Chapter one

Introduction and literature review

1.1 introduction

Hemostasis is one of a number of protective processes that have evolved in order to maintain stable physiology. It interacts with other body defense mechanisms, such as the immune system and the inflammatory response (Gomez and Mcvey, 2016).

The maintenance of circulatory hemostasis is achieved through the process of balancing bleeding (hemorrhage) and clotting (thrombosis). Hemostasis, the arresting of bleeding, depends on several components. The four major components are the vascular system, platelets (thrombocytes), blood coagulation factors, and fibrinolysis and ultimate tissue repair (Turgeon, 2012), the coagulation cascade consists of a complex network of reactions that are essential for the conversion of zymogens into enzymes and of inactive procofactors into cofactors. Most of these reactions take place on a membrane surface, which restricts coagulation to the site of injury. Upon initiation, these reactions serve to produce the fibrin that is necessary for the formation of a stable hemostatic plug (Bos, *et al*. 2016). Deficiencies of coagulation factors can be inherited or acquired, acquired deficiencies of plasma coagulation factors are more frequent than congenital disorders (Arruda and High, 2013) and there are several bleeding disorders characterized by the inherited deficiency of more than one plasma coagulation factor (Arruda and High, 2013).

Hemostasis and thrombosis primarily involve the interplay among three factors: the vessel wall, coagulation proteins, and platelets. Many prevalent acute vascular diseases are due to thrombus formation within a vessel, including myocardial infarction, thrombosis cerebrovascular events, and venous thrombosis (Freedman and Loscazlo, 2013).

Thrombosis is more likely to happen in conditions of increased numbers of platelets and high levels of fibrinogen, both high numbers of platelets and raised fibrinogen are present in smoking, hypertension, diabetes and hypercholesterolaemia. These are the risk factors for arterial thrombosis and atherosclerosis. Obesity is an important modifiable risk factor for VTE. Clots in the veins (VTE) are somewhat different to those in arteries; there are more risk factors but the clots are generally in only two places: the legs (deep vein thrombosis [DVT]) and the vessels of the lung (pulmonary embolus [PE]) (Blaan, 2009).

Thrombophilia is an inherited (genetic) or acquired tendency to form blood clots, the two most common inherited thrombophilias are the factor V Leiden mutation and the prothrombin G20210A Gene mutation. People can have one abnormal gene (referred to as heterozygous state or carrier state) or two abnormal genes (referred to as homozygous state). Less common inherited thrombophilias include deficiencies of the blood clotting proteins called protein C, protein S and anti-thrombin. The most common acquired thrombophilia is antiphospholipid antibody syndrome (APS) (Lim and Moll, 2015).

1.2 Literature review

1.2.1 Hemostasis

A dynamic process in which the platelet and the blood vessel wall play key roles. Platelets become activated upon adhesion to von Willebrand factor (vWF) and collagen in the exposed sub-endothelium after injury. The activated platelet surface provides the major physiologic site for coagulation factor activation, which results in further platelet activation and fibrin formation. Genetic and acquired influences on the platelet and vessel wall, as well as on the coagulation and fibrinolytic systems, determine whether normal hemostasis or bleeding or clotting symptoms will result (Konkle, 2013)

The hemostatic system consists of blood vessels, platelets, and the plasma coagulation system including the fibrinolytic factors and their inhibitors.

1.2.1.1 Platelet

Role in hemostasis. During normal circulation, platelets circulate in a resting state blood, platelets are small, a nucleate cellular fragments that play an essential as small discs. However, when challenged by vascular injury, platelets are rapidly activated and aggregate with each other to form a plug on the vessel wall that prevents vascular leakage (Joseph and Italiano, 2008).

1.2.1.2 Blood vessel

Blood vessels, especially their endothelial lining, play a critical role in the maintenance of vascular fluidity, arrest of hemorrhage (hemostasis), prevention of occlusive vascular phenomena (thrombosis), and regulation of inflammatory cell processes (Hajjar, *et al*. 2016)

1.2.1.3 Coagulation factors

Also called procoagulants, all are glycoproteins synthesized in the liver, some of them are enzymes that circulate in an inactive form called zymogens, and others are cofactors that bind, stabilize, and enhance the activity of their respective enzymes. During clotting, the procoagulants become activated and produce a

localized thrombus. In other side there are coagulation inhibitors that act as controls to regulate the coagulation process. (Fritsma and Fritsma, 2016)

1.2.1.4 Coagulation inhibitors

Human plasma contains a number of antiproteases that inhibit the activity of most of the activated coagulation factors and fibrinolytic enzymes. These inhibitors include antithrombin (AT), protein C and S, TFPI, and PAI, among others. All belong to the serine protease inhibitors. Their task is to limit thrombosis on the one side and fibrinolysis on the other side. A defect or decrease of activity of these inhibitors can thus lead to thrombosis or hyper fibrinolysis (Hiller, 2007).

1.2.2 Coagulation cascade

Coagulation is initiated after vascular injury by the interaction of the membrane bound tissue factor (TF), exposed and activated by vascular injury, with plasma factor VII. The factor VIIa and TF complex the extrinsic Xase activates both factor IX and factor X. The factor Xa, in the absence of its cofactor, forms small amounts of thrombin from prothrombin. This is insufficient to initiate significant fibrin polymerization. Amplification is needed. The extrinsic pathway is rapidly inactivated by tissue factor pathway inhibitor (TFPI) (Hoffbrand and Moss, 2016).

Thrombin generation is now dependent on the traditional intrinsic pathway. In this factor VIII and V are converted into VIIIa and Va by the small amounts of thrombin generated during initiation. In this amplification phase the intrinsic Xase, formed by IXa and VIIIa on phospholipid surface in the presence of Ca2+, activates sufficient Xa, which then, in combination with Va, PL and Ca2+, forms the prothrombinase complex and results in the explosive generation of thrombin which acts on fibrinogen to form the fibrin clot (Hoffbrand and Moss, 2016).

Thrombin hydrolyses fibrinogen, releasing fibrinopeptides A and B to form fibrin monomers. Fibrin monomers link spontaneously by hydrogen bonds to form a loose insoluble fibrin polymer. Factor XIII is also activated by thrombin and stabilizes the fibrin polymers with the formation of covalent bond cross-links.

Fibrinogen consists of two identical subunits, each containing three dissimilar polypeptide chains (α, β and γ) which are linkedby disulphide bonds. After cleavage by thrombin of small fibrinopeptides A and B from the α and β chains, fibrin monomer consists of three paired α, β and γ chains which rapidly polymerise (Hoffbrand and Moss, 2016).

Fibrinolysis the final step in the regulation of fibrin deposition is the prevention and/or rapid removal of insoluble fibrin by the fibrinolytic system. Once sufficient fibrin is generated, it binds tissue plasminogen activator (tPA), leading to the increased activation of plasminogen. This results in the formation of plasmin at the site of the fibrin clot, which breaks down fibrin into soluble fibrin degradation products. The fibrinolytic system is also subject to inhibition through the action of inhibitors of tPA and plasmin, namely plasminogen activator inhibitor (PAI)-1 and α2-antiplasmin, respectively (Gomez and McVey, 2016).

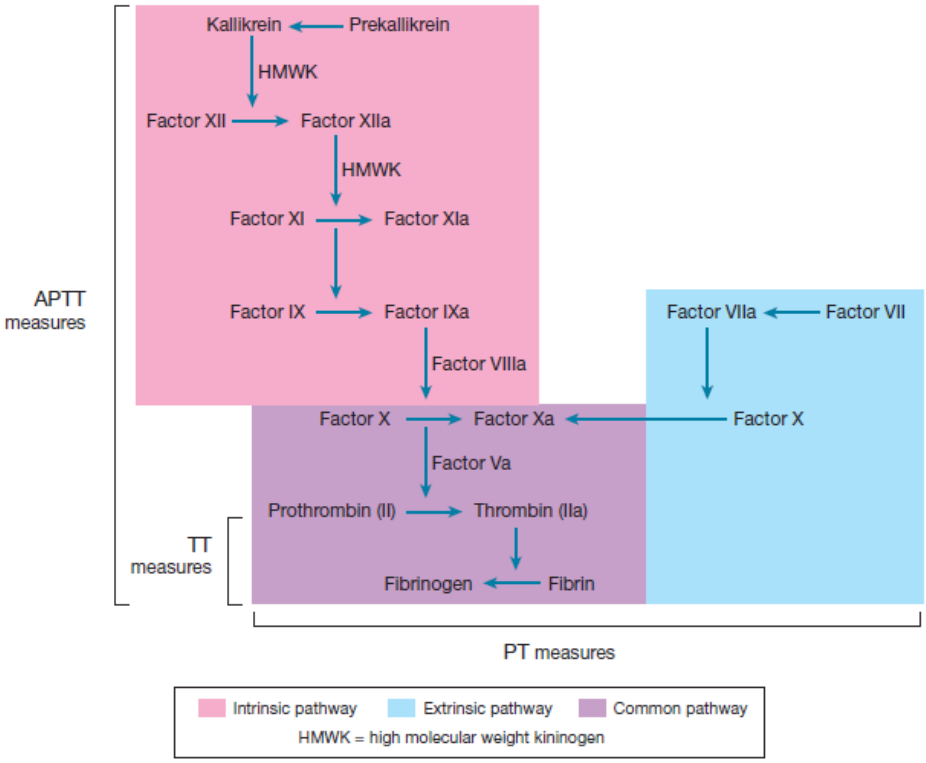


Figure 1: represents a sketch of the coagulation cascade (Hoffbrand and Moss, 2016).

1.2.3 Thrombosis

Thrombosis is a multifaceted disorder resulting from abnormalities in blood flow, such as stasis, and abnormalities in the coagulation system, platelet function, and the blood vessel wall. Thrombosis is the inappropriate formation of platelet or fibrin clots that obstruct blood vessels. These obstructions cause ischemia and necrosis (Fritsma, 2016).

Thrombophilia (called hypercoagulability) is defined as the predisposition to thrombosis secondary to a congenital or acquired disorder, it can be arterial or venous thrombosis (Fritsma, 2016).

1.2.3.1 Venous thrombosis

The primary forms of venous thrombosis are deep-vein thrombosis (DVT) in the extremities and the subsequent embolization to the lungs (pulmonary embolism), referred together as venous thromboembolic disease. Venous thrombosis occurs due to heritable causes and acquired causes.

1.2.3.1.1 Inherited thrombophilia

The most common significant inherited thrombophilias include heterozygosity for the Factor V Leiden (FVL) mutation heterozygosity, and prothrombin G20210A gene mutations. Rarer causes of inherited thrombophilias include: Antithrombin (AT) deficiency, Protein S deficiency, Protein C deficiency (Paidas, *et al.* 2011).

1.2.3.1.1.1 Antithrombin Deficiency

Antithrombin is the most important inhibitor of activated coagulation factors. A deficiency of AT is responsible for ~1 to 2% of cases of thrombosis in patients, with an apparent inherited thrombophilia in the United States. Patients with AT deficiency usually suffer from deep venous thrombosis in the lower extremities (Kern, 2002)

1.2.3.1.1.2 Protein C and protein S deficiency

Protein C is a vitamin K dependent anticoagulant protein that once activated by thrombin, will inactivate factors Va and VIIIa, there by inhibiting the generation

of thrombin. Activated protein C stimulates the release of t-PA, it is produced in the liver and is the dominant endogenous anticoagulant with an eight-hour half-life (Sirlak and Inan, 2012).

Protein S is also a vitamin K dependent anticoagulant protein that is a cofactor to activated protein C. The actions of protein S are regulated by complement C4b binding protein and only the free form of protein S serves as an activated protein C cofactor. The prevalence of protein S deficiency is about 1:500 with an autosomal dominant inheritance (Sirlak and Inan, 2012).

1.2.3.1.1.3 Factor V Leiden

Factor V Leiden is a common genetic prothrombotic defect. Among patients with venous thrombosis it is found in 20% and in around 50% of patients with familial thrombophilia. Factor V Leiden was identified after the discovery of resistance to activated protein C (APC resistance) as a major risk factor for venous thrombosis. Factor V Leiden is the result of the mutation at the location coding for one of the cleavage sites in factor V, where APC inactivates factor Va. Inactivation of the mutant procoagulant factor V occurs less efficiently and the resulting 'factor Va persistence' leads to an increased risk of thrombosis (Blann, 2009).

1.2.3.1.1.4 Mild Hyperhomocysteinemia

Homocysteine is an intermediate in the metabolism of the amino acids methionine and cysteine and participates in several metabolic pathways. Some of the enzymes involved in homocysteine metabolism are dependent on vitamin B6, folic acid, and vitamin B12, and deficiencies lead to hyperhomocysteinemia. A polymorphism in the enzyme methylenetetrahydrofolate reductase (MTHFR), c.C677T, leads to an alanine to valine substitution at position 222 resulting in a variant enzyme with reduced activity and increased thermolability, this polymorphism leads increased homocysteine levels (Middeldorp and Coppens, 2016).

1.2.3.1.2 Acquired Thrombophilia

The main acquired thrombophilic disorders are antiphospholipid antibodies and/or lupus anticoagulant (LA), malignancy, nephrotic syndrome, myeloproliferative disorders, paroxysmal nocturnal hemoglobinuria (PNH) and induction of cancer chemotherapy (Hillar, 2007)

1.2.3.1.3 Deep vein thrombosis (DVT)

A clinical disorders that characterized by clot formation in the iliac, or femoral veins of the calves and upper legs. Large occlusive thrombi also may form, although less often, in the veins of the upper extremities, liver, spleen, intestines, brain, and kidneys (Fritsma, 2016). The mean incidence of first DVT in the general population is 5 per 10,000 person-years. The incidence is similar in males and females and increases dramatically with age from 2 to 3 per 10,000 person-years at 30–49 years of age to 20 at 70–79 years of age (Freedman and Loscalzo, 2013). The prevalence of DVT in Africa varies between 2.4% and 9.6% in patients after surgery, and between 380 and 448 per 100 000 births per year in pregnant and postpartum women (Danwang, *et al*. 2017).

1.2.3.1.3.1 Predisposing factors

Immobility/bed rest, post-operative, pregnancy and post-partum, oral contraceptives, cardiac failure and disseminated cancer

1.2.3.1.3.1.1 Immobility/bed rest

Immobility increases the risk of thrombosis, due to stasis of blood flow in the venous system. Relevant settings of immobility include bed rest, plaster casts on the legs and paresis of the legs due to neurological conditions. Minor forms of immobility, such as after minor surgery or injury, have also been linked to thrombosis risk **(**Cushman, 2007).

1.2.3.1.3.1.2 Post-operative

A high risk of venous thrombosis is brought about by surgery, where for some interventions over 50% of the patients experience thrombosis in the absence of antithrombotic prophylaxis. The risk is increased most in large surgical

procedures, which may be related both to the size of the surgical wound and to the duration of the intervention and the related immobilization, but in orthopedic surgery even minor interventions, such as arthroscopy, have a sizeable effect on the risk of venous thrombosis (Rosendaal, 2009)

1.2.3.1.3.1.3 Pregnancy

Relationship between hereditary thrombophilia and pregnancy complications, including recurrent miscarriage, late pregnancy loss, preeclampsia, intrauterine growth restriction, and placental abruption, is unlikely that hypercoagulability with thrombosis of placental vasculature is the pathophysiologic substrate for an association with thrombophilia (Middeldorp and Coppens, 2016).

1.2.3.1.3.1.4 Oral Contraceptives

Women taking oral contraceptives appear to have ~3- to 4-fold increased risk of thrombosis (primarily deep venous thrombosis in the lower extremities) compared to women not taking oral contraceptives. The risk is lower in young women with no other risk factors for thrombosis, higher in older women or women with other risk factors. Oral contraceptives can interact with inherited thrombophilia, notably factor V Leiden. Women who are heterozygous for the mutation and take oral contraceptives have a ~30-fold increase in risk of deep venous thrombosis compared to women without the mutation and not taking oral contraceptives. Women who are homozygous for the mutation and take oral contraceptives have a several hundred-fold increase in risk of thrombosis (Kern, 2002).

1.2.3.1.3.1.5 Cardiac failure

Heart failure contributes to abnormalities of hemostasis through low cardiac output, the dysfunctional cardiac chambers creates area of blood stasis, then the abnormalities of hemostasis accelerates the activation of the coagulation system and fibrin formation, which makes heart failure patients more vulnerable to DVT so the heart failure is considered a major risk factor for VTE because of (Sirlak and Inan, 2012).

1.2.3.1.3.1.6 Cancer

Therapeutic interventions in patients with cancer, especially surgery and chemotherapy (such as cytotoxic drugs) further increase the risk for thrombosis (Blann, 2009).

1.2.3.1.3.2 Signs and symptoms

Clinical sign and symptoms of DVT include: Calf pain, engorged veins, swelling, edema, calf cramping, low-grade fever, warmth, erythema and pain along the course of the involved vein (Quinn, 2017).

Long-term complications associated with DVT are chronic venous insufficiency secondary to venous dilation and valvular. At 5 years post DVT, symptoms may include: Night pain, pigmentation changes, pain with prolonged standing, venous ulceration, and edema (Quinn, 2017).

1.2.3.1.3.3 Diagnosis of DVT

1.2.3.1.3.3.1 D-dimer

Measurement of D dimer level has been made possible by the development of monoclonal antibodies, which bind to epitopes on D dimer fragments that are absent on fibrinogen and non-cross-linked fragments of fibrin. The detection of the resulting complexes occurs by enzyme-linked immunosorbent assay , immunofiltration, and sandwich-type or agglutination techniques. The classic microplate ELISA technique was considered the gold standard and this technique was used in early clinical studies to assess the value of D dimer for VTE diagnosis. The sensitivity and negative predictive value were high enough to use the assay as exclusion test in diagnostic strategies for deep vein thrombosis (DVT) or pulmonary embolism (Righini, *et al.* 2009).

1.2.3.1.3.3.2 Well's Score for DVT

Based on clinical signs and symptoms can be used to screen for DVT in patients with 28 prolonged hospital admission. Patients with a score of ≥ 3 are classified as high risk, those with a score of 1 or 2 as moderate risk and those with a score of ≤ 0 as low risk. Well's score is further used to stratified patients into categories of DVT likely if the clinical score is more than 1 and DVT unlikely if the score is 1 or less (Mwandama, *et al*. 2016).

1.2.3.1.3.3.3 Doppler Ultrasound

Doppler ultrasound has very high sensitivity and specificity for diagnosing proximal DVT in symptomatic patients and >90 % for the calf veins so a negative whole leg Doppler rules out DVT (DeLoughery, 2015).

1.2.3.1.3.3.4 Contrast venography

Iodinated contrast medium is injected into a vein peripheral to the suspected DVT. This permits direct demonstration by X-ray of the site, size and extent of the thrombus. However, it is a painful invasive technique, with a risk of contrast reaction and procedure-induced DVT (Hoffbrand and Moss, 2016).

1.2.3.1.3.3.5 Magnetic resonance imaging (MRI)

MRI used to image DVT, particularly in the pelvis (100% sensitivity, 95% specificity), where it is more sensitive than venography. MRI is equally sensitive for detection of DVT in the thigh but inferior in the calf (87% sensitivity and 97% specificity) (Quinn, 2017).

1.2.3.1.3.4 Treatment of DVT

The continuous intravenous infusion of heparin has been the preferred mode of therapy in the treatment of DVT or PE. In adult patients with an average weight of 70 kg, doses of 30,000–40,000 U over 24 h (1200–2000 U/ h with a loading dose of 5000 U) are usually required. Subcutaneous heparin (e.g., 15,000 U every 12 h) can be used as an alternative to the intravenous route. The therapy must be monitored by maintaining the PTT between 1.8 and 2.5 times the normal time. Warfarin therapy can be started on day 2 after the initiation of heparin therapy.

Thus on days 5–7, one can expect that warfarin is in the therapeutic range (international normalized ratio [INR] > 2) and heparin can be discontinued. Oral anticoagulant therapy should be continued for at least three months. And longer than three months when the thrombophilic state persists (Hiller, 2007).

1.2.4 Prothrombin gene

In 1996, a mutation in the prothrombin gene was described that appears to be associated with thrombophilia**.** The gene for prothrombin is located on chromosome 2 and contains 14 exons spanning 21kb. Prothrombin is a vitamin-K dependent protein synthesized in the liver (579 amino acids, 72kDa, and plasma concentration 2μmol/L). Prothrombin is the zymogen precursor of thrombin. A single nucleotide change, guanine to adenine at position 20210 in the 3′ untranslated region of the prothrombin gene (F2G20210A), is associated with elevated plasma prothrombin levels and an increased risk of venous thrombosis (Balgin and Keeling, 2016).

1.2.4.1 Prevalence

The prothrombin gene mutation is mainly found in populations of Caucasian origin. In southern Europe the prevalence is 2–4%, nearly as twice as high as that in northern Europe. Founder effects are the likely explanation for the differences in the distribution of the prothrombin 20210 G→A mutation. The mutation is found in 6–16% of patients with unselected deep venous thrombosis and carriers have an approximately 3-4 fold increased risk of thrombosis. (Dahlbäck and Hillarp. 2005).

1.2.4.2 Function

Thrombin is usually produced in the liver in an inactive form called prothrombin, which circulates in the blood until being activated in case of injury. Potrhombin gets activated into thrombin by another clotting factor called activated factor X. The main function of thrombin is to convert fibrinogen (clotting factor I) into a fibrin clot that blocks the injured blood vessel (Jadaon, 2011).

1.2.4.3 Pathophysiology

The Factor II (Prothrombin) mutation (20210G>A) is the second most common genetic defect associated with inherited venous thrombosis. The genetic variation (Guanine to Adenine transition at position 20210) in the 3'-untranslated region of the prothrombin gene had been associated with elevated blood levels of prothrombin which leads to a state of hypercoagulability, and increased incidence of venous thrombosis. Some individuals who have Factor V Leiden mutation may also have the Prothrombin 20210 G>A mutation (Yousif, et *al*. 2017).

This Prothrombin G20210A mutation is present outside the coding region for prothrombin, and hence it does not affect the actual structure of the prothrombin molecule and it does not affect its function as a strong clotting factor when activated into thrombin. However, Prothrombin G20210A mutation was found to cause elevated levels of blood prothrombin (by one-third above normal; 133%), Which is more than 15% needed to develop VTE. Also, it has been proven that Prothrombin G20210A mutation leads to increased mRNA and protein expression for prothrombin (Jadaon, 2011).

1.3 Rationale

Prothrombin G20210A gene mutations has been reported in different population worldwide as one of the causes of deep vein thrombosis. However, in Sudan, there is no much researches regarding this issue.

In this study we are attempting to describe this genetic variant, to find the frequency of this gene mutation among Sudanese with deep vein thrombosis.

1.4 Objectives

1.4.1 General objective

To detect the prothrombin gene mutation and measure the coagulation profile among patients with deep vein thrombosis.

1.4.2 Specific objectives

1. To detect the prothrombin gene (G20210A) mutation in patients and control.
2. To calculate the prevalence of the prothrombin gene (G20210A) mutation in case and control.
3. To estimate PT, APTT and platelets in case and control.
4. To estimate frequency of risk factors (surgery, pregnancy, contraceptive pills, diabetes mellitus and hypertension).

Chapter two

Materials and methods

2.1. Study design

This is the case control study.

2.2 Study area

The study was conducted in Omdurman teaching hospital, Khartoum state, Sudan.

2.3 Sample size

Forty patients diagnosed with deep venous thrombosis (DVT) and forty healthy volunteers were included as control group. Both case and control group were unrelated and randomly selected.

2.4 Inclusion criteria

Confirmed DVT patients were included. The diagnosis done by clinical assessment of risk factors and physical findings followed by Doppler ultrasound confirmation

2.5 Exclusion criteria

Patients of other thrombosis (e.g. fatty embolism) than deep vein thrombosis were excluded.

2.6 Sampling

Blood samples were collected under sterile condition and the vein puncture was well dressed. Four milliliter of blood was drawn from each participant. 2.2ml of whole blood poured in ethylenediamine tetra acetic acid (EDTA) container which used for DNA extraction. 1.8ml was collected in sodium citrate tubes for clot based methods. Plasma and EDTA blood were stored at -70 ℃ tell the time of examinations.

2.7 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.

2.8 Data analysis and presentation

Data were analyzed using SPSS software, Version 16. Chi-square test was used and p value was calculated. Data are reported as mean ±SD for continuous variables or as percentages. Statistical significance was set at < 0.05.

2.9 Methodology

2.9.1 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. (Chirgwin, *et al,* 1979)

2.9.2 Molecular analysis

2.9.2.1 Detection of Prothrombin G20210A gene mutation

2.9.2.2 Primers design and preparation

The primers used were previously published and used by Farah P *et al*, (2012). The specificity was checked in the NCB 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. (Parveen, *et al*.2012).

2.9.2.3 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 **(**parveen, *et al*.2012**)** by using CLASSIC K960 China Thermocycle Device.

2.9.2.4 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). (parveen, *et al*.2012).

Table 2.1 Details of primers (forward and reverse) restriction enzymes and product sizes (Both before and after digestion)

Genotypic Locus	Restriction enzymes	Primers	DNA products size before digestion	DNA products size after digestion
20210	Hind III	F5′ -TCTAGAAACAGTTGCCTGGC-3′ R5′-ATAGCA CTGGGAGATGAAGC-3′	345	322 ,23

Chapter three

The Results

3.1 Demographic data

The data were collected for 80, 40 cases (20 male 25% and 20 female 25%) with documented VTE in this study and 40 control (22 male 27.5% and 18 female 22.5%) as the showed in graph(3.1). The mean age of case study group was 38.2, age range of 20-77, mean age of control group was 30.8, age range of 20-52. The variable frequencies of case group under study included: post-operative disease (POD) 30%, hypertension (HTN) 2.5%, pregnancy 35%, diabetes mellitus (DM) 7.5%, contraceptive pills 35% as showed in table (3.1).

Out of 40 patients confirmed to have DVT, 2 patients (5%) were found to harbor to mutant prothrombin gene (G 21210 A) and 38 patients (95%) had the wild gene and hence were negative for the prothrombin gene (G21210A) mutation, while there were no positivity for mutation among control group .this result showed no statistical differences in present of prothrombin mutation between case and control groups (p- value 0.152), as showed in table (3.2).

The screening tests for case and control (mean $\pm$SD) and p-value showed that platelets count in case (254.5±55.9) versus (330.8±77.3) in control and p-vlue is 0.000, PT in case (13.4±2.4) versus (12.5±1.3) in control and p-value is 0.037, APTT in case (38.1±10.1) versus (30.8±2.9) in control and p-value is 0.000.

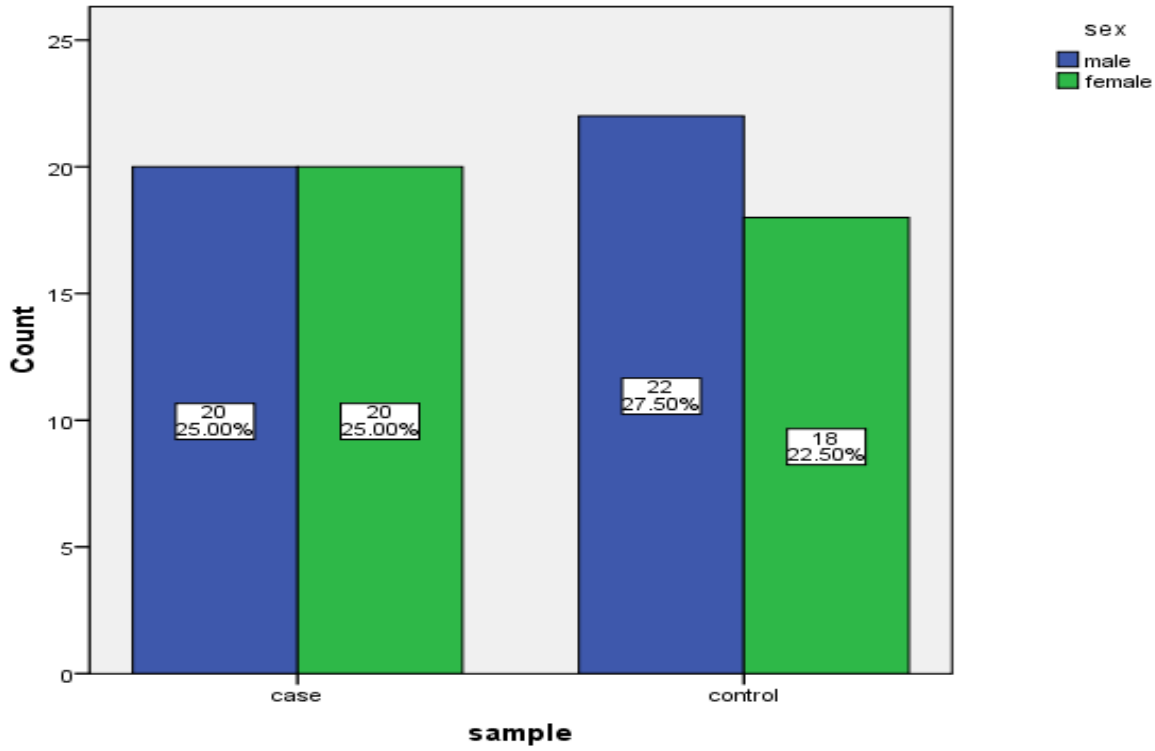


Figure (3.1): Show the frequency of sex among case and control groups:

Table (3.1): Show frequency of risk factors among case group

Risk factors	Yes N (%)	No N (%)	Total
Surgery	12 (30%)	28 (70%)	40
Pregnancy	14 (35%)	26 (65%)	40
Contraceptive	14 (35%)	26 (65%)	40
HTN	1 (2.5%)	39 (97.5%)	40
DM	3 (7.5%)	37 (92.5%)	40

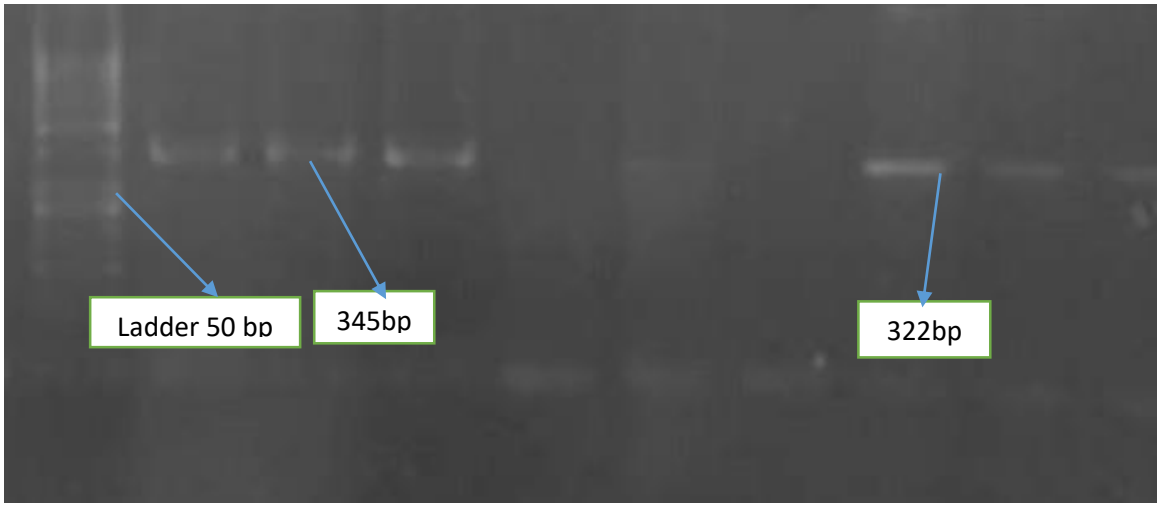
Table (3.2): Show frequency and association of prothrombin among study population

prothrombin	Sample (count, percent)		Total	P-value
	case	control		
yes	2(5%)	0	2	0.152
no	38(95%)	40(100)	78	
Total	40	40	80	

Table (3.3): Show the (mean±SD) and p-value of platelet, PT and APTT:

Tests	Case (mean±SD)	Control (mean±SD)	p-value
Platelets count	254.5±55.9	330.8±77.3	0.037
Prothrombin time PT	13.4±2.4	12.5±1.3	0.000
APTT	38.1±10.1	30.8±2.9	0.000

Figure (3.2) show PCR result:



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

Chapter four

Discussion, conclusion and recommendation

4.1Discussion

Prothrpmbin (G20210A) is the second most common thrombophilic polymorphism in Caucasians (Yousif, *et al.* 2017). On the other hand, Prothrombin G20210A mutation was found to be very rare or even absent in Asian and African populations,and in native populations of America (Amerindians) and Australia (Cushman, 2007).

The aim of the study to detection of Prothrombin gene mutation in the Sudanese patients with venous thromboembolism

In this study the mean age of case group was 38.2 and the mean age of control group was 30.8 that is mean the age consider as a risk factor and this agree with Engbers, *et al.* (2010), who founded that the incidence of venous thrombosis (VT) increases sharply with age: it is very rare in young individuals (< 1 per 10 000 per year) but increases to 1% per year in the elderly.

About the risk factors of population under study (surgery, pregnancy, contraceptive pills, diabetes mellitus and hypertension). The pregnancy was 35%, Women are up to 5 times more likely to develop DVT during pregnancy than when not pregnant (Devis and Knuttinen, 2017). Also the women who used contraceptive pills was 35%, oral contraceptive use is associated with a 4-fold increased risk of venous thrombosis, independent of the presence of other genetic or acquired risk factors. It has been reported that women with prothrombin mutation who use oral contraceptives have an increased risk of deep vein thrombosis (30-fold) in comparison with non-carriers and nonusers (Martinelli, *et al*. 1999). Then the post-operative disease was 30%, Surgery leads to a 6-fold increased risk of VT and approximately 3% of individuals undergoing lower limb arthroplasty (whose average age is over 65) develop VT even when they receive prophylactic medication (Engbers, *et al.* 2010). Also the patients with diabetes mellitus in case group was 7.5%, the other study reported that individuals with

diabetes mellitus have an almost 50% increased risk of VT compared with individuals without diabetes (Engbers, *et al.* 2010). And the hypertensive patients in case group was 2.5%, the previous study reported the patients with hypertension have been found with 2-fold increased likelihood of developing DVT the hypertension may promote the formation of DVT after orthopedic surgery (Huang, *et al.* 2016).

About the numeric data, the mean and SD and p-value of PT and APTT in case group was (13.4±2.4) and (38.1±10.1) respectively, and in control group was (12.5±1.3) and (30.8±2.9) respectively, the p-value of PT in case and control was 0.037 and p-value of APTT in case and control was 0.000, this significant differences in both results and increasing of the means of case group due to the patients on this study on heparin and warfarin treatment and this anticoagulants prolonged the PT and APTT. Also the mean and SD of platelets count in case group was (254.5±55.9) and in control group was (330.8±77.3) and p-value was 0.000 this significant differences due to decrease the platelet count in patients with deep vein thrombosis, a previously study reported that patients with deep vein thrombosis had a fall in platelet count (Monreal, *et al.* 1991).

In this study there was no association between prothrombin mutation and DVT in Sudanese patient (p-value 0.152) with Similar results conducted by Yousif, *et al.* (2017), who reported that prothrombin gene mutations totally absent in Sudan. Also new reports determined the prevalence of heterozygous prothrombin G20210A genotype among Saudi patients with DVT was 3 (5%) in agreement with those who suggested an insignificant association between prothrombin G20210A and DVT (Algari, *et al*. 2017). However, this result differs with the finding of Dolek, *et al*. (2007), they observed higher frequencies for FII G20210A mutations in Turkish thrombosis patients, and these discrepancies are most probably due to the small sample size in this study, non-equal distribution of patient's age, ethnic affiliation and different geographical prevalence of these mutations.

In Sudan, the prothrombin 20210G>A gene mutation are not associated with VTE in Sudanese patients.

4.2 Conclusion

The prothrombin 20210G>A gene mutation is not associated with venous thromboembolism in Sudanese patients and the means of PT and APTT in patients were found higher than the means of PT and APTT in control group due to the patients on treatment.

4.3 recommendation

1. There is no much researches on this study and another study should be done including large sample size and wide distribution in the country and more studies in mutations.
2. Further detectors of thrombophilia should be thought of to prevent this condition.
3. Further hereditary and acquired causes should be thought of to prevent this condition.

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Appendix 1: Questionnaire

Sudan University of Science and Technology

College of Graduate Studies

Research title: Prothrombin Mutation and Coagulation Profile as Indicators of Deep Vein Thrombosis among Patients with Thromboembolism

Degree: MSc hematology

Duration of research: from January to November

Name:…….. No:……

Gender:……

Age:……. ..

Residence:……..

Mobile number:…….....

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

Treatment:……

Pregnancy:……

Contraceptive pills:……..

Surgery:…….....

Diabetes……. ..

Laboratory results:

A) PCR

2. prothrombin G20210A …….

B) Coagulation profile:

1.PT…….

2.APTT……

3.platelets……

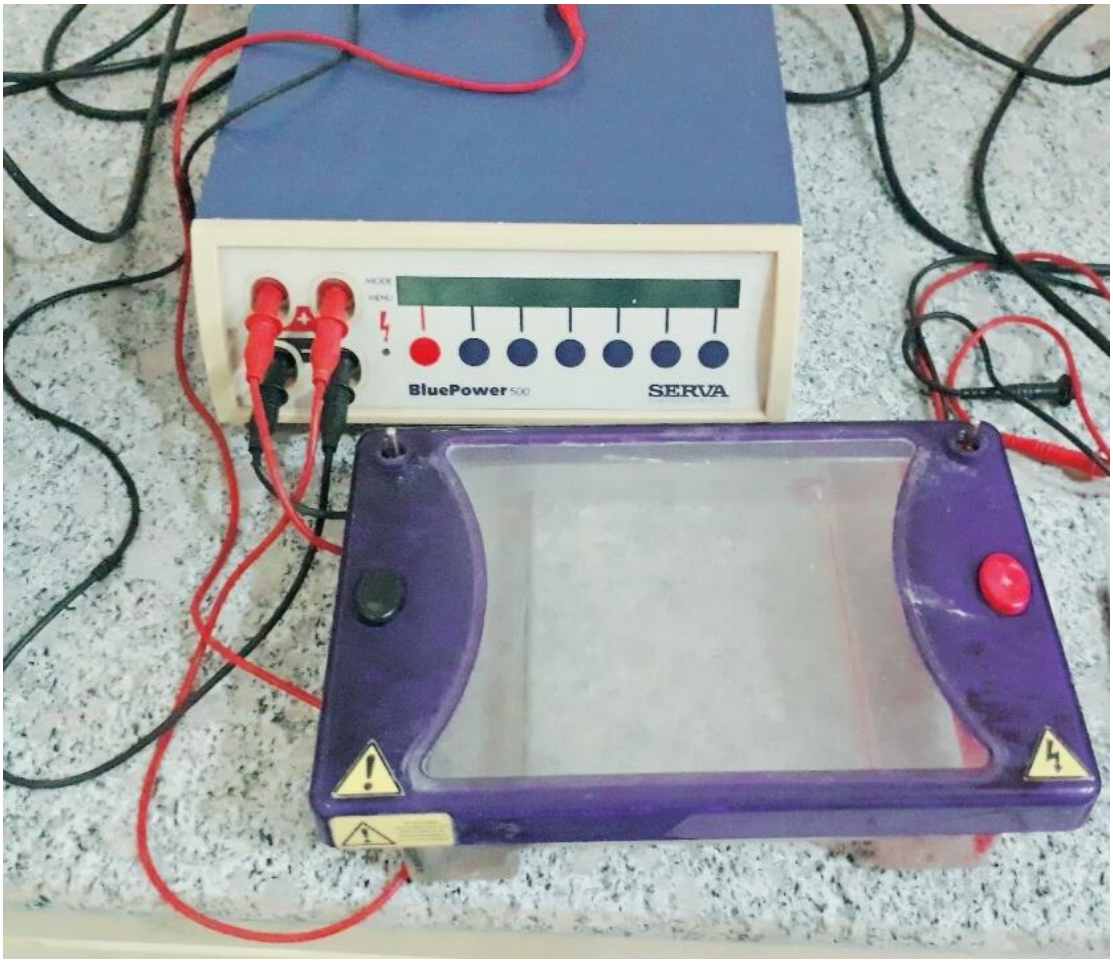
Remarks:

……
……
……
……
……

Appendix 2

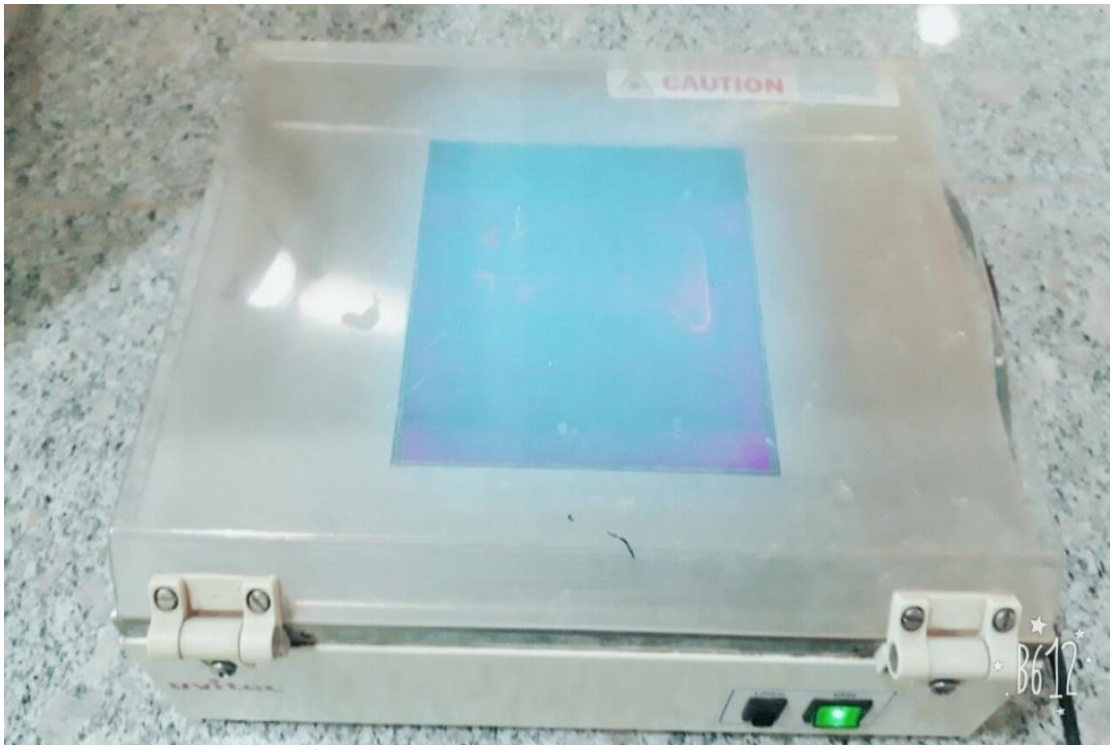
CLASSIC K960 China Thermocycle Device.

Appendix 3



Gel Electrophoresis and Power Supply Device

Appendix 4



UV Light Transilluminater Device