بسم الله الرحمن الرحيم

Sudan University of Science and Technology

College of Graduate Studies

Detection of Coagulation Factor XIII-A (Val34Leu) Polymorphism in Sudanese Patients with Deep Vein Thrombosis

الكشف عن الطفرة الوراثية (Val34Leu) لعامل التجلط الثالث عشر- أ- في مرضي سودانين مصابين بتخثر الاوردة العميقة.

A Dissertation Submitted in Partial Fulfillment for Requirements of the Master Degree in Medical Laboratory Sciences (Hematology and Immunohematology).

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الآية

قال الله تعالى :

(وَعَلَّمَ آدَمَ الأَسْمَاءَ كُلَّهَا ثُمَّ عَرَضَهُمْ عَلَى الْمَلآئِكَةِ فَقَالَ أَنْبِئُونِي بِأَسْمَاءِ هَؤُلآء إِنْ كُنتُمْ صَادِقِينَ(31) قَالُوا سُبْحَانَكَ لاَ عِلْمَ لَنَا إِلاّ مَا عَلَّمْتَنَا إِنَّكَ أَنْتَ الْعَلِيمُ الْحَكِيمُ(32)) .

صدق الله العظيم

سورة البقرة من الآية (31-32).

Dedication

I dedicate this work to my Family and my best friends….and for me.

Acknowledgment

I wish to expresses my gratitude thanks allah for helping me to achieve this work…Thanks for Dr. abdallah musa for his invaluable assistance with This work and great support…thanks for my colleagues manal altyeb and hana mohammed for their Co- operation and Technical assistance…. thanks for my supervisior Dr. hisham noraldyem for his valuable time which he gave for my study and his support… I am really appreciating the help of the research lab employees…thank you all…

Abstract

This is analytical case- control study was conducted among Sudanese DVT patients to detect the most common polymorphism of coagulation factor XIII-A (Val34Leu), the practical was performed in the research laboratory of Sudan university of sciences and technology in period between March and July 2018.

Eighty venous blood samples (2.5 ml) were collected in EDTA containers, forty samples from patients were already diagnosed with the DVT by imaging of dopplar ultra sonography, patients with different ages and both gender were included, some risk factors such as hypertension, pregnancy and contraceptives, obesity and smoking were considered, and forty samples were collected from healthy individuals as controls, all samples were kept in -20°C for DNA extraction. DNA was extracted by guanidine hydrochloride method, PCR were performed on the eighty samples and the presence of factor XIII- A (val34leu) polymorphism was determined by the use of restriction enzyme.

Four polymorphisms were detected in the patient samples, 3 were heterozygous (val/leu) and one was homozygous (leu/leu), and no polymorphism was detected in the control samples. These results were analyzed by statistical package for social sciences computer programme (SPSS), the p-value was insignificant and the study |concluded that no association between factor XIII-A (val34leu) polymorphism and DVT.

مستخلص البحث

هذه الدراسة دراسة تحليلية اجريت لتحديد الطفرة الجينية متعددة الاشكال **(val34leu)** في عامل تجلط الدم الثالث عشر-أ في مرضى سودانين اصيبوا بتخثر الاوردة العميقة وتمت التجارب العملية في معمل الابحاث بجامعة السودان للعلوم والتكنولوجيا في الفترة مابين مارس و حتى يوليومن العام 2018 .

تم جمع 80 عينة من الدم الوريدي (2.5 مليليتر) ووضعت في حاويات تحتوى على مانع التجلط ايثايلين ثنائي الامين رباعي حامض الاستيك , تم اخذ 40 عينة من مرضى شخصت حالتهم بتخثر الاوردة العميقة باستخدام دوبلر الموجات فوق الصوتية. المرضى هم من فئات عمرية مختلفة ومن كلا الجنسين, كذلك تم الاخذ بالاعتبار عوامل من الممكن ان تتسبب في حدوث تخثر الاوردة العميقة كارتفاع ضغط الدم,وجود عمليات جراحية سابقة, الحمل و موانع الحمل لدى الاناث,وكذلك السمنة و التدخين. وتم اخذ 40 عينة من متطوعين اصحاء كضوابط للدراسة, حفظت جميع العينات في 20 درجة تحت الصفر ثم استخدمت طريقة كلوريد الغوانيدين لاستخلاص الحمض النووي منزوع الاكسجين من العينات التي جمعت و اجري تفاعل البلمرة المتسلسل على الحمض النووي منزوع الاكسجين من الثمانين عينة.

استخدمت انزيمات القطع على العينات التى تمت مضاعفتها بتفاعل البلمرة المتسلسل وتم الكشف عن اربع طفرات متعددة الاشكال ثلاثة منها مختلفة الطراز (val/leu), وطفرة واحدة متماثلة (leu/leu) وذلك في عينات مرضى تخثر الاوردة العميقة. ولم يتم الكشف عن وجود اي طفرة متعددة الاشكال في عينات الضوابط. تم تحليل النتائج عن طريق برنامج الحزم الاحصائية للعلوم الاجتماعية المحوسبة, ووفقا لقيمة الاحتمال فانه لا توجد علاقة بين هذه الطفرة متعددة الاشكال ومرض تخثر الاوردة العميقة.

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Abbreviations

AT	Anti- Thrombin
APLS	Anti- phospholipids Syndrome
APTT	Activated Partial Thromboplastin Time
Arg37	Arginine in position 37 in the A chain of factor XIII
BT	Bleeding Time
BP	Base Pair
CAD	Coronary Artery Diseases
CNS	Central Nervous System
CT	Computed Tomography
DNA	Deoxribonucleic Acid
DNAases	Deoxribonucleases
DVT	Deep Vein Thrombosis
D W	Distilled Water
EDTA	Ethylin Dymin- Tetra- Acetic Acid
EPCR	Endothial Protein C Receptor
FXIII	Factor XIII
FXIIIa	Activated Factor XIII
FSF	Fibrin Stabilizing Factor
(F) Primer	Forward Primer
FVL	Factor V Leiden
Gly38	Glycine in position 38 in the A chain
G>T	Guanine substituted by Thymine in the Codon

Glu651Gln	Glutamine in position 651 replaced by Glutamate
INR	International Normalized Ratio
KHCO3	Potassium Bicarbonates
Leu564Pro	Leucine in position 564 in (A) chain replaced by Proline
MTHFR	Methyl Tetra- Hydro- Folates Reductase
MI	Myocardial Infarction
MRI	Magnetic Resonance Image
ML	Mililiter
NaCL	Sodium Cholride
NH4CL	Ammonium Chloride
NH4 Acetate	Ammonium Acetate
PCR	Polymerase Chain Reaction
PE	Pulmonary Embolism
PT	Prothrombin Time
(R) Primer	Reverse Primer
RCLB	Red Cells Lysis Buffer
RNAases	Ribo-nuclease
RPM	Round per Minutes
SDS	Sodium Dodecyl Sulfates
SPSS	Statistical Package Social Sciences
TE Buffer	Tris- EDTA Buffer
Tris- HCL	Tris- Hydrochloride
Tyr204Phe	Tyrosine in Position 204 in (A) chain substituted by Phenlalainine
UFH	Unfractionated Heparin

Val34Leu	Valine in Position 34 in (A) chain substituted by Leucine
Val650Ile	Valine in position 34 in (A) chain substituted by Isolucine
VKA	Vitamin K Antagonists
VTE	Venous Thromboembolism
WCLB	White Blood Cells Lysis Buffer

Chapter One

Introduction

1.1 Background

Normal blood physiology hinges on a delicate balance between pro- and anti-coagulant factors, and Virchow's triad distills the multitude of risk factors for DVT into three basic elements favoring the thrombus formation: venous stasis, vascular injury, and hypercoagulability. Anticoagulation is the mainstay of therapy for DVT with the goal of preventing progression to PE and recurrence of thrombosis (Stone *et al*., 2017). Risk factors for venous thrombosis include obesity, cancer, immobilization, surgery, trauma and pregnancy, and inherited tendencies such as factor V leiden, prothrombin G20210A, and deficiencies of anticoagulant factors, protein C, protein S, and anti-thrombin (Cushman *et al*., 2007).

Deep vein thrombosis is a subset of venous thromboembolism (VTE), is major preventable cause of morbidity and mortality worldwide, affect an estimated 1per 1000 people annually and DVT accounting for approximately two- third of VTE, pulmonary embolism (PE) occur in up to one third of the VTE cases (Beckman *et al*., 2010). Patients at risk must be identified and given appropriate prophylaxis to reduce VTE- related mortality. The failure to reduce this rate may be a result of uncertainly regarding risk factors for VTE and the associated difficulty in recognizing individual at risk (Gader *et al*., 2008)

Deep vein thrombosis occurs at an incidence ranging from 45 to 117 in 100,000 adults per year (Heit, 2015), the prevalence of DVT in Africans varied between 380 and 448 per 100,000 birth per year in pregnant and postpartum women, and between 2.4%and 9.6% .in postoperative patients (Zaki *et al*., 2014; Ibrahim *et al*., 2018).

Activated factor XIII cross links between fibrin monomers, thus increasing the clot stability and resistance to fibrinolysis, congenital factor XIII deficiency causes severe bleeding diathesis, factor XIII- A val34leu polymorphism is a common polymorphism in factor XIII- A gene, a substitution in exon2 of the A subunit resulting a valine to leucine substitution at position 34 (val34leu), the amino acid substitution is located 3 amino acids from the thrombin cleavage site (Arg37-Gly38), it appears to increase activation of FXIII and lead to a higher dissociation index of A and B subunits, factor XIII that has val34leu allele results in fibrin clots looser and more susceptible to fibrinolysis, many studies identified these polymorphism as a protective factor against both arterial and venous thrombosis (Hancer *et al*., 2006; Cushman *et al*., 2007).

1.2 Rationale

Deep vein thrombosis (DVT) and venous thromboembolism (VTE) is a serious health problem with high mortality worldwide. The thrombi that had been developed in DVT obstruct the venous outflow and could be a source of emboli if untreated, pulmonary embolism is a serious complication of DVT when the blood clot in the deep vein travels to the lungs, patients at risk must be identified to given the treatment and reducing VTE development. VTE as other complex and multifactorial diseases is influenced by genes as well as environmental factors. Analyzing genetic polymorphism of FXIII- A val34leu allele in DVT patients to detect any possible association in between and assessing the protective effect if present, where there are no information available about this polymorphism in the Sudanese population and the identification of such variants is also useful in establishing risk profiles to identify those at risk of developing VTE.

1.3 Objectives

1.3.1 General objective

- Detection of Factor XIII Val34Leu polymorphism in DVT patients among Sudanese population.

1.3.2 Specific objectives

- To investigate the presence of FXIII Val34Leu polymorphism in DVT patients and controls by using restriction enzyme.
- To assess the protective effect of factor XIII-A val34leu allele in DVT and detect any possible association if found.
- To assess interaction of these variant with many common VT risk factors

Chapter Two

2.1 Deep Vein Thrombosis (DVT)

Deep vein thrombosis occur when a blood clot or thrombus developed in the large veins frequently occur in the lower limps, DVT is a subset of venous thrombo-embolism (VTE), affects an estimated 1 per 1000 people annually and DVT accounting approximately two- third of these events, about 60-70% of patients with asymptomatic VTE develop DVT (White, 2003; Beckman *et al*., 2010; Moheimani and Jackson, 2011).

DVT usually initiate in the calf area of the leg and the thrombi form in the deep veins below the popliteal- trifurcation and this most likely resolve spontaneously with no symptoms (Ho, 2010) , DVT lead to complications such as post-phlebitic syndrome, pulmonary embolism, but much of the morbidity of DVT result from the development of post- thrombotic syndrome which occur in 20% to 50% of the DVT patients and the symptoms including leg pain, swelling, and in severe cases venous ulcers (Kahn, 2011; Stone *et al*., 2017), DVT may reoccur in about 10% pf the patients who may develop severe post- thrombotic syndrome within 5 years (Kearon, 2003).

2.2 Pathogenesis

The pathogenesis of DVT depend mainly on the presence of three contributing factors that predispose to formation of the clot including venous thrombosis, vascular injury, and hypercoagulability, but venous stasis is the most consequential of the three factors, the recurrent presence of all increases the risk for the clot

formation. Those three factors are elements of Virchow's Traid (Kumar *et al*., 2010; Stone *et al*., 2017).

Venous thrombosis develop in areas with decreased blood flow such as the pockets adjacent to valves in the deep veins of the leg, this valves help to promote blood flow through the venous circulation they are also potential location for venous stasis and hypoxia, as the blood flow slow, oxygen tension declines with coincident increase in hematocrit and the hypercoagulable environment may down regulate certain antithrombotic proteins that expressed on venous valves including thrombomodulin and endothelial protein C receptor (EPCR), also hypoxia reducing the expression of certain pro-coagulant, Venous thrombosis has two components an inner platelets rich white thrombus forming that what called lines of Zhan surrounded by an outer red cells dense fibrin clot (Stone *et al*., 2017).

2.3 Risk Factors of DVT

Risk factors are both hereditary and acquired which are important in the development of DVT, the acquired risk factors including trauma, surgery, chemotherapy, congestive heart failure, hormone replacement therapy, oral contraceptive therapy, pregnancy, postpartum, family history of VTE, previous DVT, advanced age, varicose veins. The inherited risk factor including deficiencies of some natural coagulation inhibitors such as anti-thrombin (AT), protein C, protein S, elevated level of factor VIII, factor V leiden (FVL), prothrombin 20210A, methyl tetra- hydrofolate reductase (MTHFR) (Anderson and Spencer, 2003; Rosendal and Reitsma, 2009).

2.4 Clinical Signs and Diagnosis

The clinical presentations of DVT varies according to extent and location of the thrombus, the cardinal signs and symptoms include asymmetrical swelling, warmth

or pain in an extremity. The initial diagnosis, clinical assessment, for suspected DVT, wells scoring criteria and D-dimer assay used to identify the DVT patients, D-dimer is a prominent fibrin degradation product which generated by fibrinolytic response to thrombus formation in the body, D-dimer assay is highly sensitive but with lower specificity for the diagnosis of DVT, and there increase is not unique to thrombosis but help to diagnose DVT patients and with wells scoring criteria (Wells *et al*., 2003). The diagnostic imaging is often employed to confirm the presence of DVT, the clot can be characterized with real time imagining such as duplex and color- flow Doppler, other diagnostic imaging used for DVT like computed tomography (CT), and magnetic resonance image (MRI) venography (Stone *et al*., 2017).

2.5 Management

Anticoagulation is an essential component of therapy of DVT, to prevent thrombus extension and decrease of the risk of recurrent thrombosis and subsequent death, unfractionated heparin (UFH), was the initial pharmacological approach in DVT patients, it is catalyzing the inactivation of thrombin and other clotting factors by binding to anti-thrombin III, but UFH has high nonspecific binding affinities to endothial cells, platelets factor 4, and platelets lead to heparin induced thrombocytopenia and osteoporosis. Low molecular weight heparin replaced the UFH, which it is safer and can be administered in a fixed weight adjusted dose. Vitamin K antagonists (VKA) such as warfarin are the most oral anticoagulant for prevention and treatment of VTE. During acute phase patients administered LMWH and VKA to reduce the symptoms and risk of further thrombus formation or progression (McRae and Eikelboom, 2007; Moheimani and Jackson, 2011).

The therapeutic level are determined by activated partial Thrombo-plastin time (a PPT), and the dose modified according to international normalized ratio (INR) (Stone *et al*., 2017).

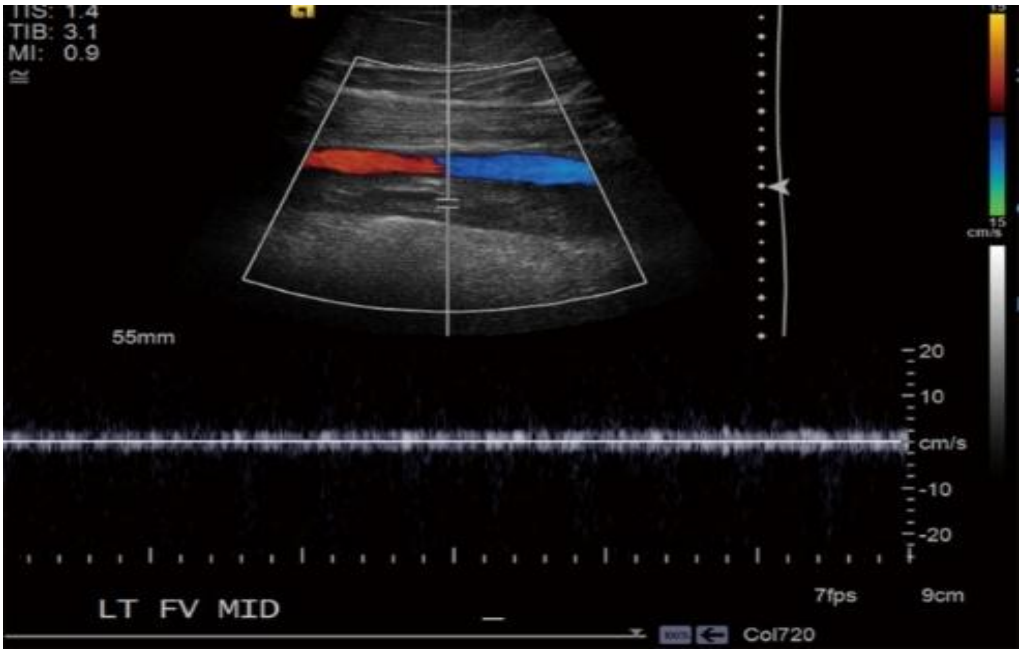

Figure 2.1 Venous color- flow Doppler. US Doppler imaging of the left femoral vein showing complete occlusion by a heterogeneous thrombus with dilation of the vein at the site of thrombosis (Stone *et al*., 2017).

2.6 Coagulation

Coagulation contains soluble protein components of thrombin generating pathways, the generation of thrombin lead to conversion of soluble plasma fibrinogen, forming insoluble fibrin polymers or clot, this provides the physical consolidation of vessel wound repair following injury. Factor XIII (FXIII) is a Transgulaminase found that cross links newly formed fibrin to other proteins of the clot generated a stable structure able to stop bleeding and prevent blood loss (Kampell *et al*., 2005).

2.7 Factor XIII

2.7.1 Historical Background

First was observed the formation of insoluble fibrin occurred with calcium and an unknown (serum factor), many years later these finding confirmed and cocludedb that the factor was a plasma protein, there have been several names for that protein such as fibrin stabilizing factor (FSF), laki- lorand factor (L-L), fibrinase, pro-transglutaminase, and fibrin polymerase, the International Committe of Blood Clotting Factors formally chose the designation of factor XIII (Hsieh and Nugent, 2008).

2.7.2 Factor XIII Biology

Factor XIII a pro-transglutaminase found in the cells and plasma, it is a one of 9 members of transglutaminase super family, the cellular forms of FXIII are present in multiple cells types including monocytes, megakaryocytes, osteoblasts and platelets, plasma FXIII is unique because it circulates as two catalytic subunits (FXIII-A2), and two non- catalytic subunits (FXIII-B2), arranged in a non-covalent heterotetramer (FXIII-A2B2) (Byrnes and Wolberg, 2016).

Factor XIII activity is associated with the structure of fibrin clot, factor XIII turn into XIII-A which is an active transglutaminase, in the results phase of coagulation cascade together with calcium and thrombin activation (Yilidirim *et al*., 2017). The cleavage of the 37 amino acid N- terminal peptide from the FXIII-A subunit by thrombin results in the formation of activated FXIII-a transgluaminase the cross links γ-glutamyl-ε-lysyl bonds between adjacent fibrin molecules, FXIII is the a key regulator of fibrinolysis protecting newly formed fibrin chains in addition FXIIIa is a key regulator of fibrinolytic mechainry by binding α2 plasmin inhibitor to the fibrin meshwork (Hancer *et al*., 2006; Bereczky and Muszbek, 2011), also participates in other physiological processes including, clot retraction, cell migration, and wound healing (Francis, 2002).

2.7.3 Factor XIII Structure and Function

Plasma FXIII is a hetero-tetramer (FXIII-A2B2), the gene coding for the FXIII-A subunit (F13A) is located on chromosome 6 p24-25, spanning 160 kb and consist of 15 exons interrupted by 14 introns encoding a mature protein of 731 amino acids. FXIII-A is divided into the activation peptide, β- sandwich, catalytic core, β-barrel1 and β- barrel 2. Liver is the major site of synthesis for FXIII-B subunit and the FXIII-B gene (F13A) is located on chromosome 1 q31-32 and spans approximately 28 kb in length and is composed of 12 exons interrupted by 11 introns encoding the mature protein of 641 amino acids, FXIII-B subunit composed of 10 tandem repeats called glycoprotein-1 or such domain because of their shape, B subunit is important in the stabilization and transport of the hydrophobic A2 subunit in the aqueous environment of human plasma (Hsieh and Nugent, 2008).

Platelets factor XIII is a homo-dimer of catalytic subunits (FXIII-A2), present in high concentration, most platelets FXIII derived from megakaryocytes during platelets production, in un-activated platelets FXIII-A2 is primarily associated with cytoplasmic fraction (Mitchell *et al*., 2014).

2.7.4 Factor XIII Activation

During activation of plasma FXIII-A2B2 thrombin fist catalyzes the cleavage of activation peptide from the N- termini of the FXIII-A subunit, and calcium then promotes the dissociation of the FXIII-B subunits from FXIII-A subunits yielding activated FXIII-A2 (FXIIIa) and fibrinogen facilitate dissociation of the FXIII-B subunit, activation of the platelets FXIII-A2 can occur after thrombin cleavage of the activation peptide, but the presence of high calcium concentration FXIII-A2 can also be activated without activation peptide (Byrnes and Wolberg, 2016). FXIII inactivation has been attributed to reversible oxidation, proteolytic digestion by thrombin and proteolytic enzyme released by granulocytes (Bagoly and Muszbek, 2007).

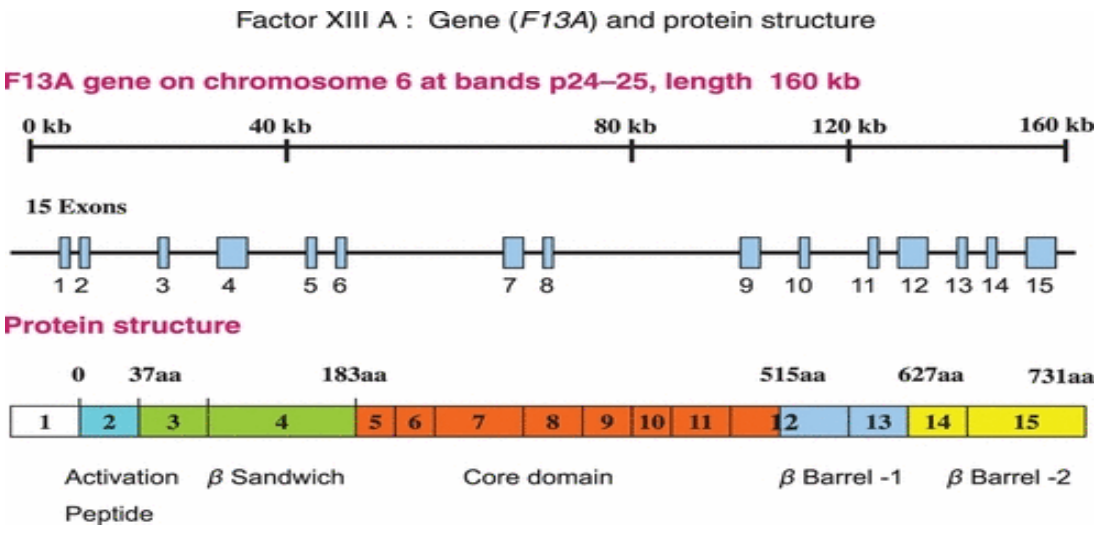

Figure 2.2 Factor XIII- A gene and protein structure (Hsieh and Nugent, 2008).

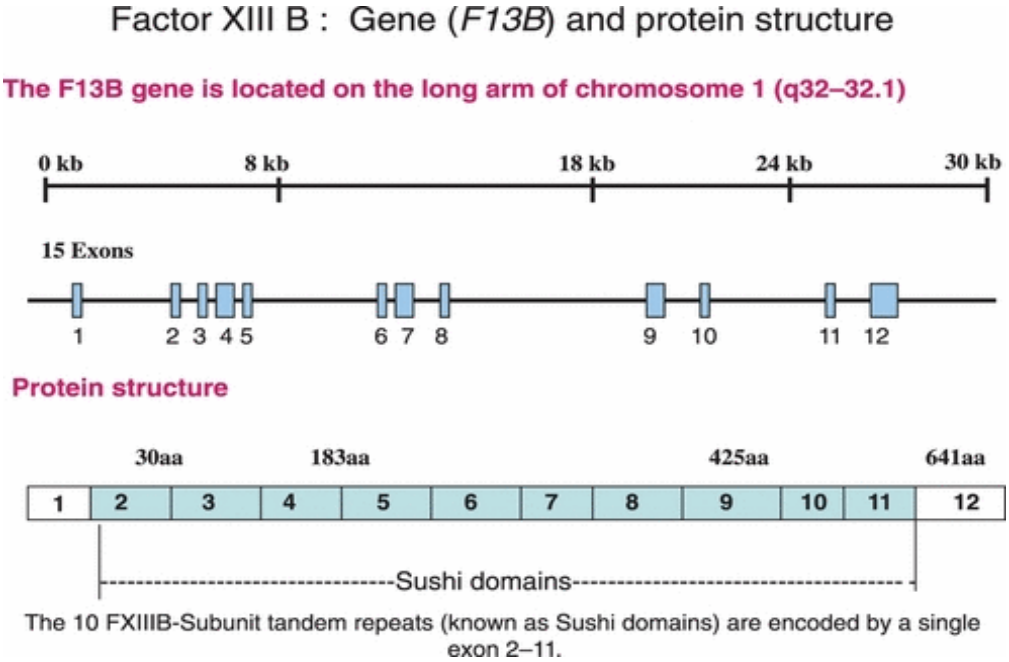

Figure 2.3 Factor XIII- B gene and protein structure (Hsieh and Nugent, 2008).

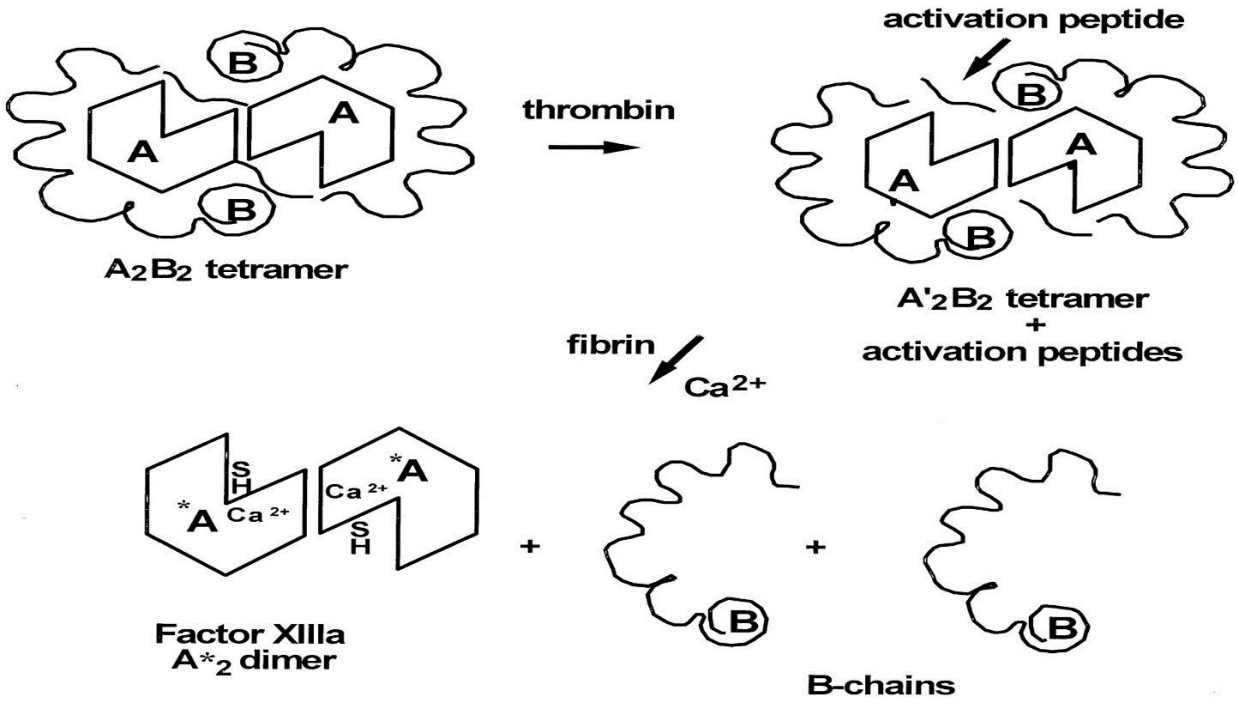

Figure 2.4 Factor XIII tetramer structure and activation (Ariens *et al*., 2002).

2.7.5 Factor XIII Deficiency

Congenital FXIII deficiency can be due to defects in either FXIII-A genes (type 2 defect) or FXIII-B genes (type 1 defect), in severe factor XIII-A deficiency the A subunit is usually absent from plasma, platelets, monocytes and placenta (Hsieh and Nugent, 2008). The affected individuals may show delayed wound healing, CNS bleeding, recurrent miscarriage in women in childbearing age, but umbilical cord bleeding is the most common clinical manifestation among FXIII deficiency patients (Dorgalaleh *et al*., 2015)

The incidence of sever FXIII deficiency is one in 3-5 million people is inherited in an autosomal recessive pattern, this rare disorder affect people of all races and there is often history of consanguinity within certain families of FXIII deficient patients, Iran have the most global incidence of these disorder with the most sever FXIII deficiency cases. The routine laboratory clotting tests or first screening tests such as Bleeding Time (BT), Prothrombin Time (PT), activated Partial Thromboplastin Time (a PTT) and platelets count are normal in FXIII deficiency disease (Dorgalaleh *et al*., 2015).

2.8 FXIII-A Gene Polymorphisms

The FXIII-A gene has several common single nucleotide sequence variations, which encode amino acid substitution. Five common coding polymorphism have been identified in the FXIII-A subunit: Val34Leu, Tyr204Phe, Pro564Leu, Val650Ile and Glu651Gln. The Val34Leu variant is the most studied polymorphism, with amino acid substitution occurring in the activation peptide sequence, three amino acids upstream from the thrombin cleavage site, and the studies have shown that the val34leu allele variant promotes accelerated cleavage of the activation peptide by thrombin (Hsieh and Nugent, 2008; Amin *et al*., 2013).

Try204Phe and Pro564Leu have been linked with increased risk of hemorrhagic stroke in young women, the Tyr204Phe substitution has been reported to be associated with an increased risk of miscarriages, and the Glu651Gln and Val650Ile polymorphisms are the least studied single nucleotide polymorphism (Ariens *et al*., 2002; Hsieh and Nugent, 2008).

2.9 Factor XIII-A val34leu polymorphism

A common polymorphism in FXIII-A, G100T substitution in exon 2resulting in a valine to leucine at position 34, three amino acid from thrombin cleavage site which occur at Arg37- Gly38 (Francis, 2002)

Factor XIII-A val34leu variant is common in white population with the frequency varies among ethnic group, with the lowest (0.01) in Japanese and highest (0.40) in Pima Indians (Wells *et al*., 2006a), FXIII-A val34leu polymorphism has a significant ethnic heterogeneity, the incidence of val34leu allele is quit high in western countries and this polymorphism is rare in Asian population, the incidence of FXIII val34leu polymorphism was reported as high as 48% and 51% in England 45.8% in the USA , 43% in Italy , 45.1% in hungary ,50.2% in france , 28.9% in brazillians and Africans , 2.5% in japanses and 0% in Koreans, the incidence of FXIII val34leu in turkey 37% (Hancer *et al*., 2006; Yilidirim *et al*., 2017).

2.9.1 Pathophysiology

Factor XIII-A val34leu polymorphism is G> T transition in exon 2 of FXIII-A gene resulting in substitution of leucine for valine at amino acid 34, Factor XIII-A val34leu polymorphism dose not result in a change may modify FXIII activity, activation of FXIII-A by thrombin was found to be proceed two-three fold more rapidly in plasma of FXIII-A val34leu variant carrier, this action has an effect on clot stability since the catalytic efficiency of thrombin- induced cleavage of FXIII-

A alter the structure of the cross- linked fibrin, such that fibrin fully cross- linked by FXIII-A val34leu and result in an faster rate of fibrin stabilization and the product has a finer structure with thinner fibers and smaller pores, the thin clot are very resistant against fibrinolysis and associated with increased risk of thrombosis (Wells *et al*., 2006a; Amin *et al*., 2013; Yilidirim *et al*., 2017).

It has been demonstrated that carrier of the FXIII-A val34leu variant activation and depletion of the plasma FXIII-A subunit is more rapid , this action results in less stable clots and indeed may result in decrease in FXIII-A available for stabilization of the clot and thus could provide a protective effect against VTE (Wells *et al*., 2006a).

Factor XIII-A val34leu polymorphism affect the function of FXIII by increasing the rate of activation which result in faster rate of fibrin stabilization (Shemirani *et al*., 2010), this effect appear to be genetic dependent, leu/leu genotype is associated with elevated activity of FXIII, the heterozygous carrier exhibit intermediate activity. The effect of FXIII- A allele might be variable in different populations according to the levels of fibrinogen and thrombin in plasma (Amin *et al*., 2013).

A meta- analysis of a published data demonstrate a moderate but statistically significant protection by the FXIII- A allele (Voko *et al*., 2007). Some result suggest that a significant gene- covariate interaction exist between FXIII-A genotype and fibrinogen (Bereczky *et al*., 2007). The protective effect of the FXIII-A val34leu variant is specific to condition in which fibrinogen levels are high, that levels known to be associated with an increased risk of VTE, at higher fibrinogen levels clots in leu/leu person were more permeable and looser than in val/val person, characterstic associated with more breakable clots, similar change in permeability were also observed for a fibrinogen variant, thus the joint effect of

genetic variants and hemostatic trait concentrations is likely to be important in predicting the risk of VTE (Wells *et al*., 2006a).

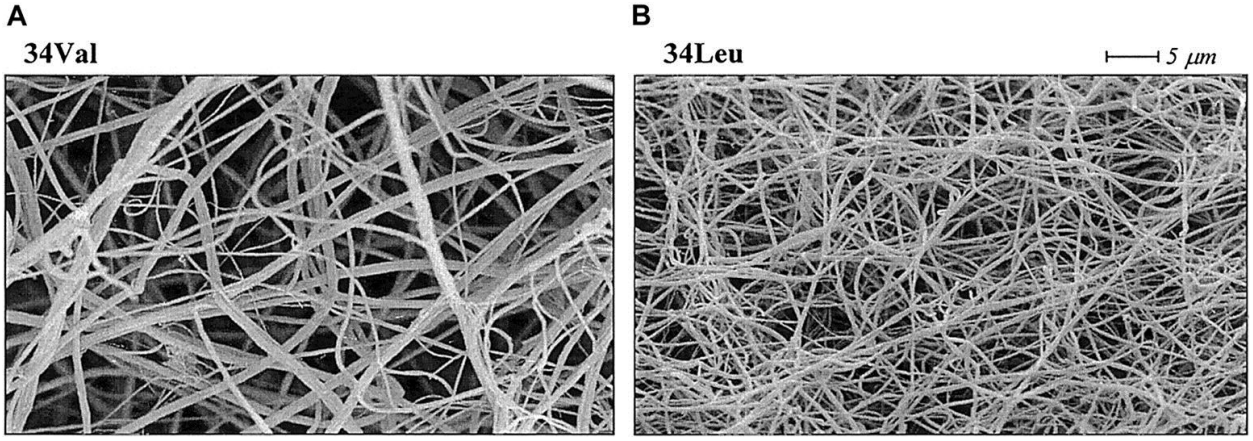

Figure 2.5 Effect of FXIII-A val34leu polymorphism on cross- linked fibrin structure (Ariens *et al*., 2002).

2.10 Previous Studies

Several- case control studies suggested a protective effect of the FXIII-A polymorphism against VTE. Most studies had sample size insufficient to detect statistically significant difference, while a few other reported no effect of this variant on the risk of VTE, variant with a protective effect are of interest because they provide insight into the beneficial effect against VTE, some studies reported no effect of this polymorphism on the risk of deep vein thrombosis (Wells *et al*., 2006a).

FXIII-A TT allele genotypes were present in less than 10% of cases and controls and heterozygote individuals were more abundant, factor XIII TT were more prevalent among controls than cases, factor XIII GT was not clearly associated with reduced risk of venous thrombosis (VT) compared with GG genotype was associated with a non- significant 55% lower risk with an 18% lower risk with the

TT genotype, the FXIII-A val34leu variant was not as clearly associated with a lower risk of VT (Cushman *et al*., 2007).

A study conducted on 127 patients who were monitored due to thrombosis and other 102 healthy people without thromboembolism were included as controls, the cases were compared with the controls, there were no significant differences in terms of age, and no statistically significant differences have been found between the study patients and the control group in terms of factor XIII-A gene polymorphism, they concluded that no consensus has been achieved regarding the correlation between FXIII val34leu polymorphism and venous thrombosis (Yilidirim *et al*., 2017)

Many studies investigated the association of FXIII-A val34leu polymorphism in MI patients and found that this polymorphism was less frequent in the MI patients than control group, then suggested that FXIII-A val34leu variant may have a protective role in MI (Hancer *et al*., 2006).

Early studies were confounded by the use of FXIII activity assays that influenced by factor XIII-A val34leu polymorphism, this polymorphism causes accelerated release of the FXIII activation peptide 2-5 fold earlier FXIII activation and fibrin cross- linking in vitro. The effect of FXIII-A val34leu polymorphism are modulated by complex gene- environment interaction, plasma fibrinogen level and epidemiological analysis to find the association between FXIII and the thrombosis risk in certain population (Byrnes and Wolberg, 2016).

In meta- analytical studies in the association of FXIII-A polymorphism and MI, they suggested that this polymorphism may be associated with the risk to MI (Chen *et al*., 2014; Wang *et al*., 2014). Another meta- analytical study was investigated the association between FXIII-A val34leu polymorphism and coronary artery diseases risk, concluded that Val/Val genotype increased risk for CAD (Jung *et al*., 2017).

Chapter Three

Materials and Methods

3.1 Study Design

Analytical case- control study.

3.2 Study Area

The study was conducted in Khartoum state in Omdurman Teaching Hospital during March to July 2018.

3.3 Sample Size

Forty patients were diagnosed with DVT and forty healthy volunteers as control, the total is eighty.

3.4 Inclusion Criteria

The study included thrombosis patients were diagnosed with DVT according to the clinical signs that suggestive of thrombosis and imaging by Dopplar Ultra sonography.

3.5 Exclusion Criteria

The study excluded other venous thromboembolism patients were not diagnosed with DVT.

3.6 Sample collection

Whole venous blood sample (2.5ml) collected in EDTA containers and stored at -20°C for DNA extraction.

3.7 Data collection

Data were collected with a non-self- questionnaire about demographic data and risk factors.

3.8 Data Analysis

Data were analyzed by SSPS version 25, chi- square test was performed and the frequencies of FXIII val34leu allele were determined among cases and controls, and percentages (%) in qualitative data, mean values and standard deviation (SD) in quantitative data were presented. A p-value considered as significant at the 0.05 level (2- tailed).

3.9 Ethical Considerations

The study was approved by College of graduate studies, Sudan University of Sciences and Technology, and all participants were informed that their diagnosis and collected samples could be used for research purpose.

3.10 Methodology

3.10.1 DNA Extraction

DNA was extracted from EDTA venous blood samples which had been preserved at -20 °C, and extracted by 5M Guanidine Hydrochloride method. All blood samples were taken and placed in Eppendorf tubes and 10 ml of red cell lysis buffer was added RCLB contains 8.3 gm of NH4CL ,1gm of KHCO3, 1.8ml of 5% EDTA, and all were dissolved in 1 liter of Distilled Water, after adding the buffer mixed well and centrifuged at 6000 rpm for 5 minutes , and these step repeated until a clear pellet of white blood cell appear or the suspension is clear , after a clear pellet had been gotten the samples centrifuged at high speed 3000 rpm for 10 minutes to collect the pellet , then 2ml of white blood cell lysis buffer added,

WCLB contains 1.5 gm of tris-HCL , 1.0 gm of EDTA , 0.2 gm NACL , 0.2% SDS, and all dissolved in 100 ml of distilled water, after that 10 microliters of proteinase K which it is enzyme used in various protocols to clear glycoprotein and inactivates RNA-ses and DNA-ses these work on the cells digestion, then 1ml of guanidine chloride added guanidine salts used to disrupt the cells and inactivate cellular enzymes, after guanidine was added, 300 ml of ammonium acetate NH4 acetate provide negative charge to the phosphate backbone and added to clear the pellet , the mixture was incubated for 2 hours at 65°C, then cooled at room temperature, 2ml of pre- chilled chloroform transferred to 15 ml falcon tubes, was mixed very well and centrifuged at 2500 rpm for 5 minutes, the upper layer was collected in new tubes and 10 ml of absolute ethanol added, ethanol used as solvent to precipitate the nucleic acid in concentration of 70-80% also work on remove the excess salts, the mixture was vortexed well and kept at -20°C for 2 hours, after that centrifuged at 3000 rpm for 20 minutes , and the supernatant was carefully drained and the tubes were inverted on a tissue paper for 5 minutes, the pellet was washed with 4 ml of 70% ethanol and centrifuged at 300 rpm for 15 minutes, the supernatant was discharged and the pellet re- suspended in 100 ml of Tris- EDTA, the Tris- EDTA buffer contains 48.4 gm , 30 gm of boric acid , 5 gm of EDTA dissolved in 100 ml of distilled water to prepar5% EDTA ,40 ml of 5% EDTA added, and all dissolved in 100 ml of distilled water , these buffer that was prepared was a stock buffer (10X). (Jeanpierre, 1987).

2.10.1.1 DNA storage:

The buffer is commonly used for long term DNA storage to prevent it from being damaged by nucleases, and provides a safe PH 7-8, and EDTA chelates divalent ions used in nucleases activity and counteract oxidative damage from heavy metals, the obtained DNA was aliquot as stock solution and stored in -20°C.

3.10.2 Preparation of Pre- Mix of PCR

The primers was firstly used according to the manufacturer instructions. Stock solution formed, and for the pre-mix preparation only 5 microliters was taken and by 95 microliters of sterile D.W, 1microliter of (F) primer and 1microliter of (R) primer were used from the working primers solution, 12 microliters of D.W added, then 6 microliters from the extracted DNA were added, the total volume was 20 microliters in each master mix tube, maxime PCR premix kit-i- tag were used from (iNtRON Biotechnology) company was used.

3.10.3 Preparation for Restriction

Take 0.2 microliters of *Hinf*1 from (New England Bio- labs), and 1 microliter buffer with 3.8 microliters of D.W were used, all mixed together to made working enzyme solution, used the PCR products (5 microliters from the working enzyme solution added to 5 microliters of PCR products), this mixer incubated overnight, after that the results were observed on 1.5% agarose gel electrophoresis and by ultra violet light illumination. The restriction enzyme cut were G/T found in the amplified sequence, in wild type (val/val) the PCR product remained undigested, and in heterozygote both the PCR product and the mutant detected, and homozygote mutant (leu/leu) 94 bp long detected.

3.10.4 Electrophoresis:

The agarose gel prepared by 1.5 gm agarose powder that was dissolved in 100ml TE buffer, 10ml of 10X TE Buffer added to 90 ml of distilled water, to prepare 1.5 % agarose gel, the microwave was used to dissolved the agarose for about 1:30 minute, added 2 microliters of ethidium bromide to the molten agarose before it cooled down, and applied directly to the gel plate, lefted for 10-15 minutes to be

solidified. TE buffer was used as runner buffer, both samples and marker were applied into the gel, 100bp DNA marker was used, current was 100 volts for 25 minutes.

Table 3.1 Primers Sequences (Hancer *et al*., 2006).

Primer	**Primer sequences**	**Product length**
(F) Primer	5'-ACTTCAGGACCGCCTTTGGAGGC-3'	114 bp
(R) Primer	5'-GTTGACGCCCCGGGGCACCG-3'	

Table 3.2 Preparation of the PCR pre-mix.

PCR Reaction Mixture	**The Pre- Mix Add**
Template DNA	6 microliters
(F) Primer	1 microliter
(R) Primer	1 microliter
D.W	12 microliters

Table 3.3 PCR Protocol (Hancer *et al*., 2006)

PCR Cycle	**Temperature**	**Time**	**Cycles**
Initial Denaturation	95 °C	5 min	1
Denaturation	95 °C	1 min	40
Annealing	55 °C	1 min	40
Extension	72 °C	1 min	40
Final Extension	72 °C	5 min	1

Chapter Four

Results

Table 4.1 Mean age Distribution in males and female

Gender	Number	Age (Mean)	SD	P. value
Male	42	36.61	12.649	0.553
Female	38	32.39	11.274	

Table 4.2 Demographic characteristics of DVT patients and control group.

Variable	Cases Male/Female	Controls Male/Female	P. value
Age (Mean)	46.08 / 36.57	31.03 / 23.0	0.073
Gender	12 / 28	30 / 10	0.000

Table 4.3 Frequencies of Val34Leu Allele in Cases and Controls.

Val34Leu Allele	**Cases**	**controls**	**Total**	**P. value**
Wild type (Val/Val)	36 (45 %)	40 (50 %)	76 (95 %)	0.122
Heterozygous (Val/ Leu)	3 (3.7 %)	0 (0 %)	3 (3.7 %)	
Homozygous (Leu/Leu)	1 (1.2 %)	0 (0 %)	1 (1.2 %)	

P. value= 0.122> 0.05 considered as insignificant association.

Table 4.4 Effect of risk factors of DVT in cases group (males and females).

Risk Factor	**Males**	**Females**	**Total**	**P. value**
Obesity	2 (5 %)	4 (10 %)	6 (15 %)	0.328
Smoking	4 (10 %)	0 (0 %)	4 (10 %)	0.051
Hypertension	0 (0%)	1 (2.5%)	1 (2.5 %)	0.290
Recurrence	2 (5 %)	2 (5%)	4 (10 %)	0.918
Pregnancy	--	7 (17.5 %)	7 (17.5%)	--
Contraceptives	--	17 (42.5 %)	17 (42.5 %)	--
Surgery	4 (10 %)	11 (27.5%)	15 (37.5 %)	0.026

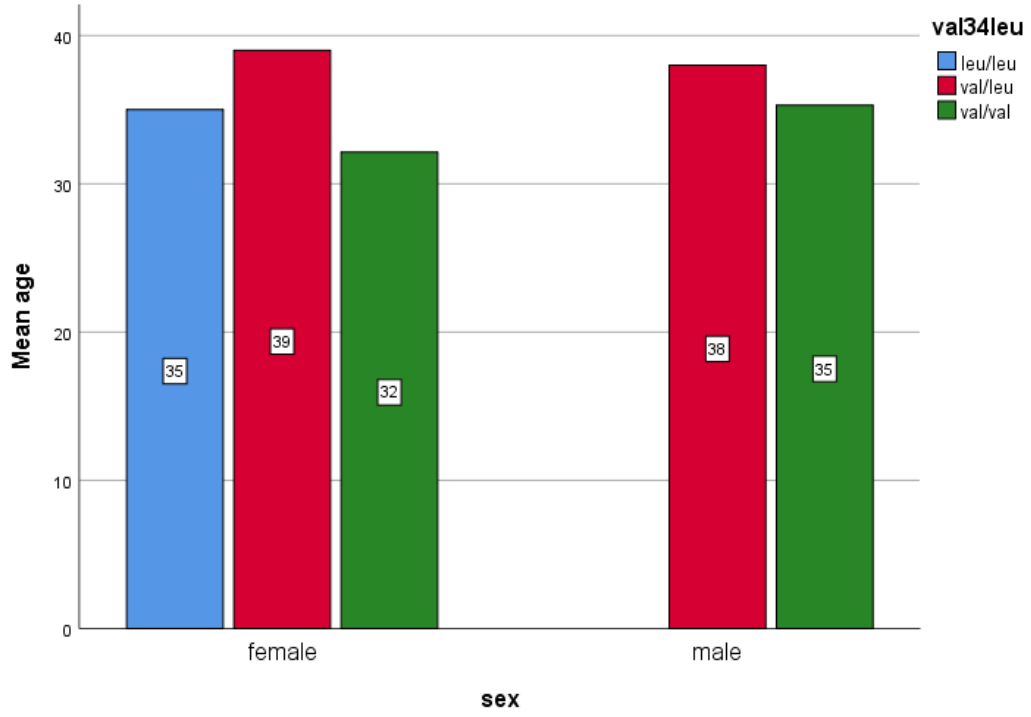

Figure 4.1 Mean Age Distribution of Males and Females in the Cases and Controls

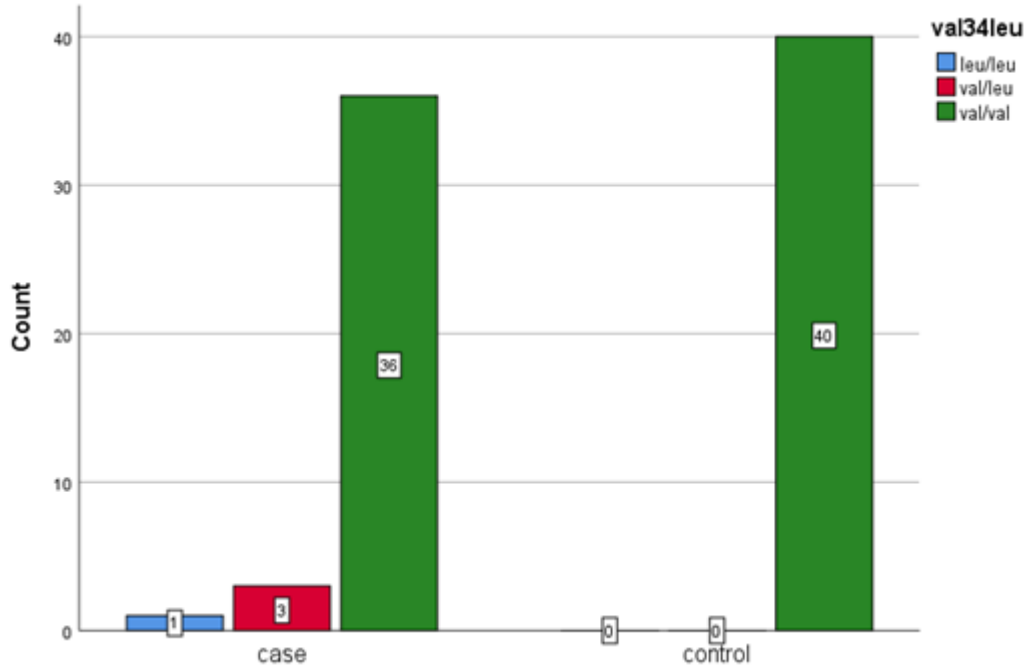

Figure 4.2 Frequencies of val34leu allele in the cases and controls.

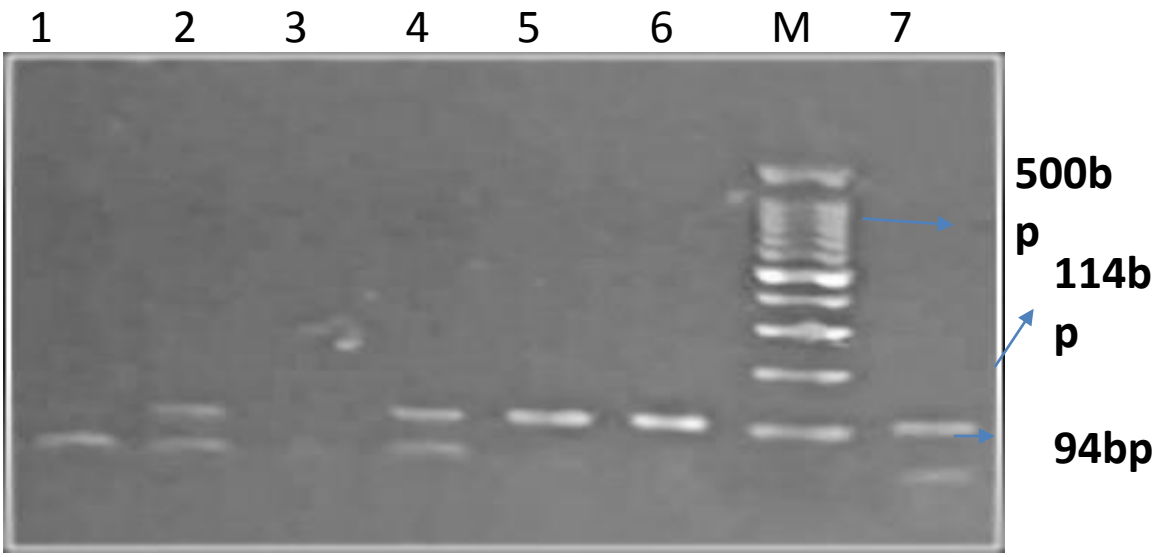

Figure 4.3 PCR product after digestion with Hinf1 the results analyzed by 1.5% gel agarose electrophoresis. Lane M: DNA Marker 100b, Lanes 2&4 and 7: Heterozygous genotype (val/leu), Lane 1: Homozygous genotype (leu/leu) 94 bp long, Lanes 5, 6: wildtype genotype (val/val) 114 bp long, Lane 3: No Band detected.

Chapter Five

Discussion, conclusions and Recommendations

5.1 Discussion

This study conducted on DVT patients to detect the presence of factor XIII-A val34leu polymorphism were 4 mutation detected, 3 were heterozygous (V/L) and one was homozygous (L/L), were no mutation had been detected in the control group, in cases group the homozygote wild type (V/V) 95 %, and heterozygote (V/L) 3.7%, homozygote mutant (L/L)1.2%, while in the control group only wild type variant were detected (100 %). Statistically no significant difference was found of FXIII-A variant when compared between patients and control group (p=0.122). No statistically significant difference found according to the age of the cases and control group (p= 0.073), DVT risk factors percentages were determined and P-values showed no significant association. The results of this study showed that FXIII-A val34leu has no association with the Sudanese DVT patients.

The studies which investigated the protective effect of FXIII-A val34leu variant and had a contradictory results and their finding agreed with this study results, Yilidirim et al. (2107) found no statistically significant differences between the study patients and control group in term of factor XIII-A gene polymorphism (p= 0.787), also no significant differences found when compared the ages of patients and control group (p=0.071), the patients were categorized as arterial and venous and compared with control group in terms of gene polymorphism but no statistically significant difference was found (p= 0.624), no results obtained as to whether this variant increases the predisposition to thrombosis or plays a protective

role in the patients with thrombosis, concluded that no correlation between FXIII-A val34leu polymorphism and venous thrombosis in the South-Est of turkey.

In a case- control study conducted on Caucasian Canadian population, FXIII-A leu/leu allele genotype was present in 4.9% of cases and 6.5% of controls, their results did not support an independent association of FXIII-A val34leu polymorphism with idiopathic venous thromboembolism (Wells *et al*., 2006b)

Cushman et al.(2007) studied the effect of FXIII-A T allele in venous thrombosis patients and compared to age, sex and race, but found that FXIII-A T allele was not clearly associated with reduced risk factor of VT, and reported no reduced risk of VT with this variant (ORs 0.97 for FXIII-A GT, 0.73 for TT).

Balogh et al.(2000) found no association of val34leu polymorphism with risk of thrombosis in Hungarian population case- control study, the leu allele found in 25.4% of population, among control subjects the frequency of wild-type (54.9%), heterozygous (38.5%), and homozygous (6.6%),which they did not differ from that in the patients in whom the frequency of wild-type (52.7%),heterozygous (40.7%), and homozygous (6.6%) the study reported no protective effect of val34leu allele against venous thrombosis.

In contrast to this study finding many studies found a significant association between FXIII- A val34leu allele with cases and control group when compared, wells et al.(2006b) in meta- analysis included 12 studies about the protective effect of this variant against venous thromboembolism, found that FXIII-A val34leu variant confers a small but protective effect against VTE. Van Hylkama Vlieg et al. (2002) found that the risk of deep vein thrombosis was reduced by 10% (OR 0.9) for heterozygous carriers of FXIII-A val34leu allele and by 30% (OR 0.7) for

homozygous carriers, this finding suggested a weak protective effect, this effect increased with age and restricted to men.

5.2 Conclusions

The study concluded that FXIII-A val34leu polymorphism has no effect in the recruited DVT patients, but may be associated with other risk such as MI or CAD.

5.3 Recommendations

- Additional studies are needed to determine FXIII genetic polymorphism and measure FXIII antigen level and activity, to integrate their effect in thrombosis.
- Large sample size must be included
- Take in considering the presence of other risk factors that may confounding with val34leu polymorphism predisposing to thrombosis, MI, and CAD, which should be studied.
- Further in-vitro studies are required to evaluate the effect of FXIII-A val34leu polymorphism in venous thrombosis.

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APPENDICES

Appendix A

Name:…….. No:……

Gender:……

Age:…… ..

Mobile number:…….....

Residence:……..

Duration of the disease (DVT):…….

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

Recurrence:……..

Obesity:……..

Smoking:……..

Surgery:…….....

Hypertension:……..

Other:……

Laboratory results:

PCR :

FXIII-A val 34 leu……

Appendix B


Heal Force
1 2 3 Esc
4 5 6 Tab
7 8 9 Run
0 +/- Stop
Enter
CLASSIC
K960
F1 F2 F3 F4


CLASSIC
K960 - Thermal Controller
Model No: K960
Power Supply: AC110V/220V 50Hz/60Hz
Input Power: 605VA
Fuse: AC250V F10A
Safety Classification OF Equipment
Manufacturing Code: GM-02-1988
Manufacturing Date: 2017.07
Manufacturer: HangZhou Jingle Scientific
Instrument Co.Ltd.
Heal Force

Appendix C


PS 300 T
Biometra


T2A
HIPOT TESTED
Produced for:
Biometra GmbH
Rudolf-Wissell-Str. 30
37079 Goettingen, Germany
Model : PS300T
Serial No. : 131023097
110 - 240V ~
50-60 HZ
2A
Made in Taiwan

Appendix D