to be done in Sudan to detect this mutation. Turkish study conducted by Diz-Kucukkaya *et. al.*, 2004 sustained thsese results. They found the mutant allele in 3.3% of the cases and the mixed allele in 20% of the cases. They concluded that this polymorphism may not prevent the development of thrombosis [(167)]. Another study that is in accordance with recent findings, done in Hungary by Balogh *et.*

al., 2017. They found the mutant allele in both study group and control group (27.6% and 24.3%, respectively). They presumed that, no protective impact of the mutation could be observed [(168)]. In Saudi Arabia El-Tarras *et al.*, 2012 found the mixed allele in 4% of their study group. They didn't find the mutant allele in any of their participants. They concluded that the role of this mutation depends on the ethnic group [(169)].

In general, it is believed that the discrepancy between results of genetic association studies like those encountered here could be due to many reasons including population genetic variation (background) unrelated to the alleles, presence of nucleotide polymorphism somewhere else in the examined allele e.g., in the untranslated or intronic regions, epigenetic alterations and linkage disequilibrium to other sequence variants in the vicinity of the studied loci.

The antigen fibrin D.dimer is the primary enzymatic degradation product of cross linked fibrin by plasmin. D.dimer is an index of fibrin turnover in the circulation. It is well known that D-dimer level is increased in thrombophilia. Recent study showed results that are in accordance to this fact. The difference between cases and control is statically significant (P =0.014), indicating the strong relation between the disease and D-dimer. Findings of this study are similar to results of some researches done in different countries although of most them used D-dimer as predictor for thrombophilia. For instance, Abd Elkarim and Khalil, 2015 measured D-dimer in Sudanese smokers. They found a significant variation between case and control group (P =0.01) [(170)]. Alhassan and Guafri, 2017 found that the D-dimer levels were statistically significantly higher in pulmonary tuberculosis patients compared to the normal healthy control groups (P= value 0.00) [(171)]. Other studies showed different results which disagreed with results of this study. Dafalla *et. al.*, 2016, found no difference between the study group and the control group (P = 0.305) [(172)].

Recent study observed a higher plasma fibrinogen concentration in the study group (mean 474.11 mg/dl) than in control group (mean369.3 mg/dl), but the difference was statistically insignificant (P =0.109). Kloviate *et. al.*, 2013 found results that are similar to those have been reported in this study. They concluded a statistical difference between study group and control group when they examined fibrinogen in patients with DVT and PE (P =.0001). The DVT group showed insignificant difference P =0.4). They reported

that elevated plasma fibrinogen levels are associated with increased risk of PE in combination with DVT but not with DVT alone [173]. A study done in Oxford by Tibbutt *et. al.*, 1975 found a significant difference in fibrinogen level between DVT patients and normal individuals ($P < 0.05$). They suggested that fibrinogen level can be good indication for DVT if other known causes of a raised level of the antigen are absent [174]. Measurement of plasma fibrinogen in Sudanese patients by Osman and Muddathir, 2013 revealed a statistical difference between the study group and control group (*P*-value=0.00). These findings were contradicting with current study results [175]. Fibrinogen is known to be an acute phase protein. The insignificance association between fibrinogen level and DVT might partly be explained by a concomitant inflammation at time of blood sampling for both patient and control group.

This study revealed that there is a deficiency in protein C and protein S among patient group. The mean in patient group was 93.19 mg/dl and in control group was 97.06 mg/dl for protein C. The difference was insignificant ($P= 0.263$). While the mean for protein S was 75.28 mg/dl for patient group and 79.27 mg/dl for control group. The difference between patient and control group was statistically insignificant ($P= 0.534$). Shen *et. al*., 1997 found the deficiency of protein C and S in 59% of the population they studied [176]. Data reported by Suehisa *et. al.* 2001 suggested that the Japanese population have an extremely high frequency of PC and PS deficiency [177]. Athanasiadis *et. al.* 2011 stated that Individuals with protein C or protein S deficiency present a dramatically increased incidence of thromboembolic disorders [178]. Discrepancy in results of protein C and protein S shown in this study might probably be due to the method we used which depends mainly on measuring the antigen not the activity.

Measuring of APTT exhibited that the means was 36.79 and 31.64 seconds for patient group and control group, respectively. The difference was statistically significant ($P= 0.000$). The results were incompatible of the results observed by Garg *et.al*., 2011 who found low APTT in postoperative thrombophilic patients [179]. It is well known that the APTT is affected directly by heparin and to some extent by warfarin. Because of that it is believed the APTT is prolonged in the study group, since the majority of patient receiving heparin or warfarin.

This study revealed an elevation of PT in the study group. The difference was statistically significant (P= 0.001). The mean of PT was 13.58 seconds in patient group and 12.78 seconds for the control group. Garg *etal*., 2011 described that the mean of PT decreased in postoperative individuals, indicating hypercoagulable state although the change was insignificant (*P* =0.06) [(179)]. It is assumed that the increment of PT in this study is mainly due to the anticoagulant therapy.

In this study low platelets count is observed in the patient group. The mean platelets count was $256x10^9$/L in patient group while it was $331.4x10^9$/L in the control group. The difference was statistically significant (*P*= 0.000). These findings are supported by Habib *et al*., 2012 who found a significant decrease in platelets count among DVT patients when compared to healthy individuals (P <0.001) [(180)]. Monreal *et. al.,* 1991 confirmed the reduction in platelets count in patients with PE (P-value<0.008), but not in DVT. They advised that lung scan should be done for DVT patients when platelets count is lesser than the standard [(181)]. Since the instrument used in this study for platelets count is calibrated and standardized, it is definite that the significant association between platelets count and the disease is due to the fact that platelets are consumed in the clot formation.

4.2. Conclusions

Study concluded that PAI-1(C667T) mutation was one of the most common inherited defects causing thrombophilia in this study. Factor XIII val 34leu doesn't have a role in preventing the development of the disease. Other mutations don't have impact on this study.

Study demonstrated that D-dimer was significantly high. Fibrinogen level was relatively high in patient group, but the elevation is statically insignificant.

Protein C and protein S were deficient in the study group.

PT and APTT were high in the study group when compared to control group.

Platelet count was markedly decreased in patient group.

4.3. Recommendations

- Performing a larger study to investigate the impact of these mutations in other states of Sudan.
- Performing DNA sequencing to know if there is any SNP in other parts of the genes.
- Performing further studies to investigate the relation between other polymorphisms in these six and other genes related to thrombophilia and DVT in particular.
- Coagulation proteins should be considered as an important tool for the diagnosis of DVT.

Chapter five
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Appendices

Appendix I: Questionnaire

Shendi University

College of Graduate Studies

Questionnaire

Research Title: Evaluation of Mutations and Changes of Coagulation Profile Associated with Deep Venous Thrombosis in Sudan

Researcher: Abdalla Musa Abdalla

Supervisor:Prof.Babikir Ahmed Mohammed

Co. Supervisor:Dr.Hamdan Sidig Siraj

Duration of research:

Name:…….. No:……

Gender:……

Age:…… ..

Residence:……..

Mobile number:……..

Duration of the disease (DVT):…….

Clinical and lab finding at the time of diagnosis:…….

Treatment:……

Recurrence:……..

Pregnancy:……

Contraceptive pills:……..

Obesity:……..

Smoking:……..

Trauma:…….. …….

Surgery:…….....

Diabetes…… ..

Others (If any) :……

Laboratory results:

A) <u>PCR</u>

1.Factor V leiden :……..,…

2. prothrombin G20210A …….

3. (MTHFR) C677T……

4.Beta fibrinogen 455G/A……

5.Plasminogen activator inhibitor type-1 4G/5G 675……

6.XIII val 34 leu……

B) <u>Proteins</u>

1.protein C ……

2.Protein S …….

3.D-dimer ……..

4.Fibrinogen……

C) Coagulation profile:

1.PT…….

2.APTT……

3.Platelet count……

Remarks:……

……

……

……

……

Appendix II: Preparation of reagents

10 X TBE buffer

Formula in grams per liter

Tris base……. 108 gm

Boric acid…….……..…..55gm

EDTA……..…...40 ml of 0.5M

Deionized water……..……..…1 liter

Preparation

Amount of 108 gm. Tris base were weighed and added to 55gm of boric acid and 40 ml of 0.5M EDTA then dissolved into 1 liter deionized water pH 8.0.

1X TBE buffer

Formula in ml per liter

10 X TBE…….10 ml

Deionized water…….90 ml

Preparation

Ten ml of 10 X TBE buffer was added to 90 ml deionized water and heated until completely dissolved.

Ethidium bromide solution

Formula in grams per 1ml

Ethidium bromide……10 mg

Deionized water……I ml

Preparation

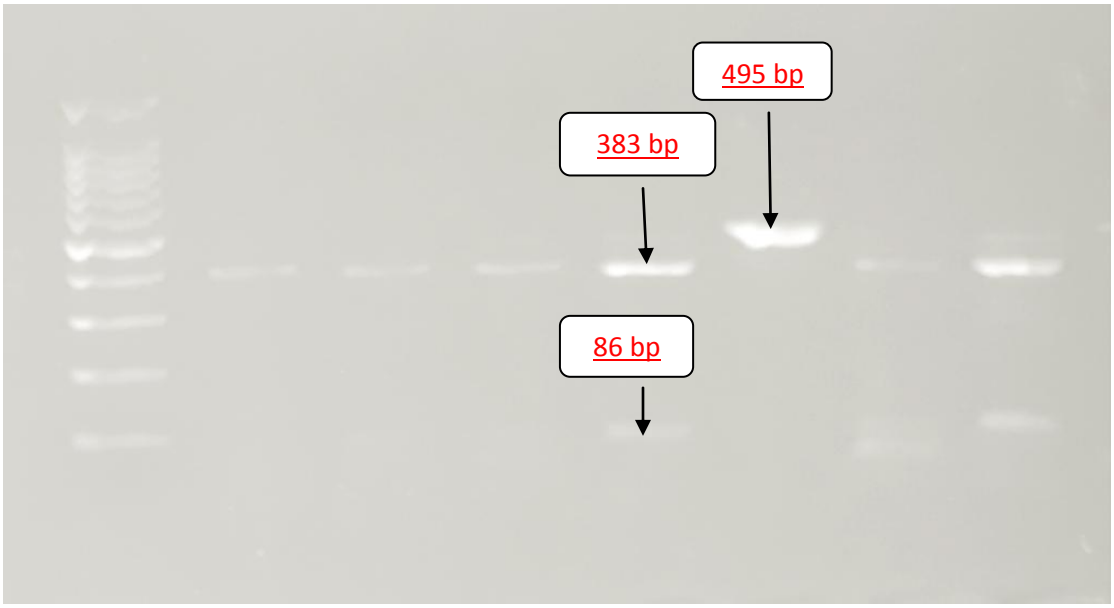
Twenty milligrams of ethidium bromide powder were dissolved into 1000 µl deionized water, and kept into brown bottle

Agarose gel

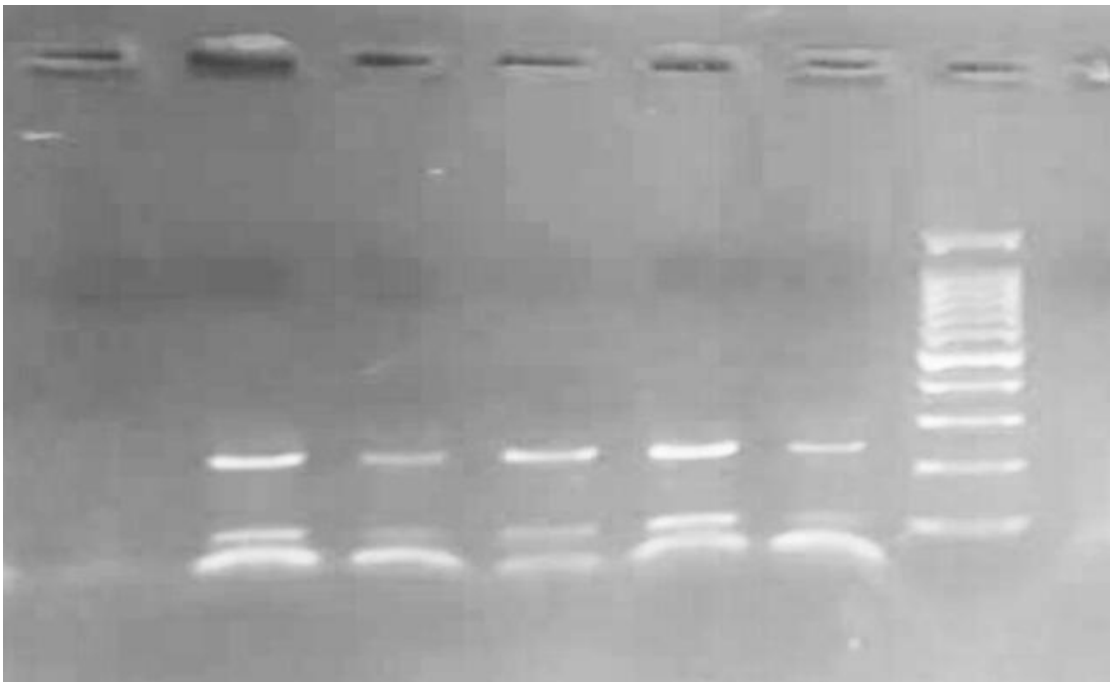
Preparation

Amount of 2 gm of agarose powder dissolved by boiling in 100 ml 1X TBE buffer, then was cooled to 55°C in water bath, then, 1.5 µl of Ethidium bromides stock (10 mg/ml) per 100 ml gel solution for a final concentration of 0.5 ug/ml were added, mixed well and poured on to the casting tray that has been taped up appropriately and was equipped with suitable comb to form well in place. Any bubbles were removed and the gel was allowed to set at room temperature. After solidification, the comb was gently removed and the spacer from the opened sides was removed.

Appendix III:Electrophoresis

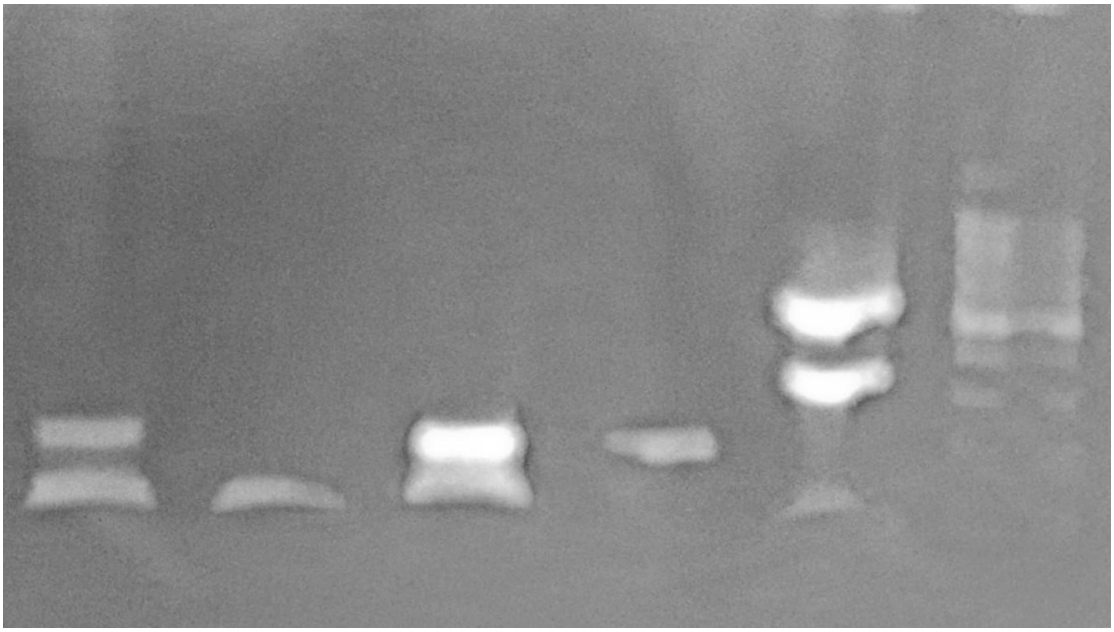


Gel electrophoresis with ethidium bromide. Hae III enzyme digested the wild type of beta- fibrinogen into (383, 86, 26 bp). The mutant gene remains undigested (495bp).



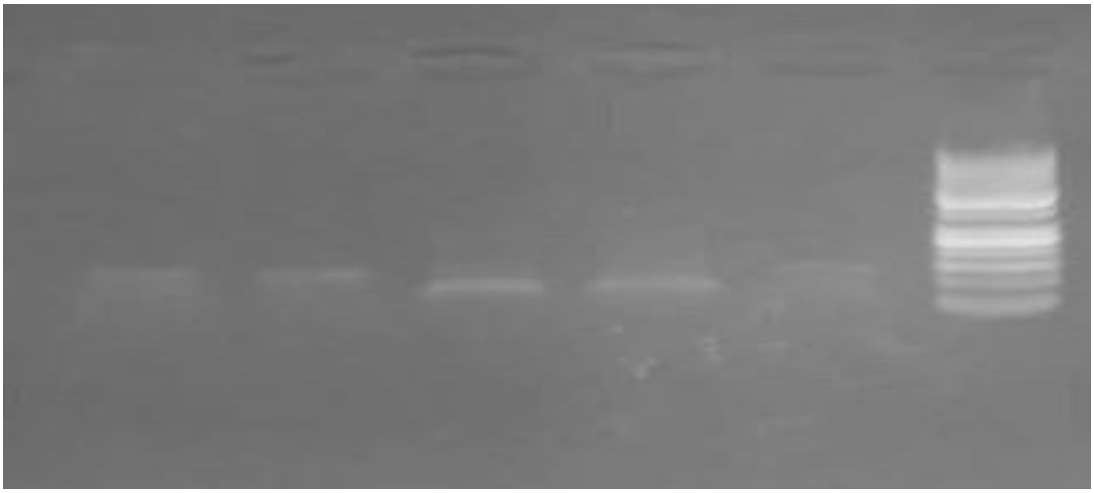
1 2 3 4 5 M

Digestion pattern of PCR product (388 bp) on electrophoresis with ethidium bromide. MNL 1 restriction enzyme digested the wild type of Factor V gene into three fragments (246, 105, 37). 1, 2, 3, 4, 5 are wild type. M is 100 bp marker.



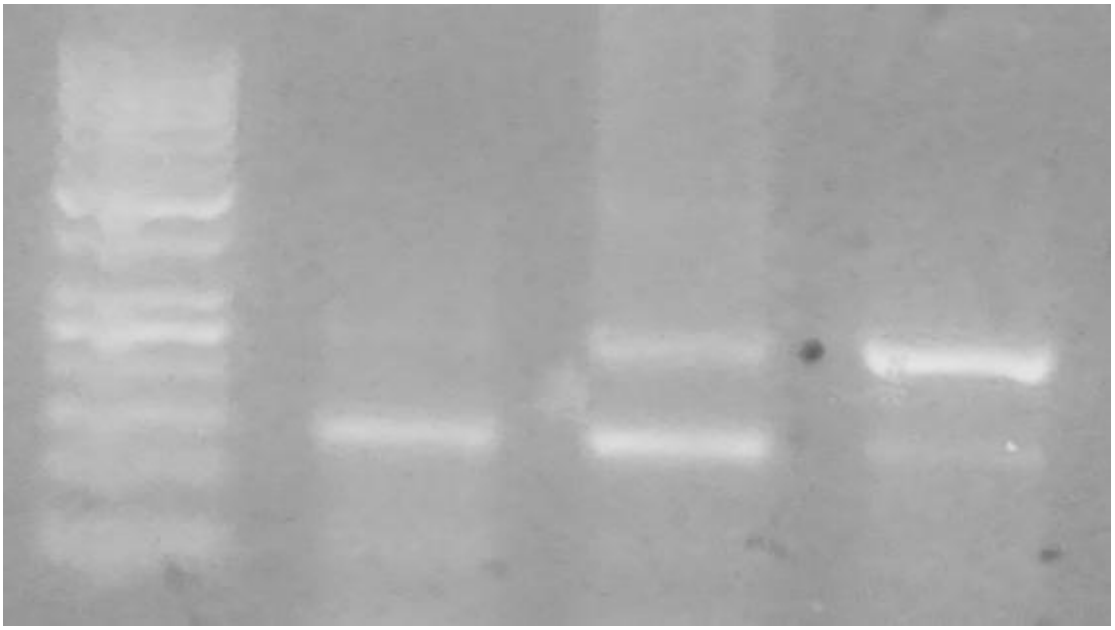
1 2 3 4 M

Restriction enzyme Hinf1 digested MTHFR mutant gene into (175, 23bp). The wild type remains undigested (198bp). 1 and 3: heterozygous type, 2: control positive. M: marker 100 bp.



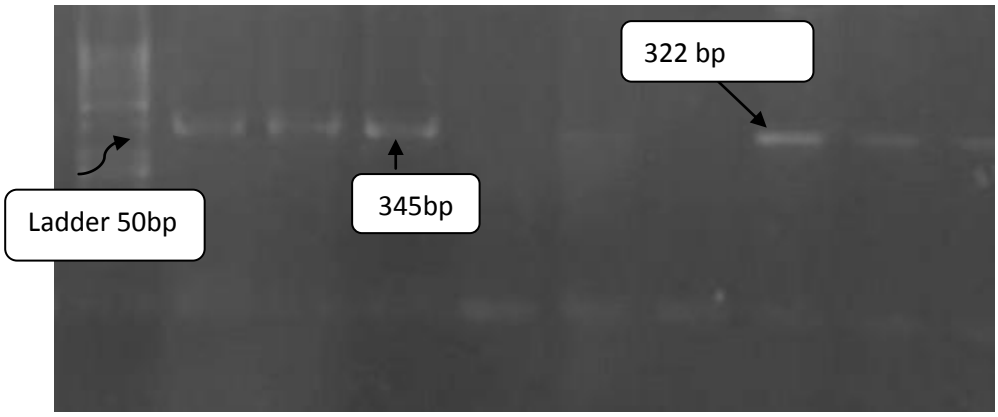
1 2 3 4 5 M

Gel electrophoresis with ethidium bromide. Hinf1 enzyme restriction results in two types of factor XIII gene, 114 bp for the wild type and 94 bp for the mutant type. 1, 2, 5: wild type. 3, 4: mutant type.



M **1** **2** **3**

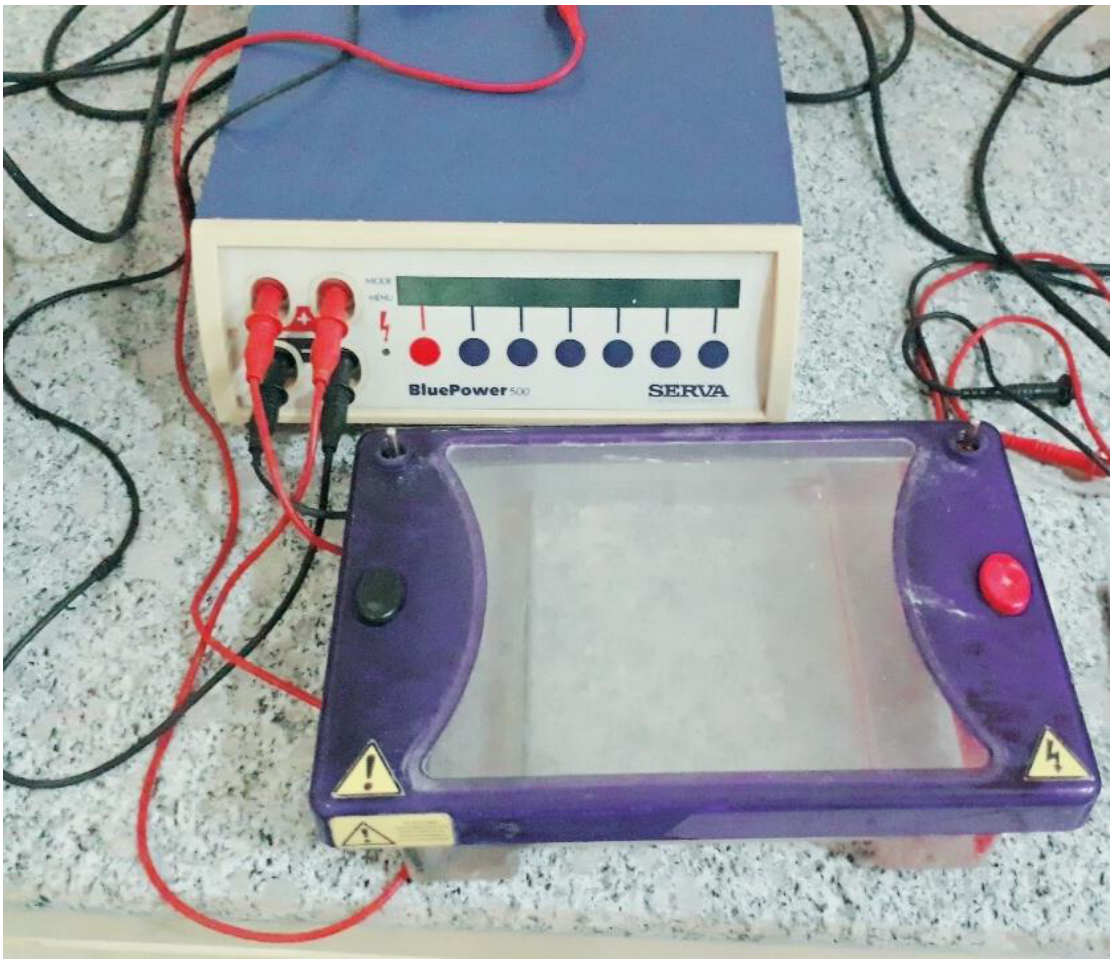
Gel electrophoresis. Allele specific PCR for PAI-1. Lane 1: 5G/5G (139bp), lane 2 4G/4G (139bp) and lane 3: 4G/5G (257bp). M: marker 50 bp



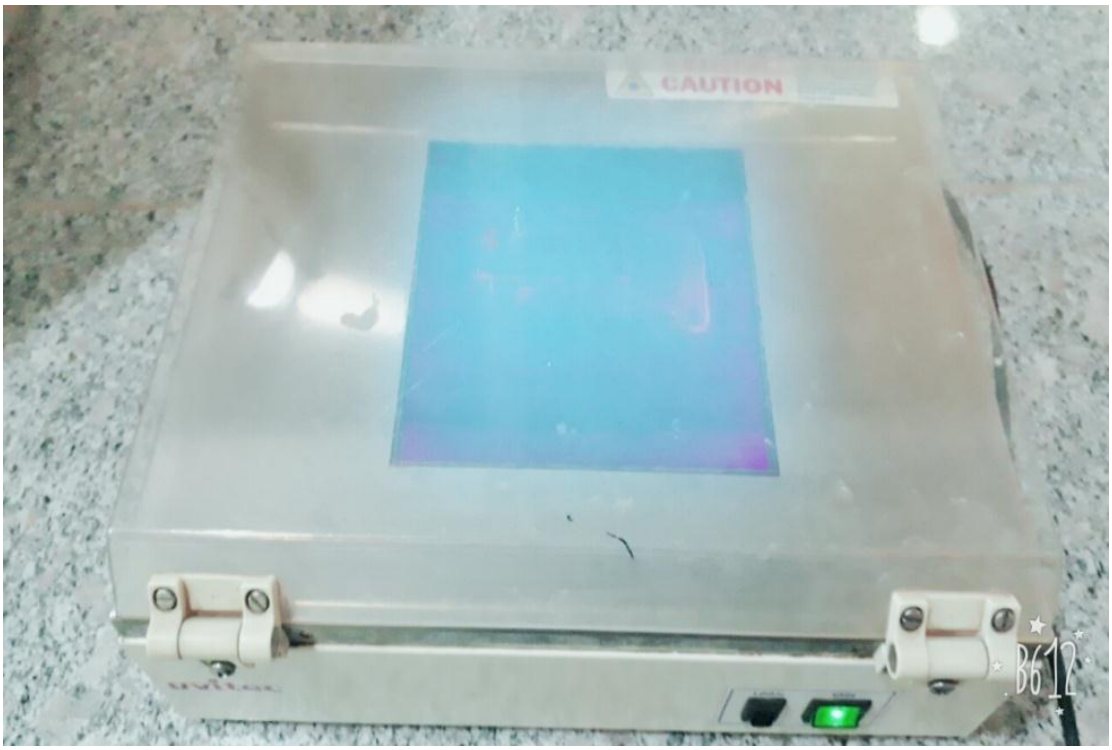
Gel electrophoresis with ethidium bromide. Factor II mutation G20210A. Mutant gene digested into (322, 23bp), while the wild type undigested with Hind III (345bp).

Appendix IV:Instruments

CLASSIC K960 China Thermocycle Device.



Gel Electrophoresis and Power Supply Device



UV Light Transilluminater Device

Hosptex coagulometer

Mispa-i2

Appendix V :PCR kits

Maxime PCR PreMix Series *Research Use Only*

ISO 9001/14001 Certified Company

Maxime PCR PreMix Kit (*i*-Taq)

for 20μl rxn / 50μl rxn

Cat. No. 25025(for 20μl rxn, 96 tubes) **Cat. No. 25026**(for 20μl rxn, 480 tubes)
Cat. No. 25035(for 50μl rxn, 96 tubes)

DESCRIPTION

iNtRON's *Maxime* PCR PreMix Kit has not only various kinds of PreMix Kit according to experience purpose, but also a 2X Master mix solution.
Maxime PCR PreMix Kit (*i*-Taq) is the product what is mixed every component: *i*-Taq™ DNA Polymerase, dNTP mixture, reaction buffer, and so on- in one tube for 1 rxn PCR. This is the product that can get the best result with the most convenience system. The first reason is that it has every components for PCR, so we can do PCR just add a template DNA, primer set, and D.W.. The second reason is that it has Gel loading buffer to do electrophoresis, so we can do gel loading without any treatment. In addition, each batches are checked by a thorough Q.C., so its reappearance is high. It is suitable for various sample's experience by fast and simple using method.

STORAGE

Store at -20℃; under this condition, it is stable for at least a year.

CHARACTERISTICS

- High efficiency of the amplification
- Ready to use: only template and primers are needed
- Stable for over 1 year at -20℃
- Time-saving and cost-effective

CONTENTS

- *Maxime* PCR PreMix (*i*-Taq, for 20μl rxn) 96 (480) tubes
- *Maxime* PCR PreMix (*i*-Taq, for 50μl rxn) 96 tubes

Component in	20 μl reaction	50 μl reaction
i-Taq™ DNA Polymerase(5U/μl)	2.5U	5U
dNTPs	2.5mM each	2.5mM each
Reaction Buffer(10x)	1x	1x
Gel Loading buffer	1x	1x

Note : The PCR process is covered by patents issued and applicable in certain countries. iNtRON Biotechnology does not encourage or support the unauthorized or Unlicensed use of the PCR process. Use of this product is recommended for persons That either have a license to perform PCR or are not required to obtain a license.

PROTOCOL

1. Add template DNA and primers into *Maxime* PCR PreMix tubes (*i*-Taq).
   Note 1 : Recommended volume of template and primer : 3μl~9μl
   Appropriate amounts of DNA template samples
   - cDNA : 0.5-10% of first RT reaction volume
   - Plasmid DNA : 10pg-100ng
   - Genomic DNA : 0.1-1ug for single copy

   Note 2 : Appropriate amounts of primers
   - Primer : 5-20pmol/μl each (sense and anti-sense)
2. Add distilled water into the tubes to a total volume of 20μl or 50μl .
   Do not calculate the dried components

Example **Total 20μl or 50μl reaction volume**

PCR reaction mixture	Add	Add
Template DNA	1 ~ 2μl	2 ~ 4μl
Primer (F : 10pmol/μl)	1μl	2 ~ 2.5μl
Primer (R : 10pmol/μl)	1μl	2 ~ 2.5μl
Distilled Water	16 ~ 17μl	44 ~ 41μl
Total reaction volume	**20 μl**	**50 μl**

Note : This example serves as a guideline for PCR amplification. Optimal reaction conditions such as amount of template DNA and amount of primer, may vary and must be individually determined.

3. Dissolve the blue pellet by pipetting.
   Note : If the mixture lets stand at RT for 1-2min after adding water, the pellet is easily dissolved.
4. (Option) Add mineral oil.
   Note : This step is unnecessary when using a thermal cycler that employs a top heating method(general methods).
5. Perform PCR of samples.
6. Load samples on agarose gel without adding a loading-dye buffer and perform electrophoresis.

SUGGESTED CYCLING PARAMETERS

PCR cycle		Temp.	PCR product size		
			100-500bp	500-1000bp	1Kb-5Kb
Initial denaturation		94℃	2min	2min	2min
30-40 Cycles	Denaturation	94℃	20sec	20sec	20sec
	Annealing	50-65℃	10sec	10sec	20sec
	Extension	65-72℃	20-30sec	40-50sec	1min/Kb
Final extension		72 ℃	Optional. Normally, 2-5min		

EXPERIMENTAL INFORMATION

- ***Comparison with different company kit***

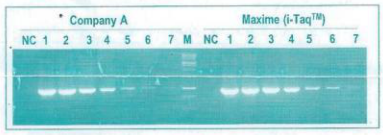


Fig.1. Comparison of *Maxime* PCR PreMix (*i*-Taq) and Company A's PreMix system by amplifying 1 Kb DNA fragment .
After diluting the λDNA as indicates, the PCR reaction was performed with *Maxime* PCR PreMix (*i*-Taq) and company's A product.
Lane M, SiZer-1000 DNA Marker; **lane 1,** undiluted λDNA; **lane 2,** 200 ng λDNA; **lane 3,** 40 ng λDNA; **lane 4,** 8 ng λDNA; **lane 5,** 1.6 ng λDNA; ; **lane 6,** 320 pg λDNA; **lane 7,** 64 pg λDNA; **lane NC**, Negative control

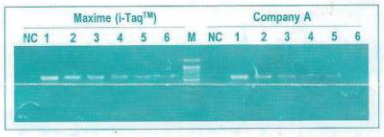


Fig.2. Comparison of *Maxime* PCR PreMix (*i*-Taq) and Company A's PreMix system by amplifying 570 bp DNA fragment (GAPDH).
Total RNA was purified from SNU-1 using easy-BLUE™ Total RNA Extraction Kit (Cat. No. 17061). And then, the first strand of cDNA was synthesized using Power cDNA Synthesis Kit (Cat. No. 25011). After diluting the cDNA mixture as indicates, the RT-PCR reaction was performed.
lane M, SiZer-100 DNA Marker; **lane 1,** undiluted cDNA; **lane 2,** 1/2 diluted cDNA; **lane 3,** 1/4 diluted cDNA; **lane 4,** 1/8 diluted cDNA; **lane 5,** 1/16 diluted cDNA; **lane 6,** 1/32 diluted cDNA; **lane NC**, Negative control

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SiZer™ DNA Markers

DESCRIPTION

iNtRON supplies a wide range of products for accurate size and mass estimations (quantitation) of nucleic acid fragments. Nucleic Acid Markers are available for sizing linear, or supercoiled DNA and single-stranded RNA fragments. A variety of these markers are available in the ready-to-use SiZer™ formats.

SiZer™ DNA Markers are ideal for determining the size of double-stranded DNA from 60~10,000bp base pairs. The SiZer™ DNA Markers consist of 7 ~ 15linear double-stranded DNA fragments. Several fragments are present at increased intensity to allow easy identification. All fragments are precisely quantified and mixed during the production.

For 5 µl loading, all fragments except typical band DNA fragments are 40 ng. The typical band of DNA fragments is 100 ng. These ladders are pre-mixed with loading dye and are ready to use.

All DNA Markers can be stained with RedSafe™ Nucleic Acid Staining Solution, ethidium bromide (EtBr) or other DNA stains.

CHARACTERISTICS

- Ideal for determining the size of DNA
- Stable for more than 12 months at - 20 ℃
- Ready to use without any handlings.

KIT CONTENTS

Product	Contents	Cat. No.
SiZer™-20 DNA Marker	0.3 ml	24071
SiZer™-50 plus DNA Marker	0.5 ml	24072
SiZer™-100 DNA Marker	0.5 ml	24073
SiZer™-1000 DNA Marker	0.5 ml	24074
SiZer™-1000 plus DNA Marker	0.5 ml	24075
SiZer™-15K DNA Marker	0.5 ml	24076
SiZer™- λDNA/HindIII DNA Marker	0.5 ml	24077

STORAGE

- Store at 4 ℃ and stable for more than 6 months. For more stable use, should be eliquoted and then stored at -20 ℃. (stable for more than 12 months)

GENERAL USE

- No DNase and RNase detected.
- Load 5 µl per each well of Agarose gel.

QUALITY CONTROL

Well-defined bands are formed during agarose gel electrophoresis. The DNA concentration is determined spectrophotometrically.

The absence of nucleases is confirmed by a direct nuclease activity assay.

ELECTROPHORESIS

- The 5 µl of ladder DNA was loaded, and then electrophoresed for 1hr at appropriate concentration of gel

PRODUCT USE LIMITATION

This product is developed, designed and sold exclusively for research purposes and in vitro use only. The product was not tested for use in diagnostics or for drug development, nor is it suitable for administration to humans or animals.

NOTICE BEFORE USE

- Do not heat before loading
- For quantification, adjust the concentration of the sample to equalize, it approximately with the amount of DNA in the nearest band of the ladder.
- Visualize DNA by staining RedSafe™, ethidium bromide (EtBr) or other DNA stains.

DETAIL INFORMATION

	Size range (bp)	Conc. (ng/µl)	Typical bands	Other bands	Loading Vol.	Band number	Contents
SiZer™ -20	60-300	128	100ng/5µl	40ng/5µl	5µl	13	60,80,**100**, 120,140, 160, 180,**200**,220, 240,260, 280,300
SiZer™ -50plus	50-500	128	100ng/5µl	40ng/5µl	5µl	13	50,100,150, 200, **250**, 300, 400,**500**, 600, 700,800,900,1000
SiZer™ -100	100-1500	100	100ng/5µl	40ng/5µl	5µl	11	100,200,300, 400, **500**, 600, 700,800, 900, 1000,1500
SiZer™ -1000	250-10000	120	100ng/5µl	40ng/5µl	5µl	12	250, 500, 750, 1000,1500, **2000**, 3000,4000, **5000**, 6000,8000, 10000
SiZer™ -1000 plus	100-10000	144	100ng/5µl	40ng/5µl	5µl	15	100,200,300, 400,**500**, 700, 1000,1500,2000, **3000**,4000,5000, 6000,8000, 10000
SiZer™ -15K	250-15000	85	125ng/5µl	50ng/5µl	5µl	7	250, 1000, **2500**, 5000, 7500, 10000, 15000
SiZer™ - λDNA/HindIII	125-23130	100	350ng/5µl	-	5µl	8	125, 564, 2027, 2322, 4361, 6557, 9416, 23130

RELATED PRODUCTS

Product Name	Cat.No.
RedSafe™ Nucleic Acid Staining Solution (20,000x)	21411
DNA-spin™ Plasmid DNA Extraction Kit	17096/17097/17098
MEGAquick-spin™ Total Fragment DNA Purification Kit	17286 / 17287/17288
Maxime™ PCR PreMix (i-StarTaq)	25165
Maxime™ PCR PreMix (i-pfu)	25185