

# Chapter One

## 1. Introduction and Literature Review

### 1.1 Introduction

DVT refers to the formation of blood clots in 1 deep veins, usually of the lower or upper extremities, causing considerable morbidity and mortality (Beckman *et al.*, 2010, Kahn *et al.*, 2014). Hereditary factors responsible for deep vein thrombosis are factor V Leiden 1691G>A and Prothrombin 20201G>A genes mutations (Khan and Dickerman, 2006). Factor V Leiden is common among Caucasians and Middle-Easterners, and with a moderate prevalence is found in Hispanic Americans, Indians, and Africans (Bertina *et al.*, 1994, Poort *et al.*, 1996). The second most common genetic risk factor for hereditary thrombophilia is the prothrombin gene mutation, which is due to the substitution of G to A at nucleotide at position 20210 of the prothrombin gene leading to an increased prothrombin production. The defect is associated with an increased risk of venous thrombosis by three fold (EGAPP, 2011).

The genetic thrombosis risk factors include a sequence variant in the prothrombin gene (20210G>A) and factor V Leiden 1691G>A (Machac *et al.*, 2006). These single nucleotide polymorphisms can be diagnosed with restriction fragment length polymorphism (RFLP) analysis, for reason that factor V Leiden 1691G>A and prothrombin 20210>A are co-inherited more often than expected (Robetorye and Rodgers, 2001).

The major non-genetic risk factors for VTE includes: old age, obesity, history of previous thromboembolism, cancer, bed-rest for more than 5 days,

surgery, congestive heart failure, bone fracture, hormonal treatment, and pregnancy. Risk of VTE is exceeded in case of combined acquired and genetic risk factors (Anderson and Spencer, 2003).

Deep vein thrombosis remains a major health problem with not only the immediate potentially fatal risk of pulmonary embolism but also the longer term sequelae of post-thrombotic syndrome, which can lead to chronic venous insufficiency and leg ulceration (Khan and Dickerman, 2006, Biondi *et al.*, 2010). It is important to identify individuals at risk and to offer them adequate treatment and/or prophylaxis (Bertina, 1997).

## **1.2 Literature review**

### **1.2.1 Hemostasis**

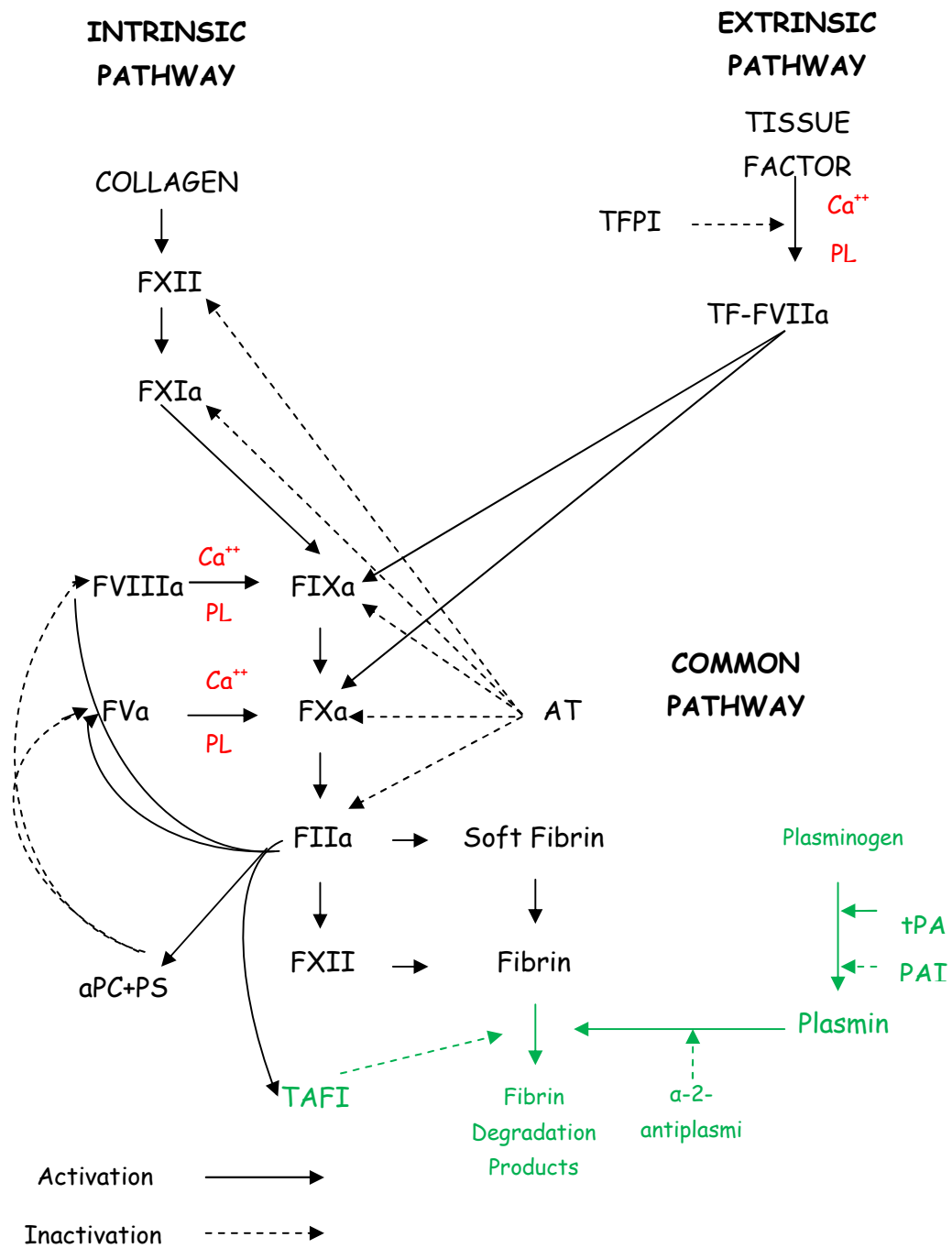
The main factors maintaining the balance between bleeding and thrombosis are the vessel wall, platelets, coagulation system, and fibrinolytic system. At the site of a vessel wall injury, platelets serve as the first hemostatic plug by adhering to exposed collagen directly and through von Willebrand factor. Aggregated and activated platelets support local coagulation by providing a negatively charged phospholipid surface for the coagulation cascade, which eventually forms a stable fibrin clot. Coagulation is regulated by natural anticoagulant mechanisms to limit the process at the site of injury. Finally, the clot is dissolved by the fibrinolytic system (Goodnight and Hathaway, 2001).

#### **1.2.1.1 Coagulation cascade**

Figure 1.1 presents a sketch of the coagulation cascade. The procoagulant coagulation cascade is composed of serine protease enzymes and their cofactors. The end point of this cascade is the formation of active thrombin. The coagulation cascade occurs on a phospholipid surface, mainly on the activated platelets or the injured endothelium, in the presence of  $Ca^{++}$ . The coagulation process begins when tissue factor (TF) is exposed to blood and binds with F VIIa, which pre-exists in trace amounts in the blood. F VIIa needs to be bound to TF to gain proteolytic activity. TF - F VIIa complex activates F IX and more efficiently F X. The first small amounts of F Xa activate F V, and together they form a prothrombinase complex to activate prothrombin to thrombin (Duckers *et al.*, 2009). After this initiation phase, the newly formed thrombin activates F V, F VIII, and F XI, thereby

accelerating its own activation and leading to a very efficient propagation phase of coagulation. F IXa, with its now activated cofactor F VIIIa (tenase complex), activates efficiently F X, and then F Xa, with its cofactor F Va (prothrombinase complex), activates prothrombin to thrombin. F XIa serves as another activator for F IX to ensure the efficiency of the thrombin formation process (Mann, 2003). Thrombin converts the soluble fibrinogen into insoluble fibrin, which forms a network in and around the platelet plug. Thrombin also activates F XIII, which cross-links fibrin molecules to form a stable clot (Goodnight and Hathaway, 2001). In addition, thrombin further activates platelets, ensuring excellent conditions for coagulation to proceed on the phospholipid surface (Mann, 2003).

Anticoagulant mechanisms regulate the coagulation cascade rigorously to limit thrombosis at the site of vessel wall trauma. Limiting factors include several phenomena: adhered, activated platelets remain at the site of injury, serine proteases involved in the process need to be proteolytically activated, and physiologic anticoagulants—tissue factor pathway inhibitor (TFPI), antithrombin, and the protein C system—control critical points of the coagulation cascade (Mann, 2003). Platelet factor 4 released from platelets increases protein C activation rate and this may also limit thrombus formation outside the site of injury (Esmon, 2003). TFPI neutralizes stoichiometrically the TF - F VII complex (Mann, 2003). Antithrombin can neutralize all the procoagulant serine proteases by binding to them (Mann, 2003), the primary targets being thrombin, F Xa, and F IXa. The protein C system regulates the coagulation process dynamically by responding to the presence of thrombin (Lippi *et al.*, 2009).



**Figure 1.1 Coagulation cascade**

### **1.2.1.2 Anticoagulation and protein C anticoagulant pathway**

When thrombin is formed, it down-regulates its own formation through the thrombin-thrombomodulin-protein C system (Mann, 2003). When thrombin binds to thrombomodulin present on the surface of the intact endothelium, it loses its procoagulant activity. Thrombomodulin-bound thrombin is not only efficiently inactivated by antithrombin and other inhibitors, but it also activates protein C to activated protein C (APC) (Esmon, 2003). Endothelial cell protein C receptor (EPCR), also present on the endothelium, presents protein C to the thrombin-thrombomodulin complex enhancing protein C activation (Esmon, 2003). APC, with its cofactor protein S, inactivates F Va and F V IIIa by cleaving certain peptide bonds in them. FVa is cleaved at least at the sites R306, R506, and R679 and F VIIIa at the sites R336 and R562 (Dahlbäck, 2008). This inactivation of central factors in the propagation phase of the coagulation cascade efficiently reduces the formation of thrombin and eventually also the formation of APC. APC is slowly inactivated by protein C inhibitor and alfa-1 antitrypsin (Dahlbäck, 2008). The thrombin-thrombomodulin complex efficiently activates also thrombin activatable fibrinolysis inhibitor (TAFI), which renders fibrin clot more resistant to lysis (Lippi *et al.*, 2009).

Disorders of this physiological anticoagulant system entail deficiencies of antithrombin (AT), protein C and S systems and they are all well-established, often congenital, causes of thrombophilia and especially commonly associated with VTE is the FV Leiden mutation, the principal cause of APC resistance (Svensson *et al.*, 1997).

### **1.2.1.3 Fibrinolysis and D-Dimer formation**

The central purpose of the coagulation process is to generate a stable fibrin plug/ thrombus which is the natural seal of a vascular injury. In the coagulation process, adequate concentrations of thrombin are eventually generated and are able to cleave fibrinogen (Shapiro, 2003). Fibrinogen is converted into fibrin by enzymatic cleavage of the fibrinopeptides A and B by thrombin, producing the soluble fibrin monomer. These monomers are then assembled with end-to-end and side-to-side association to form fibrin polymers (Shapiro, 2003).

When the aggregation phase ends, linkage of these monomers by F XIIIa results in the dimerization of adjacent D-domains and an insoluble cross-linked fibrin clot. The generation of a thrombus helps to solve an acute issue, but on the longer term, there is of course the risk of obstructing blood flow causing ischemia and necrosis in the affected areas. Therefore, once a fibrin clot has formed in vivo, it is modified by the fibrinolytic system, which constitutes the enzymatic process that leads to solubilisation of the clot by plasmin originating from tightly fibrin-bound plasminogen. Endothelial cells release tissue plasminogen activator (t-PA) into the blood stream as a result of blood flow stasis and fibrin formation. Fibrin serves as a cofactor for the activation of plasminogen by a proteolytic cleavage mediated by t-PA. Plasmin-mediated degradation of cross-linked fibrin generates fibrin degradation products of different molecular sizes, the smallest ones being dimeric fragments of the D-domain with a molecular weight of ~180kDa (Marder and Francis, 1983). These circulating degradation products can serve as diagnostic markers of thrombin and / or Factor XIIIa plus plasmin action that reflect prior clot formation and ongoing fibrinolysis (Thurlow,

1988). D dimers (DD) represent only a minor fraction of what the monoclonal antibodies, in DD assays, recognize as an antigen (Pfitzner *et al.*, 1997, Rylatt *et al.*, 1983).

Small amounts of products containing DD are detectable in the plasma of healthy individuals since 2-3% of plasma fibrinogen is physiologically converted to cross-linked fibrin and then degraded. The concentration is increased in all conditions associated with enhanced fibrin formation and subsequent degradation by plasmin. Examples are: VTE, disseminated intravascular coagulation (Whitaker *et al.*, 1980), infection/ inflammation (Roumen-Klappe *et al.*, 2002), cancer, old age (Masotti *et al.*, 2000), pregnancy (Chabloz *et al.*, 2001) and trauma (Gaffney *et al.*, 1995). The converse is also true, DD concentration decreases in response to anticoagulant therapy and the resolution of VTE symptoms. The half-life of DD is approximately 8 hours and clearance occurs mainly via the kidneys and reticulo-endothelial system (Nieuwenhuizen *et al.*, 1982, Elf *et al.*, 2009).

### **1.2.2 Factor V**

Factor V (F V), which was discovered by Paul Owren in 1943 (Stormorken, 2003), has proved to be an important regulator of the hemostatic balance with both procoagulant and anticoagulant properties. The gene of F V is on the chromosome 1 (1q23), and this single-chained glycoprotein of 2,196 amino acids is synthesized in the liver. Of the total F V, 20% is stored in platelet  $\alpha$ -granules, the rest circulates in plasma (Duckers *et al.*, 2009). The F V in platelets is of plasma origin, but it is already modified in platelets by partial proteolysis, giving it considerable F Xa-cofactor activity (Duckers *et*



*al.*, 2009). This seems to be an efficient way to ensure that this important factor is immediately present at the site of vessel wall injury and ready to function. F V is activated by F Xa or thrombin to F Va by the cleavage of three peptide bonds (Arg709, Arg1018, Arg1545) (Duckers *et al.*, 2009). The inactivation of F Va is mediated through APC, which cleaves the F Va at the sites Arg506, Arg306, and Arg679, usually in this order (Dahlbäck, 2008). The Arg506 is the preferred site for proteolysis, but protected by F Xa in prothrombinase complex when coagulation is in process. However, protein S accelerates the slower proteolysis at the site Arg306 (Rosing *et al.*, 1995) and helps APC to reach the Arg506 site (Lippi *et al.*, 2009). After cleavage at the site Arg506, F Va still has partial procoagulant activity, which is abolished when the Arg306 and Arg679 peptide bonds are cleaved (Kalafatis *et al.*, 1995). F V has procoagulant as well as anticoagulant properties. In its activated form, F Va serves as an essential cofactor for F Xa (the prothrombinase complex) in the formation of thrombin (Duckers *et al.*, 2009). On the other hand, the intact F V acts as a cofactor in the protein C system by stimulating the cofactor activity of protein S in the inactivation of F VIIIa by APC (Shen and Dahlback, 1994). This anticoagulant activity appears after the cleavage of a peptide bond at the Arg506 by APC (Dahlbäck, 2008). Mutations in the F V gene may lead to hemorrhagic and thrombotic tendencies (Dahlbäck, 2008).

### **1.2.3 Venous thromboembolism**

Venous thromboembolism includes deep vein thrombosis (DVT) and pulmonary embolism (PE) (Rosendall and Reitsma, 2009). Venous thrombosis can be seen as a classic example of complex common disease

which is caused by interaction of acquired and inherited risk factors (Reitsma and Rosendaal, 2007). Thrombosis occurs when many risk factors are simultaneously present. Each risk factor increases the thrombotic potential and eventually a trigger point for thrombosis is exceeded. The risk of thrombosis increases with age. Therefore, in young adults more risk factors are needed for thrombosis to occur than in old age. Among women of fertile-age the incidence of thrombosis is about 1:10,000 women per year (Rosendaal, 1999).

According to Virchow's triad from the 1856, the emergence of thrombosis is due to changes in the vessel wall, in blood, and in the velocity of blood flow (Dahlbäck, 2008, Lippi and Franchini, 2008). Venous and arterial thromboses only partly share the same risk factors and both also have their own risk factors (Martinelli *et al.*, 2010).

#### **1.2.4 Deep Vein Thrombosis (DVT)**

Deep vein thrombosis (DVT) and pulmonary embolism (PE) are known collectively as venous thrombo-embolism (VTE), a common and serious disorder, with genetic and acquired risk factors (Rosendall and Reitsma, 2009).

VTE often constitute an unique clinical picture in which PE follows DVT (Beckman *et al.*, 2010, Previtali *et al.*, 2011). Thrombosis can take place in any section of venous system, but commonly manifests as deep-vein thrombosis of the leg (Lewis *et al.*, 1994, Rosendaal, 1999, Kyrle and Eichinger, 2005). Although VTE is a common disease, the underlying pathogenic mechanisms are only partially known, particularly in comparison to those of atherothrombosis (Previtali *et al.*, 2011).

Worldwide known races and ethnicities are affected by VTE, as are both genders and all age groups. With many of the known risk factors (advanced age, immobility, surgery, obesity) increasing in society, it is an important and growing public health problem. Yet, until recently, this condition has received little attention from the public health community (Beckman *et al.*, 2010). In many cases, VTE is preventable; thus, the importance of research and prevention of VTE is being increasingly recognized (Ma *et al.*, 2014).

#### **1.2.4.1 Pathogenesis and classification**

Thrombus formation preferentially starts in the valve pockets of the veins of the calf and extends proximally (Kesieme *et al.*, 2011). This is especially true for those that occur following surgery (Nicolaidis *et al.*, 1971). Though most thrombi begin intraoperatively, some start a few days, weeks, or months after surgery. Lending its support to the origin of thrombus in valve pockets is a recent hypothesis of an increased expression of endothelial protein C receptor (EPCR) and thrombomodulin (TM) and a decreased expression of Von Willebrand factor (vWF) noted in valve sinus endothelium compared with vein luminal endothelium. This means an upregulation of anticoagulants (EPCR, TM) and a down regulation of procoagulant (vWF) properties of the valvular sinus endothelium (Brooks *et al.*, 2009). Thrombus is composed predominantly of fibrin and red cells (red or static thrombus). Venous thrombus must be differentiated from postmortem clot at autopsy. Postmortem clots are gelatinous and have a dark red dependent portion (formed by red cells that have settled by gravity and a yellow chicken fat supernatant resembling melted and clotted chicken fat) (Mitchell, 2005). They are usually not attached to the underlying wall. This

is in contrast to the venous thrombi which are firmer. They almost always have a point of attachment to the wall and transection reveals vague strands of pale gray fibrin (Mitchell, 2005).

DVT in the lower limb can be classified as a) proximal, when the popliteal vein or thigh veins are involved or b) distal, when the calf veins are involved. Clinically, proximal vein thrombosis is of greater importance and is associated with serious chronic diseases such as active cancer, congestive cardiac failure, respiratory insufficiency, or age above 75 years, whereas distal thrombosis is more often associated with risk factors such as recent surgery and immobilization. Fatal PE is far more likely to result from proximal DVT (Kearon, 2003).

#### **1.2.4.2 Incidence and prevalence**

Despite advances in the primary and secondary prevention of DVT, the incidences of DVT-associated mortality and morbidity among hospital patients have declined only minimally (Kahn and Ginsberg, 2004, Heit, 2005). DVT affects 1 to 3 of 1000 people in the general population annually and associated with a mortality rate of 10% (Kahn *et al.*, 2014, Stone *et al.*, 2017).

VTE incidence varies among the different ethnic groups (ISTH, 2014). Compared to Caucasians, incidence rates for African Americans are VTE 1.38, DVT 1.07, and PE 0.40, and those for Asian/Pacific Islanders are VTE 0.26, DVT 0.22, and PE 0.07 (Stein *et al.*, 2004, Raskob *et al.*, 2014a). The risk of recurrence in Caucasians is lower than that of African-Americans and Hispanics (Keenan and White, 2007).

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). The incidence of VTE in Sudan is unknown. In a study conducted in Sudan during the period between April 2007 and March 2008, a total of 14,490 deliveries occurred during the study period. The rate was 448 DVT per 100,000 births/year. Only four of these 65 patients were pregnant and the rest presented at postpartum. Cesarean section deliveries showed higher risk of DVT (Gader *et al.*, 2009). An extensive literature review and epidemiologic studies commissioned by the WHO, which were conducted in 26 hospitals across 8 low and middle-income countries in the Eastern Mediterranean and the regions of North Africa Including Sudan, and in 35 hospitals across 5 countries in Latin America, number of VTE events associated with hospitalization during 2009 for 117.8 million hospitalizations among 1.1 billion citizens of high-income countries, and for 203.1 million hospitalizations among 5.5 billion citizens of low- and middle-income countries (Aranaz-Andres *et al.*, 2011, Wilson *et al.*, 2012, Raskob *et al.*, 2014b). The study reported incidences of VTE per 100 hospitalizations of 3.3 (95% confidence interval (CI), 1.9–4.8) in high income countries, and 3.0 (95% CI, 1.0–4.8) in low- and middle-income countries (Jha *et al.*, 2013, Raskob *et al.*, 2014a). The estimated annual number of cases of VTE was 3.9 million (95% CI, 1.9–6.3) for the high income countries, and 6.0 million (95% CI, 1.2–12.8) for the low- and middle-income countries (Raskob *et al.*, 2014a).

### **1.2.4.3 Diagnosis of deep vein thrombosis**

The patient may present with symptoms of pain and swelling of the leg (Kyrle and Eichinger, 2005). Classic symptoms include limb swelling and pain, which occasionally are confused for findings of cellulitis, bruising, or muscle strain. Homans' sign is pain on dorsiflexion of the ankle and stretching of the calf muscle. This is a classically described bedside finding, although use of this sign as a test for DVT is not reliable (Bloch *et al.*, 2004). DVT can be diagnosed in asymptomatic patients as a result of routine screening in high-risk groups such as postoperative orthopaedic patients (Kahn, 2006). An accurate diagnosis and adequate treatment is important for the prevention of a pulmonary embolism, or recurrent venous thrombosis.

#### **1.2.4.3.1 Ultrasonography**

DVT can be effectively diagnosed using duplex ultrasound (Pomero *et al.*, 2013, Theerakulpisut *et al.*, 2018). Venous ultrasound imaging is considered the first line investigation for DVT diagnosis (Bloch *et al.*, 2004, Pomero *et al.*, 2013). Other techniques that are available for the objective diagnosis of deep-vein thrombosis are contrast venography, and radionuclear scans (Theerakulpisut *et al.*, 2018).

#### **1.2.4.3.2 Markers for coagulation and fibrinolysis**

Ongoing blood coagulation and fibrinolysis can be measured by different markers of blood coagulation activation, fibrin formation and fibrin degradation. After activation of the blood coagulation system, prothrombin is converted to thrombin by the splitting of the prothrombin fragments F1+2

(F1+2)(Satoshi *et al.*, 2008). The resulting enzyme, thrombin, is rapidly inactivated by AT, forming the thrombin-antithrombin complex (Satoshi *et al.*, 2008). Quantification of F1+2 makes it possible to monitor even a minor degree of blood coagulation activation (Satoshi *et al.*, 2008). Only one type of assay for F1+2 is commercially available. This is an enzyme immunoassay for the *in vitro* determination of human F1+2 and is based on the sandwich principle (Satoshi *et al.*, 2008). Free thrombin cleaves fibrinogen to fibrin monomer in a multi-step process that proceeds via a series of intermediate products. At an early stage of fibrin formation, soluble fibrin (SF) complexes are formed between fibrin monomers and fibrinogen molecules. Later, if thrombin remains active, more SF is created and forms a fibrin gel in which the subunits are cross-linked by FXIII. Plasmin, the most important fibrinolytic enzyme, may degrade fibrinogen, SF and cross-linked fibrin. When crosslinked fibrin is digested, fragments containing dimerized D-domains, including D-dimer, are formed. The concentration of D-dimer is primarily a measure of both intravascular and extravascular fibrin formation and, to a lesser extent, of fibrinolytic activity (Dempfle, 2002, Dempfle *et al.*, 2004). A large number of test kits based on different techniques are commercially available for both SF and D-dimer. There is no simple way to compare results from different assays. Correlation between different SF assays is weak (Dempfle *et al.*, 1995), as their degree of cross-reactivity with fibrin degradation products, varies considerably (Dempfle *et al.*, 1995).

Assays for D-dimer, using monoclonal antibodies against conformational epitopes of fibrin compounds containing dimerized D-domains, differ concerning specificity for cross-linked fibrin, with a preference for either high-molecular-weight crosslinked fibrin derivatives or for low- molecular-

weight fibrin degradation products. There is no simple linear relationship between different assays (Gosselin *et al.*, 2002, Dempfle, 2003). Therefore, only D-dimer assays tested in appropriate clinical trials should be used to rule out VTE. The main source of D-dimer antigen reactivity is not the proteolysis of clots but cross-linked fibrin in the circulating blood (Dempfle, 2005). D-dimer enzyme-linked immunosorbent assays (ELISA) have been considered to be the gold standard. However, they are cumbersome, labor-intensive and unsuitable for routine emergency use and have thus been replaced by latex-based systems in the clinical setting (Dempfle, 2005).

APC resistance can be evaluated in plasma using different commercially available methods, many of which are derived from the original Coatest APC Resistance test method, available since 1993 (Kjellberg *et al.*, 2010b). These tests, classic APC resistance assays, measure the classic APC ratio as clotting time in the presence of APC, divided by clotting time in the absence of APC. These assays have been shown to have some shortcomings; many haemostatic variables can interfere because the test is based on the activated partial thromboplastin time (aPTT). The assays are susceptible to platelet contamination and to clinical conditions such as age; gender; body mass index (BMI); smoking; blood group; aPTT; fibrinogen, homocysteine, triglyceride and total cholesterol levels; all conditions giving rise to high levels of plasma FVIII (Kjellberg *et al.*, 2010b).

Low classic APC ratios have been found among individuals with the metabolic syndrome (Tosetto *et al.*, 1997), during oral contraceptive use and pregnancy (Cumming Anthony *et al.*, 1996, Mathonnet *et al.*, 1996) and in individuals with lupus anticoagulants (LA) (Nojima *et al.*, 2009). In one study a correlation was found between the classic APC ratio and the protein



C level (Mahieu *et al.*, 2007). The APC ratio is not useful during treatment with heparin or oral anticoagulants (OAC). It has also been shown that the APC ratio increases in plasma samples with only slightly reduced levels of FII, FVIII and FX (Bertina, 1994).

The condition with a classic APC ratio below the lower limit of the reference interval in the absence of a FV gene mutation is called acquired APC resistance. Studies have shown that there is a correlation between this condition, blood coagulation activation and subsequent incidences of VTE (de Visser *et al.*, 1999).

Modified APC resistance tests are based on pre-dilution (1:4) of the sample plasma with FV-deficient plasma prior to analysis, which is then performed according to routine. This procedure makes the method much more specific for alterations in the FV molecule, resulting in a very high discrimination of the FV R506Q mutation as well as the ability to analyse plasma from heparin-treated individuals and pregnant women (Jorquera *et al.*, 1996). The modified APC resistance test used in the research underlying this thesis has been reported to have 100% sensitivity and 100% specificity for the FVL mutation and distinguishes homozygous from heterozygous individuals (Hall *et al.*, 1998).

More recently, APC-R assays have been developed that use Russell viper venom (RVV) from the snake *Daboia russelli* (Kadauke *et al.*, 2014). The RVV time is analogous to the aPTT, except that the clotting cascade is initiated by the RVV factor X activator (RVV-X). This sidesteps the contact pathway and thereby eliminates interference by deficiency or elevations of factors VIII, IX, XI, and XII. The ProC-Ac-R assay (Siemens, Washington,

DC) involves a dilute RVV time performed with and without APC (treating the specimen with a snake venom from *Agkistrodon contortrix* activates endogenous protein C) (Kadauke *et al.*, 2014).

#### **1.2.4.4 Etiology and risk factors of DVT**

Venous thrombi are formed in the setting of low flow and low shear stress and mainly consist of fibrin strands, red blood cells, and few platelets. Usually, thrombi form in the valve pockets of calf veins and extend to the proximal veins. Once an occlusive thrombus obstructs the deep venous system, the superficial venous system may be insufficient to drain the venous blood (Bloch *et al.*, 2004). The raised venous and capillary pressure that occurs after thrombus formation increases the transcapillary filtration rate, resulting in localized oedema (Kyrle and Eichinger, 2005).

Venous thrombosis is a multicausal disease (Rosendaal, 1999, Lippi and Franchini, 2008). An individual's thrombotic potential may be described as being dynamic age-dependent with inherited and acquired risk factors increasing the risk for venous thrombosis (VT) in an additive or synergistic fashions (Rosendaal, 1999). The strongest risk factor for VT is being affected by a previous VT. After 5 years of follow-up a recurrence rate of approximately 25% have been reported (Hansson *et al.*, 2000). Age is another strong risk factor for VT (Cushman, 2007, Crous-Bou *et al.*, 2016). The risk of VT increases approximately 50 times from 20 to 70 years of age (Rosendaal, 1999). Many acquired and environmental causes risk factors have been well established (Lijfering Willem *et al.*, 2010). Some are transient or reversible, such as surgery, trauma (Geerts *et al.*, 2008), minor injuries of the leg (van Stralen *et al.*, 2008), immobilization (Gibbs, 1957,

Crous-Bou *et al.*, 2016), Overweight (Rosendaal, 2005), lengthy travel (Rosendaal, 2005), hormone replacement therapy (Hoibraaten *et al.*, 2000, Cushman, 2007), Central venous catheter (Lee and Kamphuisen, 2012) and pregnancy (Treffers *et al.*, 1983, Eichinger *et al.*, 2013), whereas others, such as old age (Rosendaal, 1999), cancer (Wun and White, 2009) and the antiphospholipid syndrome (de Groot *et al.*, 2005), are persistent or irreversible. VTE can be classified as provoked (secondary) or unprovoked (idiopathic), depending on the presence or absence of associated risk factors (Zhu *et al.*, 2009).

Genetic risk factors for VT lead to hypercoagulability, whereas the acquired causes are either associated with stasis or related to hypercoagulability. Thrombophilia are detectable in more than 50% of patients with a first unprovoked VT (Kyrle *et al.*, 2010).

There is an intriguing question about why certain exposures yield higher risks for disease occurrence than others (Lijfering Willem *et al.*, 2010). The thrombosis potential model, first described by Rosendaal, may serve well to illuminate such findings (Rosendaal, 1999). The idea is that an individual is at risk for venous thrombosis throughout life, which is reflected in the 'thrombosis potential' and that each risk factor contributes to the potential. Only when the combination of thrombosis risk factors reach a certain potential, will venous thrombosis occur, crossing of the thrombosis threshold (Lijfering Willem *et al.*, 2010).

## **1.2.4.5 Acquired and environmental risk factors**

### **1.2.4.5.1 Age and gender**

While VTE can occur at any age, incident VTE more commonly occurs in older individuals. In young adult life until approximately midlife, VTE occurs at a low rate of 0.5 to 1 event per 1,000 person-years (Silverstein *et al.*, 1998). This rate increases in midlife and by age 80, VTE incidence is substantially higher, occurring at a rate of approximately 5 to 7 VTE events per 1,000 person-years (Silverstein *et al.*, 1998, Bell *et al.*, 2016). Although the role of older age as an independent risk factor for VTE is not well understood, it has been proposed that blood coagulability may increase with age (Luxembourg *et al.*, 2009). In addition, age effects are likely mediated by a higher prevalence of provoking risk factors for VTE, such as cancer, immobility, hospitalization, and surgery (Crous-Bou *et al.*, 2016).

VTE incidence across the lifespan also differs by sex, with a higher age-adjusted incidence rate among men (130 per 100,000 person-years) than women (110 per 100,000 person-years) (Silverstein *et al.*, 1998). In the age-group 25–30 years old, VT occurs in approximately 1 per 10 000 py as compared with nearly 8 per 1000 py in the 85 years and older population (NÆSs *et al.*, 2007). The risk of developing VT is therefore 80-fold increased in the older population, leading to a high attributable risk. The population attributable risk is more than 90%, indicating that 90% of the total incidence of thrombosis in the population can be ascribed to aging. The life-time risk (cumulative incidence) of VT is up to 15% to age 90, and approximately 60% of all VT events occur in those aged 70 years and older (NÆSs *et al.*, 2007).

However, it is controversial whether men are inherently at a greater risk of VTE than women (Crous-Bou *et al.*, 2016). In younger adult life, the annual incidence of VTE is slightly higher among women than men,(Silverstein *et al.*, 1998) a difference that has been attributed to hormonal exposures that impact women in their childbearing years, such as pregnancy, the postpartum period and oral contraceptive(OC) use (Roach *et al.*, 2014). Following midlife, VTE incidence increases more rapidly among men than among women, resulting in a higher VTE incidence among older men than women.(Silverstein *et al.*, 1998) Although it is unclear as to why VTE incidence is higher among older men than women, it has been proposed that there may be differences in lifestyle-based risk factors between men and women. Alternatively, this difference may be mediated by body height (Severinsen *et al.*, 2010).

Comparing the incidences of VT in the young and older population in many previous studies shows clearly that aging is one of the strongest and most prevalent risk factor for venous thrombotic disease, resulting in a high incidence of VT in the elderly population (Rosendaal, 1999, Becattini and Agnelli, 2002, NÆSs *et al.*, 2007, Lippi and Franchini, 2008, Engbers *et al.*, 2010)

#### **1.2.4.5.2 Race**

Although there is strong evidence that the prevalence of venous thromboembolism (VTE) varies significantly among different ethnic/racial groups, the genetic, physiologic and/or clinical basis for these differences remain largely undefined (White and Keenan, 2009). There are few if any population-based estimates of VTE incidence in African-origin populations outside of North America (Zakai and McClure, 2011). African-American

patients have a significantly higher rate of incident VTE, particularly following exposure to a provoking risk factor such as surgery, medical illness, trauma, etc (White and Keenan, 2009). Followed by White individuals, and the lowest risk among Asian or Hispanic individuals (Buckner and Key, 2012). Few studies have been able to appropriately evaluate the race-VTE association while controlling for appropriate confounders, however, and so the association of race with VTE remains controversial (Goldhaber, 2012). Recently, a large study combined data from three United States-based cohorts: the Atherosclerosis Risk in Communities Study (ARIC), the Cardiovascular Health Study (CHS), and the Reasons for Geographic and Racial Differences in Stroke study (REGARDS). In the CHS, blacks had an 81% greater risk of incident VTE than did whites, but in ARIC, there was no significant difference (Zakai *et al.*, 2014). In REGARDS, the only one of the three cohort studies to include a substantial population of blacks residing outside of the Southeast, there was significant interaction between region of residence (Southeast vs. rest of country) and race, suggesting that regional differences in comorbid illnesses, environmental risk factors, or in quality and access to medical care may mediate a potential race-VTE association (Zakai *et al.*, 2014). Further complicating the understanding of a race-related gradient in VTE risk is the understanding that factor V Leiden and prothrombin gene mutations are both less common among persons of Black race than White race (Zakai and McClure, 2011, Buckner and Key, 2012).

#### **1.2.4.5.3 Previous history of DVT**

Simply having a family history of VTE is a risk factor for VTE, no matter whether a thrombophilia is detectable or not (Moll and Journeycake, 2010).

This additional risk is due to unknown or unmeasured risk factors. Having 1 first-degree relative with a history of VTE increases an individual's risk of VTE 2.2-fold; having 2 affected relatives increases the risk 3.9-fold. Young age of the affected relative and the number of affected relatives more strongly indicate a predisposition to develop VTE. Thus, in clinical practice, family history is an inexpensive and useful tool for risk assessment (Moll and Journeycake, 2010).

In recent years, a positive family history of VTE has emerged as a possible independent risk factor for VTE, regardless of the presence of any of the most common thrombophilias (Bezemer *et al.*, 2009).

#### **1.2.4.5.4 Surgery**

The risk of thrombosis with surgery varies depending on the surgery type and patient characteristics (Cushman, 2007). The risk of VTE after major general surgery has been extensively documented. Although the term “major general surgery” is imprecise, most investigators apply this term to patients who undergo abdominal or thoracic operations that require general anesthesia lasting 30 minutes (Collins *et al.*, 1988, Clagett and Reisch, 1988). Other types of surgery associated with a high risk of VTE include coronary artery bypass (Reis *et al.*, 1991), surgery for gynecological malignancies (Mokri *et al.*, 2013, Barber and Clarke-Pearson, 2017), and major urological surgery (Forrest *et al.*, 2009). The risk after neurosurgery is similar (Browd *et al.*, 2004), but intracranial surgery presents a relative contraindication to anticoagulant prophylaxis (Anderson and Spencer, 2003).

Lower extremity orthopedic operations carry a particularly high risk. Without prophylaxis, approximately half of the patients undergoing elective total hip or knee replacement develop VTE (Anderson and Spencer, 2003). However, only 5% of these patients manifest symptoms of VTE (Anderson and Spencer, 2003, Bergqvist and Lowe, 2002). Whereas calf vein thrombi tend to be evenly distributed between the two legs in patients recovering from hip replacement, 90% of proximal thrombi occur on the operated side. Patients undergoing arthroscopic knee surgery are at low to moderate risk, so VTE prophylaxis is optional (Bergqvist and Lowe, 2002).

#### **1.2.4.5.5 Immobility**

Immobility increases the risk of thrombosis, presumably due to the reduced venous blood flow, particularly in the pockets of the venous valves, leading to inflammation and hypercoagulability (Bovill and van der Vliet, 2011). Relevant settings of immobility include bedrest, plaster casts on the legs and paresis of the legs due to neurological conditions (Cushman, 2007). Research-based definitions of immobility due to bedrest differ, but a duration of four days seems reasonable. Minor forms of immobility, such as after minor surgery or injury, have also been linked to thrombosis risk (Eekhoff *et al.*, 2000). Gibbs found that 15% of patients on bed rest for one week before death had venous thrombosis at autopsy, whereas the incidence rose to 80% in patients in bed for a longer period (Gibbs, 1957). Whereas prolonged bed rest or immobility alone does not provide adequate reason for prescribing prophylactic anticoagulant therapy, prolonged immobility combined with other major risk factors increases the likelihood of VTE (Anderson and Spencer, 2003).



#### **1.2.4.5.6 Obesity**

An important modifiable risk factor for thrombosis is obesity. Obesity is defined as a body mass index (BMI) above 30 kg/M<sup>2</sup>. Obesity leads to a 2 to 3-fold higher risk of venous thrombosis in men and women (Tsai *et al.*, 2002, Abdollahi *et al.*, 2003). The risk associated with severe obesity (BMI above 40 kg/M<sup>2</sup>) is even higher. The obese have a further increase in thrombosis risk when they are exposed to other thrombosis risk factors, such as exogenous contraceptive or postmenopausal hormones (Pomp *et al.*, 2007). Obese women who used oral contraceptives had a 24-fold higher thrombotic risk than women with a normal BMI who did not use oral contraceptives. Relative to non-carriers of normal BMI, the joint effect of factor V Leiden and obesity led to a 7.9-fold increased risk. For prothrombin 20210G>A this was a 6.6-fold increased risk (Pomp *et al.*, 2007).

#### **1.2.4.5.7 Pregnancy and postpartum**

Pregnancy is a risk factor for VTE. As mentioned, pregnancy is a hypercoagulable state characterized by increased levels of clotting factors and impaired fibrinolysis (Greer, 1999). Moreover, pregnancy may lead to venous stasis (Cordts and Gawley, 1996). Pregnant women have a much higher risk of VTE than nonpregnant women of similar age and the risk has been shown to be higher after cesarian section than after vaginal delivery (Bates and Ginsberg, 2001). During the third trimester and especially in the first two weeks following delivery the risk of Venous Thromboembolism (VTE) increases in women and it is the leading cause of maternal death in Western Countries (Bates *et al.*, 2008, Marik and Plante, 2008). VTE risk is five times greater during the postpartum period than during pregnancy (Heit

*et al.*, 2005). A population-based cohort study reported a 3.5-fold increased risk of VTE in the antepartum and an 11.9-fold increased risk in the postpartum period, compared to outside of pregnancy (Sultan *et al.*, 2012). In a study conducted in an African population, the documented rate was 48 DVT per 100,000 births per year (Gader *et al.*, 2009).

#### **1.2.4.5.8 Oral contraceptive using and hormonal therapy**

Combined progestogen and estrogen, as combined hormonal contraceptives (CHC) and menopausal hormone treatment (HT), increase the risk of VTE in women (Rosendaal *et al.*, 2003). The use of CHC is associated with a two- to six fold increase in risk of VTE in otherwise healthy young women (Rosendaal *et al.*, 2003, van Hylckama Vlieg *et al.*, 2009). For HT, the risk is increased two- to four fold (Canonica *et al.*, 2007, van Hylckama Vlieg and Middeldorp, 2011, Sood *et al.*, 2014).

The underlying mechanism explaining the increased risk of CHC and HT has been investigated in several studies (Koenen *et al.*, 2005, Tchaikovski and Rosing, 2010). Both CHC and HT have an influence on the coagulation system, mainly by causing an acquired resistance to activated protein C (APC) (Tans *et al.*, 2003). Additionally, use of HT and CHC is associated with increased levels of procoagulants like prothrombin, FVII, FVIII, fibrinogen and decreased levels of natural anticoagulants like protein S, protein C and TFPI (Bloemenkamp *et al.*, 1998). The induced APC resistance can work synergistically with inherited thrombophilias like the factor V Leiden and prothrombin gene mutation to cause clinically significant VTE in CHC and HT-users (Wu *et al.*, 2005a, Mohllajee *et al.*, 2006). Thrombophilia-screening of women prior to prescribing CHC or HT has

been suggested but has not proven to be cost-effective and most current guidelines today do not recommend general screening (Wu *et al.*, 2005b). The use of CHC is associated with slightly higher relative risk estimates than HT, but the absolute risk of VTE associated with HT is probably higher since women who are treated with HT are older and have a greater burden of additional risk factors than women treated with CHC (Tans *et al.*, 2003).

#### **1.2.4.5.9 Cancer**

Cancer is a well established risk factor for VTE and approximately 20 % of all fatal outcomes in VTE-cases are related to malignant diseases (Heit *et al.*, 2002, Khorana *et al.*, 2012). Patients with cancer have a 4-7 fold increased risk of VTE compared to patients without cancer. The incidence of cancer related VTE is 0.5-20 % depending of the type of cancer and occurrence of other risk factors (Blom *et al.*, 2006b, Horsted *et al.*, 2012). Cancer of the brain and pancreas has the highest risk whereas breast cancer and prostate cancer patients are at lower risk of VTE (Blom *et al.*, 2006a, Horsted *et al.*, 2012).

#### **1.2.4.5.10 Cardiovascular disease (CVD)**

Despite substantial interest, it remains unclear whether cardiovascular disease (CVD) is related to risk of deep-vein thromboembolism (DVT) and pulmonary embolism (PE). Whether CVD risk factors increase VTE risk has been a focus of many cohort and case-control studies (Wattanakit *et al.*, 2012). In general, case-control studies have found a positive association between markers of CVD and risk of VTE (Prandoni *et al.*, 2003, Hong *et al.*, 2005), while prospective cohort studies reported no association (Reich *et al.*, 2006, van der Hagen *et al.*, 2006). It is generally believed that if an

association between CVD and VTE exists, the mechanism is presumed to relate to the sharing of common risk factors between the two diseases. To date, among CVD risk factors, only obesity is consistently associated with VTE risk (Lutsey *et al.*, 2010, Holst *et al.*, 2010), whereas the roles of diabetes, hypertension, smoking, total cholesterol, high density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol, triglycerides, alcohol non-use, and physical inactivity are less clear (Ageno *et al.*, 2008, Mahmoodi *et al.*, 2009). Congestive heart failure yielded a nearly 3-fold increased risk of VTE in case-control studies (Howell *et al.*, 2001, Samama, 2000). In addition, the risk of VTE is increased in the presence of central venous catheters and pacemakers (Heit *et al.*, 2000).

#### **1.2.4.5.11 Renal failure**

There is recent interest in evaluating the role of chronic kidney disease (CKD) as a risk factor for VTE. This is not a surprise given that CKD patients have underlying hemostatic derangements predisposing to a hypercoagulable state (Wattanakit and Cushman, 2009). Epidemiologic evidence on the VTE risk in the End-stage renal disease (ESRD) population is very limited. Initial postmortem examinations suggested that VTE is a rare event in ESRD (Wattanakit and Cushman, 2009). However, the series by Wiesholzer *et al.* in 1999 was the first to suggest that VTE in dialysis patients was relatively common (Wiesholzer *et al.*, 1999).

Other studies reported that those with stage 3/4 chronic kidney disease have about 50% higher risk compared to those with normal kidney function (Mahmoodi *et al.*, 2009).

## **1.2.4.6 Genetic risk factors**

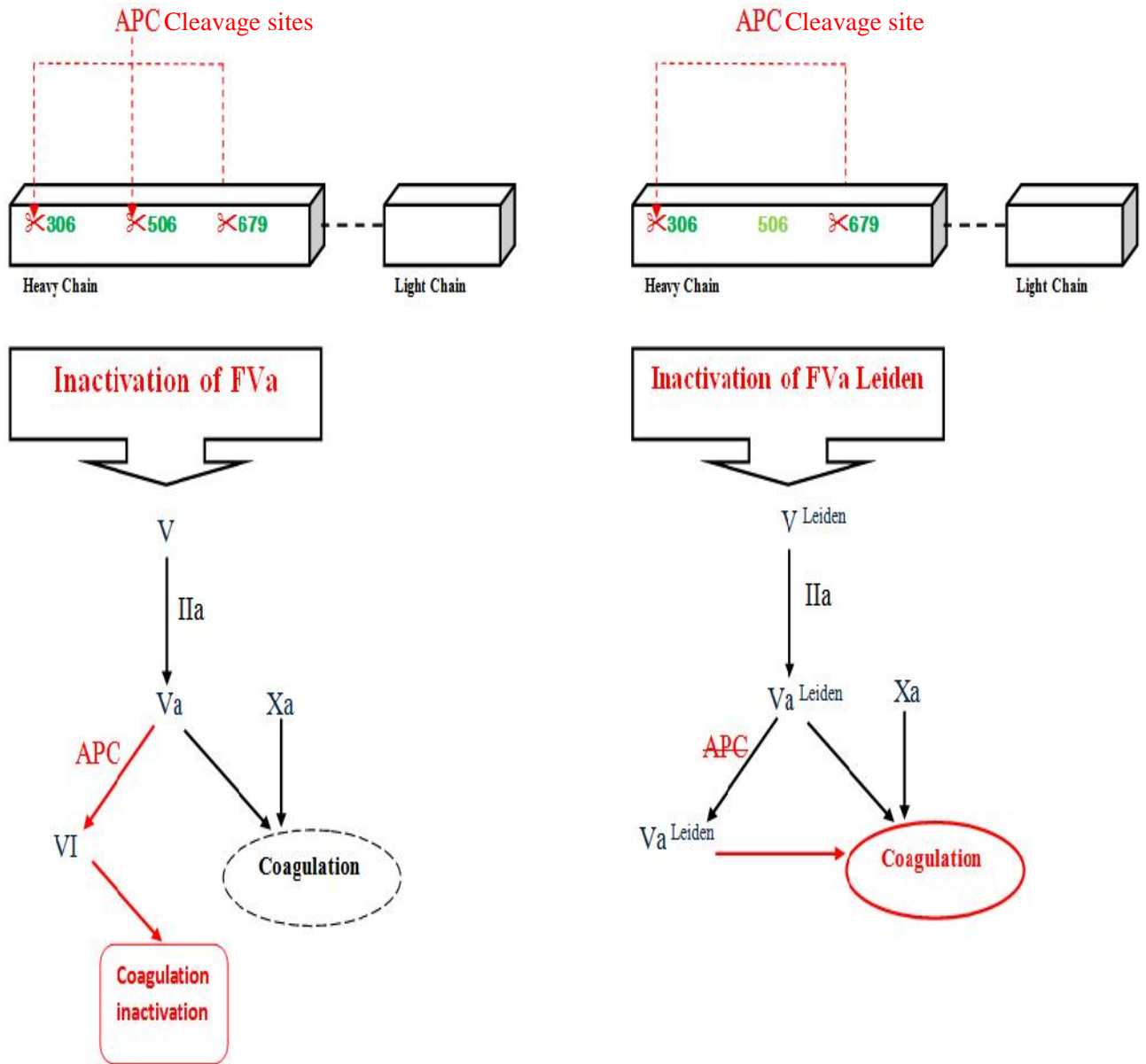
### **1.2.4.6.1 Factor V Leiden 1691G>A**

#### **1.2.4.6.1.1 History**

In 1993, Dahlbäck et al. in Malmö, Sweden described a new phenomenon, i.e., poor anticoagulant response to activated protein C, in a family with a history of venous thromboses. The phenomenon was thought to be due to the deficiency of a new protein C cofactor and the laboratory phenomenon was named APC resistance (Dahlback *et al.*, 1993). In May 1993, a commercial APC resistance test became available. APC resistance was quickly demonstrated to be a common risk factor for venous thrombosis. In the Leiden Thrombophilia Study (LETS), APC resistance was present in 21% of venous thrombosis patients and in 3 % of controls (Koster *et al.*, 1993). APC resistance was also shown to be present in over 50% of previously unexplained thrombophilic patients and concomitantly in a few with a previous diagnosis of protein C or protein S deficiency (Griffin *et al.*, 1993). In early 1994, Dahlbäck et al. reported the cause of APC resistance to be a property of factor V (Dahlback and Hildebrand, 1994).

At the same time, Bertina et al. in Leiden, The Netherlands also concluded that F V was involved. In June 1994, they published a paper showing that a single G to A substitution at the nucleotide position 1691 in the factor V gene was associated with APC resistance (Bertina *et al.*, 1994). The point mutation causes the replacement of amino acid Arg to Gln at the site 506 in factor V resulting in the inadequate inactivation of mutated F Va. The mutation was named as factor V Leiden (FV Leiden) (Bertina *et al.*, 1994). As the site Arg506 is a cleaving site for APC, it was now easy to understand

why the disappearance of this site can cause resistance to APC(Figure 1.2). Dahlbäck's initial idea of the lack of a new protein C cofactor behind APC resistance has also proved to be partially true as the FV Leiden mutation abolishes the cofactor activity of F V in the inactivation of F VIIIa (Dahlbäck, 2008). Dahlbäck and Zöller et al. found FV Leiden mutation in almost all of their fifty Swedish APC resistant families proving this mutation to be the most prevalent cause for APC resistance (Zöller *et al.*, 1994). The first large study, LETS, already proved FV Leiden to be a frequent risk factor for venous thrombosis (Rosendaal *et al.*, 1995).



**Figure 1.2 Normal factor V activation and factor V Leiden inactivation**

#### **1.2.4.6.1.2 Prothrombotic mutation**

FV Leiden is a prothrombotic mutation. It is at the same time a gain-of-function mutation and a loss-of-function mutation. First, due to disappearance of the cleavage site at the Arg506, APC is unable to inactivate F Va optimally leading to increased thrombin formation (Kalafatis *et al.*, 1995). Second, due to the disappearance of that cleavage site, APC is unable to cleave intact F V so that F V could function as cofactor for PC-PS complex in the inactivation of F VIIIa. This loss of anticoagulant function leads, again, to increased thrombin formation (Dahlbäck, 2008). However, the risk for venous thrombosis caused by FV Leiden is relatively low. This may be explained by the fact that although cleavage at the site Arg506 accelerates inactivation of F Va remarkably by exposing the cleavage sites Arg306 and Arg679 to APC, cleavage at the site Arg506 is not absolutely necessary for the inactivation of F Va (Kalafatis *et al.*, 1995).

In addition, in the prothrombinase complex, the capability of APC to inactivate F Va is similar for the wild type F Va and F Va Leiden, because the Arg506 cleavage site of the wild type F Va is protected by F Xa, and in F Va Leiden this cleavage site does not exist (Kalafatis *et al.*, 1995, Rosing *et al.*, 1995). Mechanisms that reduce the effect of this potentially injurious mutation include the acceleration of the cleavage of F Va by protein S at the site Arg306 (Rosing *et al.*, 1995).

#### **1.2.4.6.1.3 Evolutionary advantage**

According to haplotype analyses, FV Leiden is a founder mutation, which occurred about 21,000 years ago (Cox *et al.*, 1996, Zivelin *et al.*, 1997, Zivelin *et al.*, 2006). The mutation is present at a variable frequency (mean



5%) in Caucasians, but absent or nearly absent in other races (Rees, 1996, Franchini and Mannucci, 2008). This indicates that FV Leiden most likely occurred in a Caucasoid subpopulation after the separation of non-Africans from Africans, and Caucasoid populations from Mongoloid populations (Rees, 1996, Zivelin *et al.*, 1997, Franchini and Mannucci, 2008).

The high prevalence of FV Leiden in Caucasians suggests an association with evolutionary advantage and many findings support a favorable selection pressure (Franchini and Mannucci, 2008). Data exist indicating that FV Leiden might protect against peripartal bleeding (Lindqvist *et al.*, 1998, Kjellberg *et al.*, 2010a) and heavy menstrual blood loss (Lindqvist *et al.*, 2001). This could have provided considerable advantage by reducing iron depletion and by protecting against life threatening post-partum hemorrhage. However, conflicting observations about pregnancy-related blood loss exist (Clark *et al.*, 2008). Similarly to protecting against excessive bleeding in association with surgery (Donahue *et al.*, 2003), FV Leiden may have protected against excessive bleeding in association with trauma in the past. Some evidence, although partly conflicting, exists that simultaneous carriage of FV Leiden might attenuate bleeding symptoms also in hemophiliacs (Franchini and Lippi, 2010). Other possible selective advantages include a more favourable embryo implantation in carriers of FV Leiden (Gopel *et al.*, 2001, van Dunné *et al.*, 2005), and an increased fecundity (shorter time to pregnancy) in male carriers of FV Leiden (van Dunné *et al.*, 2006). This is supported by an observation of a slightly increased sperm count in male carriers of FV Leiden (Cohn *et al.*, 2010).

#### **1.2.4.6.1.4 Global prevalence of FVL-mutation**

The prevalence rate of factor V-Leiden in the general population varies from 0% to 15% according to ethnicity and geographic distribution (Montagnana *et al.*, 2010, Roberts Lara *et al.*, 2009). In a large population study, the prevalence of Factor V Leiden mutation was determined in a cohort of 4047 U.S. men and women (Ridker *et al.*, 1997). This cross-sectional study revealed the overall carrier frequency for the mutation as 3.7% and the allele frequency was 1.9%. The observed distribution of genotypes was consistent with that predicted by the Hardy-Weinberg equilibrium. Factor V Leiden mutation was found to be most prevalent in white persons (5.3%) and was significantly less prevalent in other ethnic groups (2.2% in Hispanic Americans, 1.2% in African Americans, 0.5% in Asian Americans, and 1.3% in Native Americans) (Rees *et al.*, 1995, Ridker *et al.*, 1997). The allele mutation is low in South European populations (1%–3%) (Geerts *et al.*, 2008). The highest incidence in a population has been reported from Sweden, specifically in the area where this thesis was carried out, where FVL frequency of 11% was reported (Holm *et al.*, 1996). High prevalence in the Mediterranean countries (13.6% in Syria, 12.3% in Jordan, and 13.4% in Greece) and notably uncommon in Asian and African population, a fact that may explain the decreased risk for venous thromboembolism in these groups (Burkitt, 1972, Rees *et al.*, 1995, Geerts *et al.*, 2008).

#### **1.2.4.6.2 Prothrombin 20210G>A mutation**

Poort *et al.* reported in 1996 a point mutation in the coagulation factor II (prothrombin) gene (Poort *et al.*, 1996). The mutation causes a G to A substitution at the nucleotide position of 20210 in the 3'-untranslated region

of the gene. The point mutation is associated with elevated prothrombin levels and is therefore a gain of function mutation. FII 20210G>A allele is associated with about a 2-fold increased risk for venous thrombosis (Poort *et al.*, 1996). The mutation is of single origin and is mainly found in the Caucasian population (Zivelin *et al.*, 2006). Carriers of the mutation have about 30% higher plasma levels of prothrombin than the non-carriers (Poort *et al.*, 1996) and thus, have the potential to form thrombin easily (Kyrle *et al.*, 1998) setting a pathophysiologic environment suitable for thrombosis.

#### **1.2.4.6.2.1 Global prevalence of prothrombin 20210G>A mutation**

The prevalence of the PT 20210G>A gene in the general Caucasian population is estimated to be approximately 2% to 5% (Franco and Reitsma, 2001) and its prevalence in Southern Europe is approximately two-fold higher than in Northern Europe (Robetorye and Rodgers, 2001).

In a recent systemic review, Prothrombin 20210G>A prevalence rates were organized by continent and ethnoracial ancestry. A total of 113 articles were included from six continents (Africa, Asia, Europe, Oceania, North America, and South America) and 67 countries, with a total 61,876 participants tested for prothrombin 20210G>A. Reported prevalence rates varied from 0 to 15.9% among ethnic groups, with higher rates seen in the thromboembolism affected cohort compared with the unaffected cohort (Dziadosz and Baxi, 2016). In Africa, 2354 participants were studied from seven different countries of a total possible 54 countries. They revealed carrier rates between 0 and 3.9% in healthy participants, unaffected by a history of thromboembolism (Dziadosz and Baxi, 2016). In Asia, 20 736 participants were tested from 22 of a possible 44 countries. Although rates of unaffected individual carrier rates for prothrombin 20210G>A in Japan, Singapore,

China, Oman, South Korea, and India are 0%, the Middle East populations had higher rates ranging from 2.5% to as high as 12.25% in Jordan Circassians (Dziadosz and Baxi, 2016).

#### **1.2.4.7 Management and treatment of DVT**

The aim of management of deep vein thrombosis is to prevent the sequelae of venous thrombosis which include the symptoms of acute DVT, pulmonary embolism, postthrombotic syndrome, and recurrent DVT (Bloch *et al.*, 2004, Ho *et al.*, 2005). Reducing the burden of disease due to DVT requires effective primary prevention, prompt diagnosis, appropriate treatment of acute thrombosis, and effective long-term secondary prevention (Ho *et al.*, 2005).

Anticoagulation is the mainstay of treatment of symptomatic DVT (Kearon, 2003, Ho *et al.*, 2005). The primary treatment in patients with acute DVT has been initial heparinization during the acute phase for 1-5 days, then conversion to an oral anticoagulant for 3-6 months (Bloch *et al.*, 2004). A review of this protocol shows that it reduces symptoms of acute DVT and that it has a relatively low DVT recurrence rate while minimizing the bleeding risk (Bloch *et al.*, 2004).

Heparinization is commonly achieved with subcutaneous injections of low molecular weight heparin (LMWH) or with intravenous infusion of unfractionated heparin (UFH) (Gould *et al.*, 1999). Subcutaneous LMWH has become the standard of care for the initial treatment because it is as effective and as safe as UFH and does not require continuous intravenous infusion. Low-molecular-weight heparin is more convenient to use since it

does not require regular monitoring of effect and adjustment of dose, it has a more favourable side-effect profile, and patients can be treated successfully as outpatients (Ho *et al.*, 2005).

Warfarin may be started on the first day, but LMWH or UFH should be continued until the international normalized ratio (INR) has exceeded 2.0 on at least two occasions, 24 hours apart (Ho *et al.*, 2005). Warfarin is the anticoagulant of choice for long-term treatment of most patients with DVT, because it can be given orally and is highly effective, reducing the risk of recurrent DVT and extension of the existing DVT by 80%-90% during treatment (Ho *et al.*, 2005). Although warfarin is associated with an increased risk of haemorrhage, the standard recommendation is that warfarin treatment should be continued for 3 to 6 months (Laffan and Tuddenham, 1998, Ho *et al.*, 2005).

### **1.3 Rationale**

DVT is a serious health problem and a common cause of death and disability in developing countries. The development of DVT involves the interaction of multiple genetic factors and modifiable and non-modifiable environmental influences. The determination of these risk factors heavily influences the prevention strategies of local health care agencies. Among the genetic causes of DVT, factor V Leiden 1691G>A and prothrombin 20210G>A mutations are the most common. Carriers of these polymorphisms are prone to spontaneous or secondary venous thrombotic events at any age. The main environmental risk factors for DVT include old age, obesity, history of VTE, cancer, bedrest for more than 5 days, surgery, congestive heart failure, bone fracture, hormone therapy, and pregnancy. The risk of DVT increases several folds when both genetic and environmental factors coexist.

However, whether these gene mutations cause mortality among African populations, especially the Sudanese, remains controversial. Moreover, the role of acquired and environmental risk factors in the development of DVT in the Sudanese population is poorly characterized. Therefore, The purpose of the study was to determine the prevalence of the two mutations in Sudanese deep venous thrombosis patients, and to investigate the role of environmental risk factors in the manifestation of DVT.

The awareness of DVT as an important health issue is growing, our understanding of the DVT risk factors remains limited. Blood in women is more likely to coagulate than that in men because this property of blood in women protects against excessive bleeding during pregnancy, miscarriage, and childbirth. Both genetic and acquired risk factors can further increase

the risk of thrombosis. The knowledge of genetic and environmental risk factors is crucial for the effective application of diagnostic, prophylactic, and therapeutic interventions, especially among women in childbearing age who are at a higher risk of developing DVT.

## **1.4 Objectives**

### **1.4.1 General objective**

To study factor V Leiden 1691G>A and prothrombin 20201G>A gene mutations and their frequency among Sudanese patients with deep vein thrombosis.

### **1.4.2 Specific objectives**

-To determine activated protein C resistant (APC-R), Prothrombin time (PT), fibrinogen, prothrombin fragment1+2 and D dimer level in patients with DVT compared to healthy individuals.

-To detect factor V Leiden 1691G>A and Prothrombin 20201G>A gene polymorphism using *HindIII* restriction enzyme.

-To estimate the frequency of factor V Leiden 1691G>A and Prothrombin 20201G>A gene mutations in patients with DVT compared to healthy individuals.

-To evaluate the acquired and environmental risk factors patterns in Sudanese deep vein thrombosis patients.

## **1.5 Hypothesis**

There will be a significant association between factor V Leiden 1691G>A and Prothrombin 20201G>A genes mutations and deep vein thrombosis in Sudanese population.



# Chapter Two

## 2. Materials and Methods

### 2.1 Materials

#### 2.1.1 Study design

This is an Analytical observational case control study.

#### 2.1.2 Study area

The study was conducted in Khartoum State, the capital and the largest city of Sudan. It is a tripartite metropolis located in northeast Africa near the center of Sudan and is composed of various races and tribes of the Sudan. Khartoum is located in the middle of the populated areas in Sudan, at almost the northeast center of the country between 15 and 16 degrees latitude north, and between 31 and 32 degrees longitude east. Study was conducted in Khartoum state hospitals, Omdurman Maternity Hospital, Ibrahim Malik Teaching Hospital and Khartoum Bahri Teaching Hospital.

#### 2.1.3 Study population

One hundred adult patients (75 females and 25 males) with a confirmed deep vein thrombosis diagnosis based on the results of duplex ultrasound and 92 healthy controls (61 females and 31 males). Subjects in the control group had no previous history of venous thromboembolism or other coagulation disorders.

### **2.1.3.1 Inclusion criteria**

The study included only duplex ultrasonography DVT diagnostic patients who accepted to participate and who demonstrated positive D dimer test.

### **2.1.3.2 Exclusion criteria**

Subjects who were younger than 18 years or who refused to participate.

### **2.1.4 Data collection**

The demographic and clinical data of all participants were collected using a specially designed questionnaire. Obesity was defined as body mass index (BMI) of 30 kg/m<sup>2</sup> or above. Physician's assessment was accepted in cases where weight and height data were unavailable.

## **2.2 Methods**

### **2.2.1 Specimen collection**

Five milliliter Ethylenediamine tetraacetic acid (EDTA) blood and citrated blood samples were collected by venipuncture from each individual for DNA extraction and coagulation tests, respectively. Informed consent was obtained from all subjects. Blood vacutainers were labeled with participant name, hospital record number and date of collection.

### **2.2.2 Coagulation analysis**

#### **2.2.2.1 Platelet poor plasma (PPP) preparation**

Centrifugation time to produce the recommended platelet poor plasma (PPP) with platelet counts < 10000 / $\mu$ L was 10 minutes at 2000 g, room temperature. Blood was drawn using a 21-gauge needle with minimal tourniquet application to prevent stasis and was immediately mixed by

gently inverting the tube 5 times. Citrated whole blood containers were centrifuged within one hour of collection using Nüve NF200 routine centrifuge (Nüve, Ankara, Turkey). the upper layer was collected with a blastic pipette. This collection was performed carefully to avoid disturbing the bottom layer of RBC and the buffy coat layer. Plasma was carefully removed and transferred to a nonactivating polypropylene centrifuge tubes using a plastic pipette and was centrifuged again using the same conditions (Bennett, 2015).

### **2.2.2.2 Prothrombin time (PT)**

#### **2.2.2.2.1 Principle**

The principle of the test consists of the use of calcium thromboplastin to measure the clotting time of the patient's plasma and to compare it with that of a normal standard

#### **2.2.2.2.2 Procedure**

Aliquot of PPP was incubated at 37°C with STA-Neoplastine reagent containing freeze-dried thromboplastin prepared from human recombinant tissue factor, phospholipids and heparin inhibitor. CaCL<sub>2</sub> is then added and the time required for clot formation was measured using SStart 4 Hemostasis Analyzer against control (Diagnostica Stago, Asnieres, France) at 37°C. The result was reported in seconds (prothrombin time) (Kitchen and Preston, 1999, VanCott and Laposata, 2001, Marlar, 2009).

### **2.2.2.3 Activated partial thromboplastin time (APTT)**

#### **2.2.2.3.1 Principle**

The APTT involves the recalcification of plasma in the presence of a standardized amount of cephalin (platelet substitute) and a factor XII activator (kaolin).

#### **2.2.2.3.2 Procedure**

An aliquot of PPP was incubated at 37°C with STA-C.K. reagent containing cephalin as platelet substitute and 5 mg/ml Kaolin activator suspension, followed by calcium chloride (0.025 mol/L) (all pre-warmed to 37°C). Addition of calcium initiates clotting and timing begins. The aPTT result was reported as the time required for clot formation after the addition of CaCl<sub>2</sub> and then measured by STart 4 Hemostasis Analyzer against control (Diagnostica Stago, Asnieres, France) (Marlar, 2009).

#### **2.2.2.4 Fibrinogen assay**

##### **2.2.2.4.1 Principle**

It is based on Modification of the Clauss method. Citrated plasma is brought to coagulation by a large excess of thrombin. Here the coagulation time depends largely on the fibrinogen content of the specimen.

##### **2.2.2.4.2 Procedure**

Fibrinogen assays were performed using Multifibren U reagents with BCS XP System (Siemens Healthcare GmbH, Erlangen, Germany) according to the manufacturer's instructions. Multifibren U reagent was composed of bovine thrombin, fibrin-aggregation retarding peptide, calcium chloride, hexadimethrine bromide, polyethylene glycol 6000, sodium chloride, Tris, bovine albumin and sodium azide as preservative. The expected values were between 2 to 4 g/L (Schlimp *et al.*, 2015).

### **2.2.2.5 D-dimer test**

#### **2.2.2.5.1 Principle**

Polystyrene particles covalently coated with a monoclonal antibody are aggregated when mixed with samples containing D-dimer. The D-dimer cross-linkage region has a stereosymmetrical structure. Consequently, one antibody suffices in order to trigger an aggregation reaction, which is then detected turbidimetrically via the increase in turbidity.

#### **2.2.2.5.2 Procedure**

D-dimer tests were performed by immunoturbidimetric assay using Innovance D-dimer reagent, with BCS XP System (Siemens Healthcare GmbH, Erlangen, Germany) according to the manufacturer's instructions. The expected values were between 0.17 to 4.40 mg/L FEU with BCS XP System (Bogavac-Stanojevi *et al.*, 2013).

### **2.2.2.6 APC resistance test (APC-R)**

#### **2.2.2.6.1 Principle**

Coagulation of the diluted test plasma is achieved in the presence of factor V deficient plasma and *Crotalus viridis halleri* venom. The venom acts as an activator of factor X and therefore triggers the coagulation cascade downstream from factor X, thus eliminating the influence of all coagulation factors acting upstream. The prolongation of the clotting time of a normal plasma in the presence of PCa results from the capacity of the PCa, to inactivate the factor Va of the tested plasma.

#### **2.2.2.6.2 Procedure**

The APC resistance test (APC-R) was performed using STA-Staclot APC-R kit (Diagnostica Stago, Asnieres, France). Patients' plasmas are tested

undiluted. They are loaded in the instrument. Dilutions with the buffer are automatically prepared by the instrument. Then the test to be performed was selected. Plasmas whose clotting times are equal to or greater than 120 seconds are regarded as APC-R negative, on the other hand, plasmas whose clotting times are less than 120 seconds are regarded as aPCR positive (Kadauke *et al.*, 2014). The results were expressed as a ratio and values  $\geq 2.1$  were considered normal.

### **2.2.2.7 Prothrombin fragment 1+2**

#### **2.2.2.7.1 Principle**

Sandwich enzyme-linked immunosorbent assay (ELISA) is performed for the quantitative detection of prothrombin fragment 1+2 using Human Prothrombin Fragment 1+2 ELISA Kit (Abbexa, Cambridge Science Park, UK).

Antibody specific for F1+2 had been pre-coated onto a microplate.

Standards and samples are pipetted into the wells and any F1+2 present is bound by the immobilized antibody. An enzyme-linked antibody specific for F1+2 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color developed in proportion to the amount of F1+2 bound in the initial step. The color development is stopped and the intensity of the color is measured (Hursting *et al.*, 1993, Bozic *et al.*, 2002, Suzuki *et al.*, 1993). Results  $< 9$  ng/ml are considered normal.

#### **2.2.2.7.2 Procedure**

1. Add 100 $\mu$ l of Standard, Blank, or Sample per well, cover with a plate sealer, and incubate for 90 minutes at 37°C.

2. Aspirate the liquid of each well, don't wash.
3. Add 100µl of detection antibody to each well, cover with a plate sealer, and gently agitate to ensure thorough mixing. Incubate for 1 hour at 37°C.
4. Aspirate the liquid from each well and wash 3 times. Wash by adding approximately 350 µl of Wash Buffer.
5. Add 100µl of HRP Conjugate working solution to each well, cover with a new plate sealer, and incubate for 30 minutes at 37°C.
6. Aspirate the liquid from each well and wash 5 times as outlined in step 4.
7. Add 90µl of TMB Substrate solution to each well, cover with a new plate sealer, and incubate for 15 minutes at 37°C. Protect from light and monitor periodically until optimal color development has been achieved.
8. Add 50µl of Stop Solution to each well. The blue color will change to yellow immediately. If color change does not appear uniform, gently tap the plate to ensure thorough mixing. The Stop Solution should be added to wells in the same order and timing as the TMB substrate solution.
9. Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm.

## **2.2.3 Molecular genetic testing**

### **2.2.3.1 DNA extraction**

#### **2.2.3.1.1 Principle**

Total genomic DNA was extracted from peripheral blood using a column based G-spin total DNA extraction mini kit (Intron biotechnology, Jungwon-gu, South Korea) according to the manufacturer's protocol (Kim *et al.*, 2014). The simple G-spin total DNA extraction procedure yields pure DNA ready for direct amplification in just 20 ~ 30 minutes. The purified DNA was free of protein, nucleases, and other contaminants or inhibitors. DNA purified using G-spin total DNA extraction mini kit was up to 50 kb in size, with fragments of approximately 20–30 kb predominating. DNA of this length denatures completely during thermal cycling and can be amplified with high efficiency (Kim *et al.*, 2014).

#### **2.2.3.2 DNA extraction protocol**

1. 200  $\mu$ l of whole blood was pipetted into a 1.5 ml microcentrifuge tube.
2. 20  $\mu$ l of Proteinase K and 5  $\mu$ l of RNase A Solution were added into sample tube and gently mixed properly.
3. 200  $\mu$ l of Buffer BL was added into upper sample tube and mixed thoroughly without vigorously vortexing because doing so may induce genomic DNA breakage.
4. The lysate was incubated at 56°C for 10 min, and for complete lysis, mixed 3 or 4 times during incubation by inverting tube until lysate becomes dark green.
5. The 1.5 ml tube was briefly centrifuged to remove drops from the inside of the lid.



6. 200  $\mu$ l of absolute ethanol was added into the lysate, and mixed well by gently inverting 5 - 6 times or by pipetting. After mixing, the 1.5 ml tube was briefly centrifuge to remove drops from the inside of the lid.
7. The mixture from step 6 was carefully added to the spin column (in a 2 ml collection tube) without wetting the rim, the cap was closed, and centrifuged at 13,000 rpm for 1 min. The filtrate was discarded and the spin column was placed in a 2 ml collection tube (reuse). Each spin column was closed in order to avoid aerosol formation during centrifugation.
8. 700  $\mu$ l of Buffer WA was added to the spin column without wetting the rim, and centrifuged for 1 min at 13,000 rpm. The flow-through was discarded and the collection Tube was reused.
9. 700  $\mu$ l of Buffer WB was added to the spin column without wetting the rim, and centrifuged for 1 min at 13,000 rpm. The flow-through was discarded and the column was placed into a 2.0 ml collection tube (reuse), Then again centrifuged for additionally 1 min to dry the membrane. The flow-through was discarded and collection tube altogether. It is very important to dry the membrane of the spin column since residual ethanol may inhibit subsequent reactions. Following the centrifugation, the spin column was removed carefully from the collection tube without contacting with the flow-through, since this will result in carryover of ethanol.
10. The spin column was placed into a new 1.5 ml tube, and 50  $\mu$ l of Buffer CE was added directly onto the membrane. Incubated for 1 min at room temperature and then centrifuged for 1 min at 13,000 rpm to elute.
11. Extracted genomic DNA was stored at  $-20^{\circ}\text{C}$  (Kim *et al.*, 2014).

### **2.2.3.3 DNA quantification**

DNA was quantified by measuring the absorbance at 260 nm using a NanoDrop spectrophotometer (ThermoFisher Scientific Inc, Germany) (Mierla *et al.*, 2012).

### **2.2.3.4 Polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis**

PCR-restriction fragment length polymorphism (RFLP)-based analysis is a popular technique for genotyping. The technique exploits that SNPs, MNPs and microindels often are associated with the creation or abolishment of a restriction enzyme recognition site (Narayanan, 1991). The first step in a PCR-RFLP analysis is amplification of a fragment containing the variation. This is followed by treatment of the amplified fragment with an appropriate restriction enzyme. Since the presence or absence of the restriction enzyme recognition site results in the formation of restriction fragments of different sizes, allele identification can be done by electrophoretic resolvment of the fragments. Important advantages of the PCR-RFLP technique include inexpensiveness and lack of requirement for advanced instruments. In addition, the design of PCR-RFLP analyses generally is easy and can be accomplished using public available programs .

PCR-RFLP is an extremely valuable technique for genotyping of species-specific variations. It is the most commonly used reference standard for genotyping of factor V Leiden 1691G>A and prothrombin 20210G>A (Emadi *et al.*, 2010).

In this study PCR-RFLP was performed for detection of factor V Leiden 1691G>A and prothrombin 20210G>A using two methods, standard PCR-RFLP and Primer engineered multiplex PCR-RFLP.

#### **2.2.3.4.1 Factor V Leiden 1691G>A genotyping**

Genotyping for factor V Leiden was performed using PCR-RFLP analysis with *HindIII* restriction endonuclease) (Gandrille *et al.*, 1995, Biswas *et al.*, 2009, Gupta *et al.*, 2003). A 241bp fragment encompassing the 1691G>A substitution of factor V exon 10 was PCR amplified using the forward primer 5'-TCAGGCAGGAACAACACCAT-3' and reverse primer 5'-GGTTACTTCAAGGACAAAATACCTGTAAAGCT-3' (EurofinsGenomics; Ebersberg, Germany). PCR reactions were prepared in a total volume of 25 µl, comprising 100 ng DNA, 12.5 µl of 2XGoTaq Green Master Mix (Promega, Madison, WI, USA), 1.0 µl of 10 pM/µl each primer, and 6.5 µl of Ambion nuclease-free water(not DEPC-treated) (Thermo Fisher Scientific, Waltham, MA, USA). PCR was performed using MultiGene OptiMax Thermal Cycler (Labnet International; Edison, NJ, USA) with the following conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 45 s, and a final extension at 72°C for 10 min. For RFLP analysis, 10 µl of PCR product was digested with 1.0 µl of *HindIII* (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C for at least 3 h. The digested products were separated by electrophoresis on a 2% agarose gel (Sigma-Aldrich Chemie, St. Louis, MO, USA) and stained with ethidium bromide. The expected banding patterns of digested PCR products were a 241bp fragment for the wild-type allele, 209bp and 32bp fragments for factor V

Leiden homozygotes, and all three molecular weight bands for factor V Leiden heterozygotes.

#### **2.2.3.4.2 Prothrombin 20210G>A genotyping**

Prothrombin gene polymorphism was detected according to method described by Poort *et al* (Poort *et al.*, 1996, Gupta *et al.*, 2003). Forward and reverse primers used for exon 14 were 5'-TCTAGAAACAGTTGCCTGGC-3' and 5'-ATAGCACTGGGAGCATTGAAGC-3', respectively. For RFLP analysis, PCR products were digested with *HindIII* due unavailability of *MnlI* enzyme. PCR and gel electrophoresis were performed using the same conditions as those described above for factor V Leiden genotyping. The expected banding patterns were a 345bp fragment for the wild-type allele, 322bp and 32bp fragments for homozygous mutants, and both molecular weight bands for heterozygous mutant.

#### **2.2.3.4.3 Single multiplex PCR-RFLP detection of factor V Leiden 1691G>A and Prothrombin 20210G>A**

Simultaneous detection methods were used for factor V Leiden 1691G>A and prothrombin 20210G>A mutations in which the digested amplification products were analyzed via agarose gel electrophoresis in a single gel lane and visualized by ethidium bromide staining (Huber *et al.*, 2000). A 241-bp region of exon 10 of Factor V gene, and 506 bp of the 3' untranslated region (3'UTR) of the prothrombin gene PCR amplified using gene-specific primers as previously described (Huber *et al.*, 2000), with some modifications. Mismatched antisense primers were used to amplify both products. Factor V primers were as follows: sense, 5'-TCA GGC AGG AAC AAC ACC AT-3'; antisense, 5'-GGT TAC TTC AAG GAC AAA ATA CCT GTA AAG CT-3'.

Prothrombin primers were as follows: sense, 5'-GCA CAG ACG GCT GTT CTC TT-3'; antisense, 5'-ATA GCA CTG GGA GCA TTG AAG C-3'. Briefly, each 25- $\mu$ l PCR reaction comprising of 100 ng genomic DNA, 12.5  $\mu$ l of 2X GoTaq Green Master Mix (Promega, Madison, WI, USA), 0.6  $\mu$ l of 10 pM each of the forward primers, 0.4  $\mu$ l of 10 pM each of the reverse primers, and 6.5  $\mu$ l of not DEPC-treated nuclease-free water (Thermo Fisher Scientific, Waltham, MA, USA). The PCR was performed as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, and a final extension time at 72°C for 5 min. Subsequently, 10  $\mu$ l of each PCR product was digested with 1.0  $\mu$ l of 10U *Hind*III (Thermo Fisher Scientific, Waltham, MA, USA) with 2  $\mu$ l of 10X enzyme buffer and 18  $\mu$ l of nuclease-free water at 37°C for at least 3 h. The digestion products were separated by gel electrophoresis on a 2% agarose gel (Sigma-Aldrich Chemie, St. Louis, MO, USA) and stained with ethidium bromide. Undigested products resulted in two products of 241 bp and 506 bp, representing Factor V and prothrombin amplicons, respectively. The digestion of wild-type alleles resulted in fragments of sizes 241 bp for Factor V and 407 bp + 99 bp for prothrombin. The digestion of factor V Leiden 1691G>A homozygous mutants produced 209 bp and 32 bp fragments, whereas digestion of prothrombin 20210G>A homozygous mutants produced three fragments of sizes 384 bp, 99bp, and 23bp. The digestion heterozygous individuals carrying factor V Leiden 1691G>A mutation yielded fragments of sizes 241 bp, 209 bp, and 32bp, whereas heterozygous prothrombin 20210G>A mutants yielded fragments of 407 bp, 384 bp, and 99 bp.

#### **2.2.4 Data analysis**

The Statistical Package for Social Sciences (IBM SPSS Statistics version 20; Armonk, NY, USA) was used for statistical analysis. The results were expressed as mean±SD. Continuous variables were analyzed by Student's t test. Comparison of the two groups in respect to the various categorical variables will be performed with  $\chi^2$  test. A p-value less than 0.05 were considered as statistically significant.

#### **2.2.5 Ethical approval**

This study was conducted in compliance with relevant national regulations, institutional policies, and the tenets of the Helsinki Declaration. Consent was obtained from every patient and healthy control participated in this study.

# Chapter Three

## 3. Results

### 3.1 Demographic Data

This study included a total of 100 patients (25 males, 75 females) with proven DVT diagnosis and 92 healthy controls (31 males, 61 females). No significant differences in the prevalence of DVT were detected between males and females ( $p = 0.185$ ) (Figure3.1). The mean age of the patients was  $41.60 \pm 17.28$  years, while that of controls was  $31.65 \pm 10.08$  years. Among the 100 patients studied, DVT was more prevalent at age 18 to 45 years in both males ( $n = 16$ ; 22.9%) and females ( $n=54$ ; 77.1%) (Table3.1). Among the 136 women, there was a significant difference between the mean age of DVT patients ( $40.75 \pm 16.630$  y) and controls ( $31.31 \pm 8.698$ y) ( $p=0.000$ ).

### 3.2 Clinical Data

The 100 DVT patients were divided into three age groups (18–45 years, 46–65 years, and 66–90 years) and assessed for the environmental risk factors that are generally known to predispose them to DVT (Table3.2). Risk factors, namely, immobility status and cardiovascular disease were the most significantly associated with age ( $p < 0.001$ ). Previous history of DVT was found in 73.3% of patients in the age group of 66–90 ( $n=15$ ).

Ninety-three percent of the DVTs were localized in the left leg and 7% in the right leg. Additionally, 88% of the DVTs were proximal and 12% were distal. Proximal DVT was significantly associated with age in all age groups ( $p < 0.0001$ ).

Among the 75 female patients, 29.33% were postpartum ( $n=22$ ), 9.33% were pregnant ( $n=7$ ), and 61.33% were non-pregnant( $n=46$ ). Of the 7 pregnant women, the number of women in their first, second, and third trimesters were 3(42.9%), 2(28.6%), and 2(28.6%), respectively. Of the 75 DVT women, 93.3% were present in the left leg and 6.7% in the right leg; moreover, 84% were proximal and 16% were distal (Table3.3). Among pregnant women, DVTs were always located on the proximal end of the left leg. Among postpartum women, however, DVTs were present in the right leg in 4 women and in the left leg in 8 women; all cases of DVTs were proximal among postpartum women. Table3.3 categorizes the 75 DVT patients according to various risk factors.

Additionally, the risk factors for DVT according to the age of female patients were examined (Table3.4). The 75 DVT patients were divided into three age groups (18–45 y, 46–65 y, and 66–90 y) and assessed for the environmental risk factors for DVT. Among the younger patients(18–45 y), postpartum and oral contraceptives usage were the most significant risk factors for DVT. By contrast, immobility status, cardiovascular disease, and history of DVT were the most significant risk factors affecting older patients (66–90 y).

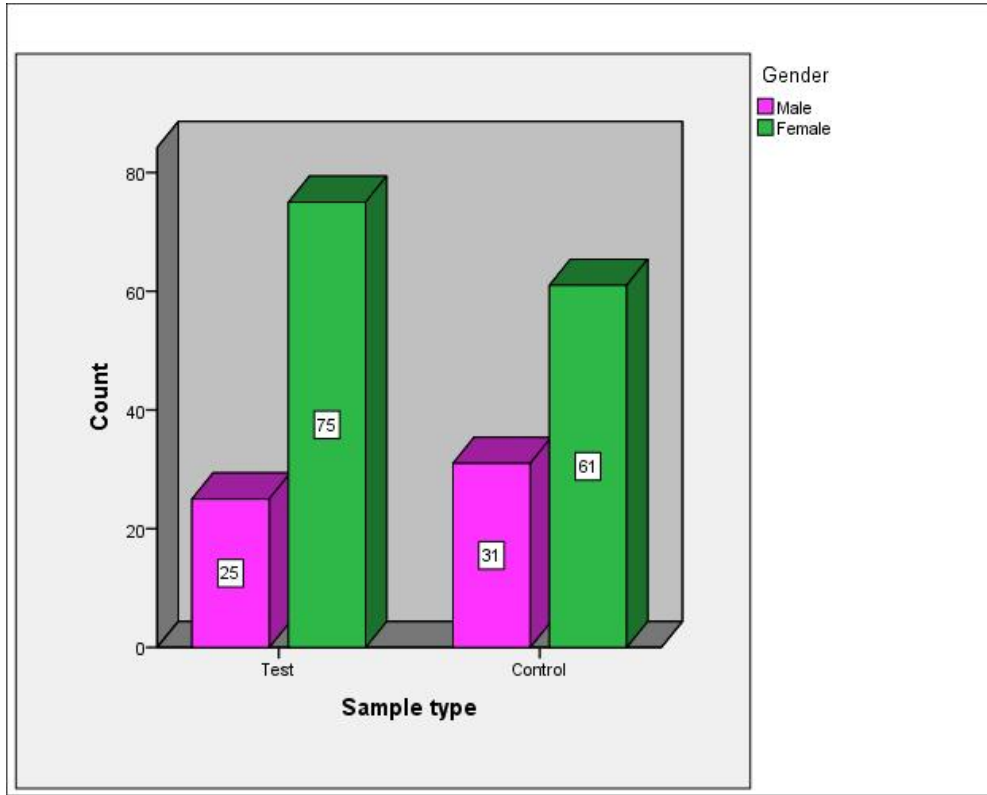
### **3.3 Laboratory Data**

Significant differences were observed between DVT patients and healthy controls in the levels of prothrombin fragment 1+2( $12.38 \pm 1.32$ ;  $4.99 \pm 2.44$ ), PT ( $23.36 \pm 9.62$ ;  $11.41 \pm 0.83$ ), APTT ( $41.82 \pm 13.59$ ;  $31.05 \pm 6.08$ ), and D-dimer ( $0.67 \pm 0.20$ ;  $0.30 \pm 0.16$ ), respectively ( $p < 0.05$ ) (Table3.5).



### **3.4 Molecular Data**

None of the 100 DVT patients tested positive for factor V Leiden 1691G>A mutation (Figure3.2) or the prothrombin 20210G>A mutation (Figure3.3) using standard uniplex PCR-RFLP. All 92 healthy controls were negative for both these mutations. Notably, using multiplex PCR-RFLP, factor V Leiden 1691G>A and prothrombin 20210G>A mutations were also absent from all individuals (Figure3.4). As a result, no correlation against the risk factors was possible.



**Figure 3.1** Distribution of study subjects according to gender using  $\chi^2$  test. ( $p = 0.185$ )

**Table 3.1 Demographic characteristics of DVT patients categorized in three age groups.**

		Age Group						p-value <sup>a</sup>
		18–45 y		46–65 y		66–90 y		
		F	%	F	%	F	%	
Gender	Male	16	22.9	3	20.0	6	40.0	0.368
	Female	54	77.1	12	80.0	9	60.0	

<sup>a</sup>P-values were determined using  $\chi^2$  test. NA: not applicable. F-frequency. \* significant.

**Table 3.2 Clinical characteristics of DVT patients categorized in three age groups.**

		Age Group						p-value <sup>a</sup>
		18–45 y		46–65 y		66–90 y		
		F	%	F	%	F	%	
Diagnosis	Proximal DVT	70	100.0	10	66.7	8	53.3	<0.0001*
	Distal DVT	0	0.0	5	33.3	7	46.7	
Previous history of DVT	yes	0	0.0	2	13.3	11	73.3	<0.0001*
	no	70	100.0	13	86.7	4	26.7	
Affected leg	Right leg	6	8.6	0	0.0	1	6.7	0.830
	Left leg	64	91.4	15	100.0	14	93.3	
Obesity	Yes	5	7.1	1	6.7	0	0.0	0.827
	No	65	92.9	14	93.3	15	100.0	
Oral Contraceptives	Yes	23	41.8	2	16.7	0	0.0	0.018
	No	32	58.2	10	83.3	9	100.0	
Cigarette Smoking	Yes	10	14.3	2	13.3	0	0.0	0.389
	No	60	85.7	13	86.7	15	100.0	
Under Surgery	Yes	30	42.9	3	20.0	4	26.7	0.167
	No	40	57.1	12	80.0	11	73.3	
Bone Fracture	Yes	3	4.3	0	0.0	0	0.0	1.000
	No	67	95.7	15	100.0	15	100.0	
Immobility Status	Yes	5	7.1	0	0.0	10	66.7	<0.0001*
	No	65	92.9	15	100.0	5	33.3	
Cardiovascular Disease	Yes	2	2.9	4	26.7	12	80.0	<0.0001*
	No	68	97.1	11	73.3	3	20.0	
Renal Failure	Yes	2	2.9	1	6.7	0	0.0	0.661
	No	68	97.1	14	93.3	15	100.0	
Cancer	Yes	3	4.3	0	0.0	1	6.7	0.766
	No	67	95.7	15	100.0	14	93.3	
Anticoagulant therapy	Heparin	44	62.9	8	53.3	1	6.7	1.000
	Warfarin	0	0.0	0	0.0	2	13.3	
	Heparin and Warfarin	26	37.1	7	46.7	12	80.0	

<sup>a</sup>P-values were determined using using <sup>2</sup> test. NA: not applicable. F-frequency. \* significant.

**Table 3.3 Risk factors in DVT women (n=75)**

	<i>n</i>	%
Age 40 yr.	35	46.7
Oral contraceptives	25	33.3
Postpartum	22	29.33
Under Surgery	18	24.0
Cardiovascular Disease	12	16.0
Previous history of DVT	8	10.7
Pregnancy	7	9.3
Immobility Status	7	9.3
Obesity	6	8.0
Renal Failure	2	2.7
Bone Fracture	1	1.3
Hormone therapy	0	0.0
Cigarette Smoking	0	0.0
Cancer	0	0.0
No risk factors	12	16.0
1 risk factor	32	42.7
2 risk factors	26	34.7
3 or more risk factors	5	6.7

**Table 3.4. Risk factors in DVT women according to age group**

		Age in groups				p-value <sup>a</sup>
		18 to 45	46 to 65	66 to 90		
		n (%)	n (%)	n (%)		
Previous history of DVT	Yes	0 (0.0)	1 (8.3)	7 (77.8)	<0.0001*	
	No	54 (100.0)	11 (91.7)	2 (22.2)		
Obesity	Yes	5 (9.3)	1 (8.3)	0 (0.0)	0.637	
	No	49 (90.7)	11 (91.7)	9 (100.0)		
Pregnancy	Yes	5 (9.3)	1 (8.3)	0 (0.0)	0.223	
	No	49 (90.7)	11 (91.7)	9 (100.0)		
Postpartum	Yes	7 (13.0)	0 (0.0)	0 (0.0)	0.002*	
	No	47 (87.0)	12 (100.0)	9 (100.0)		
Oral Contraceptives	Yes	19 (35.2)	2 (16.7)	0 (0.0)	0.059	
	No	35 (64.8)	10 (83.3)	9 (100.0)		
Under Surgery	Yes	14 (25.9)	2 (16.7)	2 (22.2)	0.787	
	No	40 (74.1)	10 (83.3)	7 (77.8)		
Bone Fracture	Yes	1 (1.9)	0 (0.0)	0 (0.0)	0.821	
	No	53 (98.1)	12 (100.0)	9 (100.0)		
Immobility Status	Yes	2 (3.7)	0 (0.0)	5 (55.6)	<0.0001*	
	No	52 (96.3)	12 (100.0)	4 (44.4)		
Cardiovascular Disease	Yes	2 (3.7)	3 (25.0)	7 (77.8)	<0.0001*	
	No	52 (96.3)	9 (75.0)	2 (22.2)		
Renal Failure	Yes	1 (1.9)	1 (8.3)	0 (0.0)	0.393	
	No	53 (98.1)	11 (91.7)	9 (100.0)		
Cancer	No	54 (100.0)	12 (100.0)	9 (100.0)	No p-value	

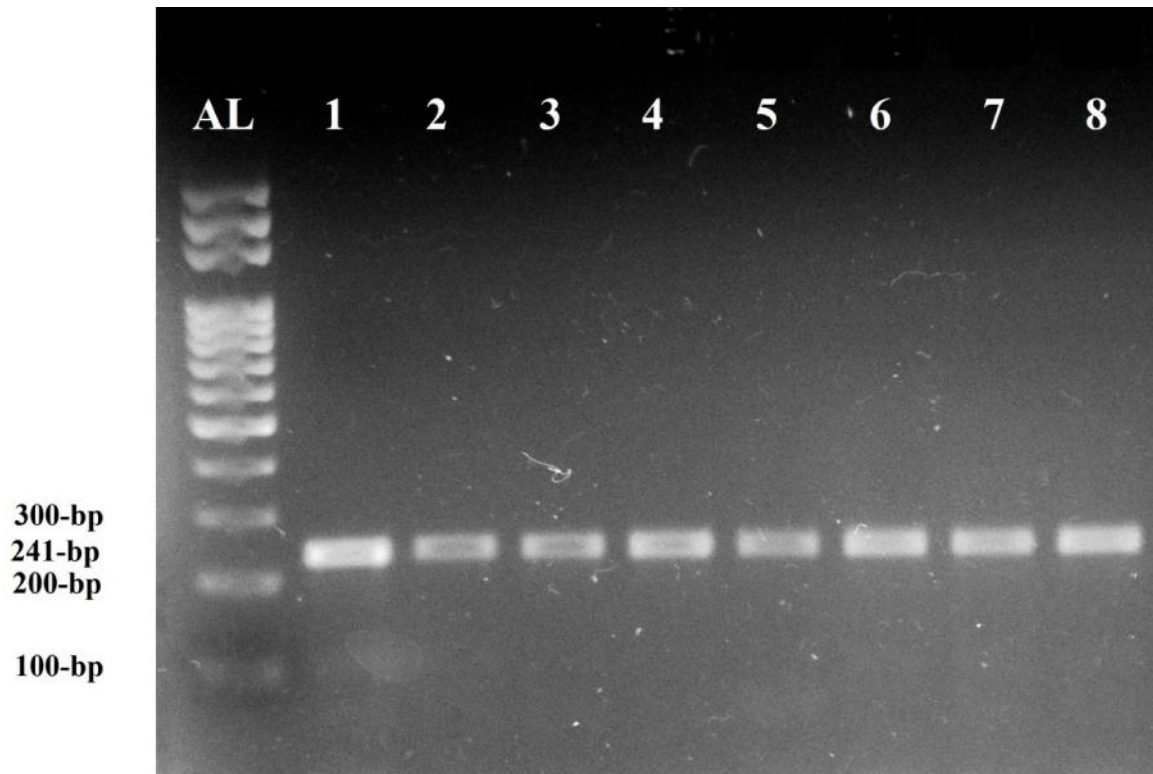
<sup>a</sup>P-values were determined using <sup>2</sup> test. NA: not applicable. F-frequency. \* significant

**Table 3.5 Coagulation characteristics in DVT patients and healthy controls group.**

Test	Mean± SD		p-value <sup>a</sup>
	Controls	Patients	
Prothrombin Time (s)	11.41 ± 0.83	23.36 ± 9.62	<0.0001*
Activated Partial Thromboplastin Time (s)	31.05 ± 6.08	41.82 ± 13.59	<0.0001*
D-dimer (mg/L)	0.30 ± 0.16	0.67 ± 0.20	0.044*
Prothrombin Fragment 1+2 (ng/mL)	4.99 ± 2.44	12.38 ± 1.32	<0.0001*
Activated Protein C resistance (Ratio)	2.67 ± 0.34	2.66 ± 0.30	0.850
Fibrinogen (g/L)	2.90± 0.49	2.93 ± 0.46	0.766

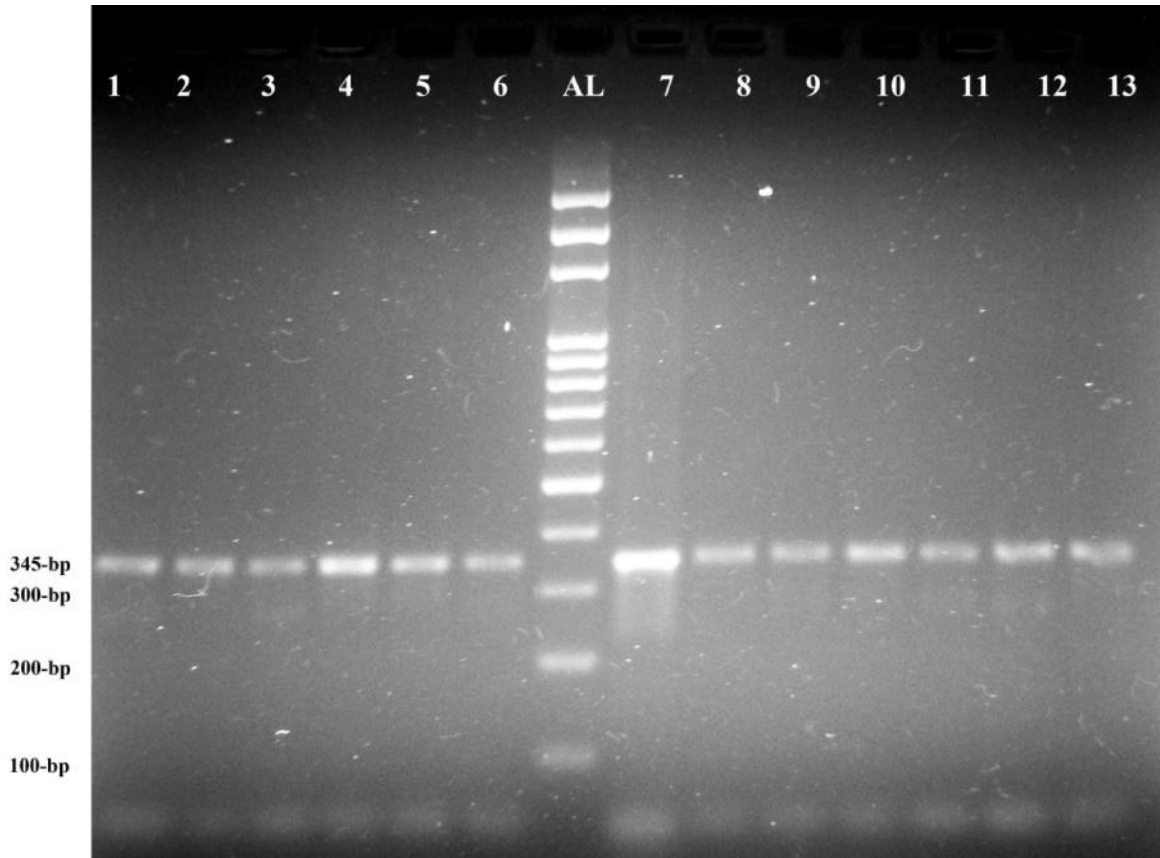
<sup>a</sup>Statistical significance of the differences between patients and controls were determined using Student's *t*-test.

\* significant.

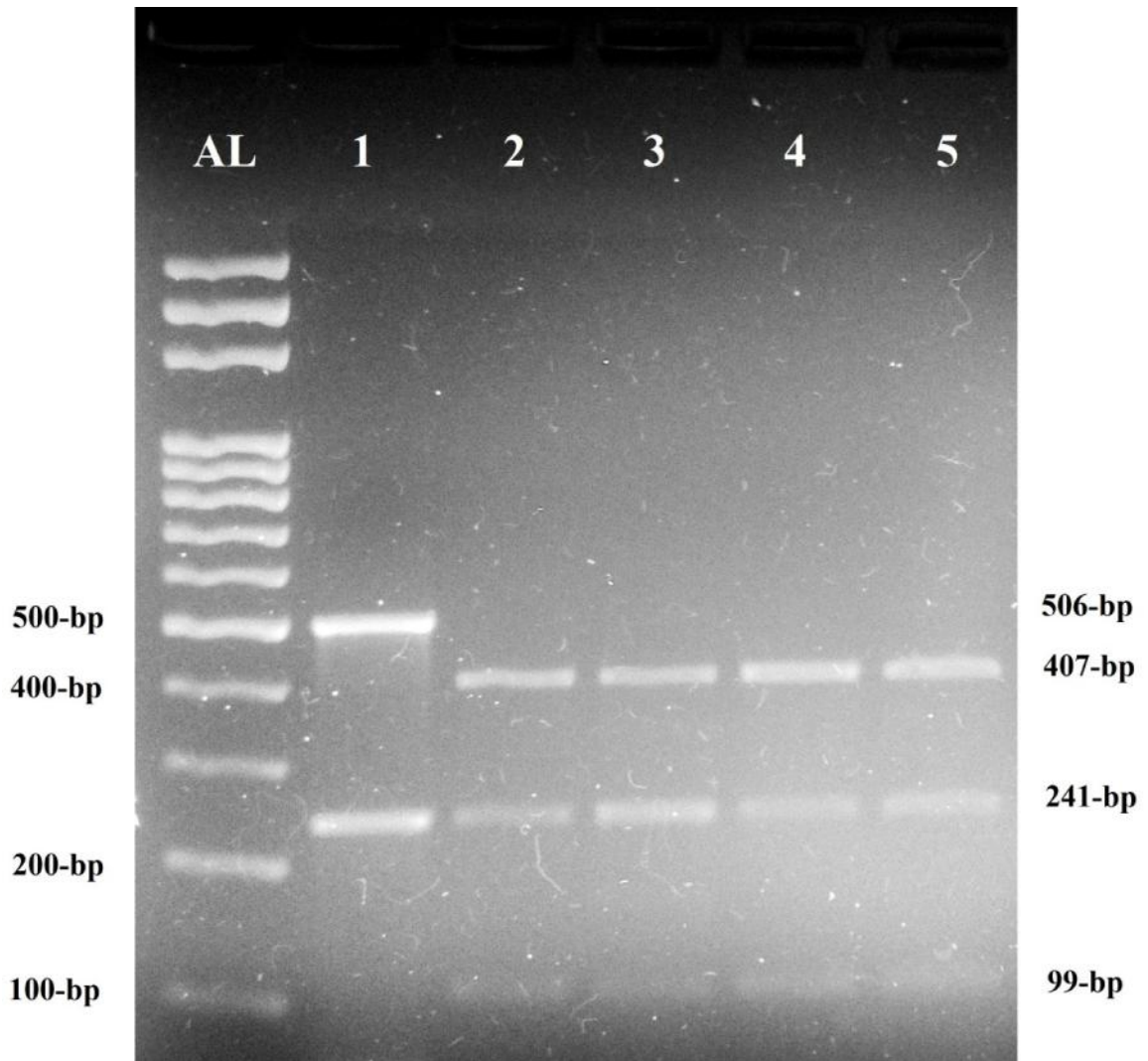


**Figure 3.2. Image of ethidium bromide-stained gel showing PCR-RFLP analysis of seven representative samples for detecting factor V Leiden 1691G>A mutation.** All samples carried the wild-type allele of factor V, indicated by the 241bp band. AL: 100 bp Allelic leader, 1: undigested PCR product (241 bp), and 2–8: PCR products digested with *HindIII*.





**Figure 3.3** Image of ethidium bromide-stained gel showing PCR-RFLP analysis of twelve representative samples for detecting prothrombin 20210G>A mutation. All samples carried the wild-type allele of prothrombin, indicated by the 345bp band. AL: 100 bp Allelic leader, 7: undigested PCR product (345 bp) amplicon, 1–6 and 8–13: PCR products digested with *HindIII*.



**Figure 3.4** Photograph of ethidium bromide-stained 2% agarose gel analysis of Primer engineered multiplex PCR-RFLP for factor V Leiden 1691G>A and prothrombin 20210G>A. AL denotes 100 bp DNA ladder. Lane 1: Undigested wild-type 506 bp (prothrombin) and 241 bp (FV). Lanes 2-5: Digested wild-type amplicons 407 bp and 99 bp (prothrombin) and 241 (FV).

# Chapter Four

## 4. Discussion, Conclusions And Recommendations

### 4.1 Discussion

In present study, a total of 192 individuals of Sudanese origin were evaluated, of which 100 were DVT patients and 92 were healthy controls. No significant differences were observed in the prevalence of DVT between male and female patients in this study in comparison with healthy subjects. However, among younger adults ( 45 years of age), DVT was more prevalent in females than in males, which is consistent with previously published data (White, 2003, Fowkes *et al.*, 2003).

Findings of this study showed that 13% of DVT patients, most of who were in the age group of 66–90years had previously had single or recurrent episodes of DVT. This is consistent with the results of Anderson *et al.* showing that the previous history of VTE is significantly associated with increased risk of DVT (Anderson and Spencer, 2003). Additionally, immobility and cardiovascular disease were found to be significantly associated with the occurrence of DVT among the elderly, which is in agreement with the results of Engbers *et al.*(Engbers *et al.*, 2010). Our findings of other environmental risk factors were consistent with previous studies (Anderson and Spencer, 2003, Cushman, 2007, Crous-Bou *et al.*, 2016), except for obesity (6%), which maybe because of dietary and behavioral differences. Our study confirms the findings of previous studies that have reported the predominance of left-sided DVT(Thijs *et al.*, 2010). The reason behind the left-side predominance is unknown. Previous studies

proposed that left-sided DVTs may be related to compression of the left common iliac vein by the right common iliac artery (Narayan *et al.*, 2012, Thijs *et al.*, 2010). Additionally, this study revealed that in most of the cases, DVTs were proximal. This was in complete agreement with a previous study (Cogo *et al.*, 1993).

Over half of all venous thrombotic events were related to pregnancy and puerperium in women of reproductive age, (Pomp *et al.*, 2008, Martinelli *et al.*, 2002). However, this study found that 29.3% of DVT women were postpartum and 9.3% were pregnant, indicating that both periods were associated with high risk of venous thrombosis. These data are in agreement with previous studies reporting approximately two and five times as many postpartum as antepartum DVT events, respectively (Simpson *et al.*, 2001, Heit *et al.*, 2005, Pomp *et al.*, 2008). Moreover, Gader *et al.* previously reported that most of the DVT events among Sudanese women occurred in the postpartum period (Gader *et al.*, 2009). Consistent with our findings, James *et al.* previously reported that the risk of DVT was higher in pregnant women in their first trimester than those in their second or third trimesters. Thus, if prophylaxis is indicated, it should be initiated early in gestation (James *et al.*, 2005). However, DVT rates in the third trimester were higher according to a previous meta-analysis (Ray and Chan, 1999). Here, we observed that in most of the cases, DVTs occurred in the lower left extremity and were proximal. This was particularly true among pregnant women; DVTs in all pregnant women occurred on the proximal end of the left leg. These observations were in complete agreement with previous studies (James, 2009, Galanaud *et al.*, 2009, Gader *et al.*, 2009, Ray and

Chan, 1999). However, why the left leg is the preferred site for DVTs is unknown (Ginsberg *et al.*, 1992).

Venous thrombosis was commonly a disease of older adults (Cushman, 2007). Our study revealed a significant difference between the mean age of DVT female patients and healthy controls. Approximately 47% of the DVT patients were more than 40 y old. However, this also means that 53% of the DVT patients were less than 40 y of age. This was expected, as the incidence rates of DVT are slightly higher in women in their childbearing age (Heit, 2015). When DVT female patients were divided into three groups according to age, we found that cardiovascular diseases and history of venous thrombosis were the most predominant risk factors among elderly women, followed by immobility, which affected more than half of the elderly women. This was consistent with a previous study (Engbers *et al.*, 2010). Among younger patients, one of the risk factor most significantly related to DVT was postpartum. This findings was in accordance with previous reports (Fall *et al.*, 2014, Gader *et al.*, 2009, Pomp *et al.*, 2008, Heit *et al.*, 2005). A significant association between the use of oral contraceptives and DVT development in female patients in the age group from 18–45 years was identified in our study, this was in the same line with previous studies on the increased risk of VTE and oral contraceptives usage in young females (Lidegaard *et al.*, 2002).

Since 1996, a large number of observational studies have consistently shown a significant (2–4-fold) increase in the relative risk of developing venous thromboembolism in HRT users compared with nonusers (Kujovich, 2004). Because none of the women involved in this study were undergoing HRT, its association with DVT in this study group was not investigated.

Fibrin-related markers, such as D-dimer and prothrombin fragment 1+2 are considered to be useful for the diagnosis of thrombosis (Satoshi *et al.*, 2008). In agreement with the results reported by Ota *et al.*(Satoshi *et al.*, 2008), significantly higher levels of plasma D-dimer and prothrombin fragment 1+2 were detected in DVT patients than those in healthy controls.

None of the 192 individuals carried factor V Leiden 1691G>A or prothrombin 20210G>A mutations, indicating the absence of a potential genetic drift due to founder effect that may be present in individuals of European origin (Jadaon, 2011, Zivelin *et al.*, 1997). Moreover, these data also showed that neither one of these gene mutations were associated with DVT in individuals of Sudanese origin, which is consistent with a previous study on an African population (Fall *et al.*, 2014, Alfatih Aboalbasher *et al.*, 2017). Similarly, factor V Leiden 1691G>A has been shown to be absent in 1600 individuals from Africa, Southeast Asia, Australasia, and the Americas (Rees *et al.*, 1995). The results of this study were consistent with a previous study in Iran reported that factor V Leiden 1691G>A or prothrombin 20210G>A mutations were not associated with thrombophilia (Karimi *et al.*, 2015). Factor V Leiden 1691G>A or prothrombin 20210G>A mutations were absent in DVT cases studied in Chinese population (Jun Z *et al.*, 2006). Moreover, Prothrombin gene mutation as risk for inherited thrombophilia was absent or occurs in a very low frequency in both thrombophilic patients and healthy controls of most South Asian populations (Gunathilake *et al.*, 2015). However, a previous meta-analysis has shown that the factor V Leiden 1691G>A mutation is associated with the risk of DVT in other populations (Dentali *et al.*, 2012). Also, the findings of this study were in contrast with a previous study in Bosnia and Herzegovina, where factor V

Leiden 1691G>A and prothrombin 20210G>A mutations were associated with DVT in Bosnian population (Jusi -Kari *et al.*, 2016). However, it is expected to get conflicting results for factor V Leiden 1691G>A when studying healthy populations (Abdullah *et al.*, 2010). Another reason for the discrepancy between studies could be the small number of subjects recruited for the study. It is noteworthy that the findings obtained in this study were reliable since two different well-designed PCR-RFLP assays were performed to eliminate sources of false negative results. Nonetheless, it is noteworthy that in Sudan, one of the economically struggling countries, it is a priority to adopt robust, cost-effective, and fast diagnostic tools for molecular analysis. The multiplex PCR-RFLP-based analysis used in this study for the simultaneous detection of both factor V Leiden 1691G>A and prothrombin 20210G>A mutations was a stable and reproducible single-tube reaction requiring no special or expensive equipment and only a small amount of standard PCR reagents.

Available data suggest that factor V Leiden 1691G>A carriers are at a 5–16-fold greater risk of developing DVT during pregnancy and puerperium than women homozygous for the wild-type Factor V, and are at a 35–100-fold greater risk among carriers using oral contraceptives(Kujovich, 2004). The risk of pregnancy-associated venous thrombosis was 15-fold higher in women with the prothrombin gene mutation than those with the wild-type prothrombin gene. Moreover, the combination of oral contraceptives with the prothrombin gene mutation has a multiplicative effect on the overall thrombotic risk, with odds ratio(OR)ranging from 16 to 59, as suggested previously(Kujovich, 2004). However, this study demonstrated a total absence of factor V Leiden 1691G>A and prothrombin 20210G>A

mutations among Sudanese women. Therefore, no association was observed between these mutations and oral contraceptives, pregnancy or puerperium in our study population.

However, There were certain limitations in this study. Specifically the relatively small sample size used. It is recommended that the future studies be performed with larger sample size to cover all parts of Sudan with an emphasis on environmental factors and other possible causes of hereditary thrombophilia other than factor V Leiden 1691G>A and prothrombin 20210G>A mutations as predisposing factors of DVT.



## 4.2 Conclusions

This study concluded that:

- No significant differences in the prevalence of DVT between male and female patients in comparison with healthy subjects.
- D-dimer and prothrombin fragment 1+2 are considered to be useful for the diagnosis of DVT.
- Previous history of DVT, immobility status and cardiovascular disease were the most significantly associated with age.
- Women who are pregnant or in postpartum period and those using oral contraceptives are at a higher risk of developing DVT than other women.
- Older women with a history of DVT, cardiovascular disease, or prolonged immobility are also at high risk of developing DVT.
- Factor V Leiden 1691G>A and prothrombin 20210G>A mutations were not associated with DVT in the Sudanese population examined in this study.

### **4.3 Recommendations**

- Despite to our findings using an efficient and cost-effective PCR-RFLP method, whenever possible, we recommend using more transient molecular methods such as real time PCR and melting curve analysis which are more suitable for larger studies.
- The multiplex PCR-RFLP analysis is a highly efficient and cost-effective method, and therefore ideal for laboratories with limited budget.
- The high rates of DVT among female patients highlights the need to consider an extended clinical and socioepidemiologic factors investigation of such patients and the need to decrease the incidence particularly in specific high risk groups during pregnancy and postpartum. Moreover, as none of the women involved in this study were undergoing HRT, further studies are needed to assess this risk group.

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# APPENDICES

## Appendix 1. Questionnaire

Date of collection: ----- Research ID number: -----  
Name of patient: ----- Age (in year) -----  
Gender: Male  Female   
Initial diagnosis: ----- Proximal  Distal   
Affected leg Right  Left   
Duration of illness: -----  
Pregnant: Yes  No   
Postpartum Yes  No   
Under surgery: Yes  No   
Immobility status: Yes  No   
Bone fracture: Yes  No   
Clotting disorder: Yes  No   
Hormone therapy: Yes  No   
Cancer: Yes  No   
Oral contraceptive : Yes  No   
Cigarette smoking : Yes  No   
Renal Failure Yes  No   
Heart disease Yes  No   
On Warfarin anticoagulant Yes  No   
On heparin anticoagulant : Yes  No   
family history of thromboembolism: Yes  No   
How many members of your family have thrombosis? .....

Sig..... Date-----



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## DESCRIPTION

- G-spin™ Total DNA Extraction Mini Kit provide fast and easy methods for purification of total DNA from cultured animal cell, animal tissue, rodent tail, fixed tissue, animal hair, gram negative bacteria, and blood samples for reliable PCR and Southern blotting. Furthermore, we have tested G-spin™ Total DNA Extraction Mini Kit to get more practical data with a lot numbers of biological samples.
- The simple G-spin™ Total DNA Extraction procedures, which are ideal for simultaneous processing of multiple samples, yield pure DNA ready for direct amplification in just 20 ~ 30 minutes. The G-spin™ Total DNA Extraction Mini Kit procedure is suitable for use with fresh or frozen whole blood and blood which has been treated with citrate, heparin, or EDTA. Pre-separation of leukocytes is not necessary.
- Purification requires no phenolchloroform extraction or alcohol precipitation, and involves very little handling. DNA is eluted in Buffer CE, TE (10:1), 10mM Tris (pH 7.5 ~ 8) or water, ready for direct addition to PCR or other enzymatic reactions. Alternatively, it can be safely stored at -20°C for later use. The purified DNA is free of protein, nucleases, and other contaminants or inhibitors. DNA purified using G-spin™ Total DNA Extraction Mini Kit is up to 50 kb in size, with fragments of approximately 20-30 kb predominating. DNA of this length denatures completely during thermal cycling and can be amplified with high efficiency.
- G-spin™ Total DNA Extraction Mini Kit provides various protocols. You can also extract genomic DNA from various your biological samples by selecting an appropriate protocol from Protocol list (see Table 1). If you need some more information in selecting a protocol, please do not hesitate to contact our Technical Assistance Team.

## CHARACTERISTICS

- **Speed** : Takes only 20 ~ 30 minutes to extract genomic DNA.
- **Smart** : High quality and quantity of DNA recovery
- **Steady** : Complete removal of inhibitors and contaminants for accurate down stream applications. And the freeze-dried formulated enzyme has been improved DNA extraction stability.
- **Stage-up** : No need various DNA Extraction kit – vast applicability. The Kit is suitable to use various kinds of biological samples. Advanced GxN technology for rapid and efficient purification of DNA without ethanol precipitation.



**INTEKRON**  
Biotechnology

G-spin™ Total DNA Extraction Kit



**INTEKRON**  
Biotechnology

Instruction Manual - Jan. 2012, B7-0105-GT104 (Rev. 2012.01)

## KIT CONTENTS

Label	Contents 50 Columns	Contents 200 Columns
Buffer CL	25 ml	90 ml
Buffer BL <sup>1</sup>	25 ml	90 ml
Buffer WA <sup>1</sup>	40 ml	160 ml
Buffer WB <sup>2</sup>	10 ml	40 ml
Buffer CE <sup>3</sup>	20 ml	40 ml
Spin Column <sup>4</sup> / Collection Tube <sup>5</sup>	50 ea	200 ea
RNase A (Lyophilized powder) <sup>6</sup>	3 mg x 1 vial	3 mg x 4 vials
Proteinase K (Lyophilized powder) <sup>6</sup>	22 mg x 1 vial	22 mg x 4 vials

1. This buffer contains chaotropic salt.

2. Before use, add 40ml (500µl) of absolute EtOH to the washing buffer.

3. DNase I/RNase free Ultra-Pure solution.

4. The columns contain silica membranes.

5. Polypropylene tube for ziti volume.

6. The lyophilized RNase A and Proteinase K can be stored at room temperature (15-25°C) until the kit expiration date without affecting performance. The lyophilized RNase A and Proteinase K can only be dissolved in D.W.; dissolved RNase A and Proteinase K should be immediately stored at -20°C. These solutions are stable at -20°C for up to 24 months and 20 times frozen-thawing until the kit expiration date.

## STORAGE


G-spin™ Total DNA Extraction Kit should be stored dry at room temperature (15-25°C). Under these conditions, G-spin™ Total DNA Extraction Kit can be stored for up to 24 months without showing any reduction in performance and quality. The lyophilized RNase A and Proteinase K can be stored at room temperature (15-25°C) until the kit expiration date without affecting performance. The lyophilized RNase A and Proteinase K can only be dissolved in D.W.; dissolved RNase A and Proteinase K should be immediately stored at -20°C. These solutions are stable at -20°C for up to 24 months and 20 times frozen-thawing until the kit expiration date.

## CONSIDERATION BEFORE USE

-  **Lyophilized RNase A:** Dissolve the RNase A in 0.3 ml of pure D.W. to each vial. The lyophilized RNase A can be stored at room temperature (15-25°C) until the expiration date without affecting performance. The lyophilized RNase A can only be dissolved in D.W.; dissolved RNase A should be immediately stored at -20°C. The RNase A solution is stable at -20°C for up to 24 months and 20 times frozen-thawing until the kit expiration date.
-  **Lyophilized Proteinase K:** Dissolve the Proteinase K in 1.1 ml of pure D.W. to each vial. The lyophilized Proteinase K can be stored at room temperature (15-25°C) until the expiration date without affecting performance. The lyophilized Proteinase K can only be dissolved in D.W.; dissolved Proteinase K should be immediately stored at -20°C. The Proteinase K solution is stable at -20°C for up to 24 months and 20 times frozen-thawing until the kit expiration date.
- Buffer WB**  
 Buffer WB is supplied as concentrate. Before using for the first time, be sure to add 40 ml (160 ml) of absolute ethanol (96 - 100%) to obtain a working solution.
- Preheat a water bath or heating block.
- Equilibrate samples to room temperature (15-25°C).
- Equilibrate Buffer IE or distilled water for elution to room temperature.
- If Buffer CL or Buffer BL contains precipitates, dissolve by heating to 70°C with gentle agitation.
- Centrifugation  
 All centrifugation steps are carried out at RT (15 - 25°C)

## SAFETY INFORMATION

All chemicals should be considered as potentially hazardous. When working with chemicals, always wear a suitable lab coat and disposable glove. Some buffer contain the chaotropic salt which may be an irritant and carcinogen, so appropriate safety apparel such as gloves and eye protection should be worn. If a spill of the buffers occurs, clean with a suitable laboratory detergent and water. If the liquid spill contains potentially infectious agents, clean the affected area first with laboratory detergent and water, then with a suitable laboratory disinfectant. Only persons trained in laboratory techniques and familiar with the principles of good laboratory practice should handle these products.

 **DO NOT** add bleach or acidic solutions directly to the sample preparation waste.



G-spin™ Total DNA Extraction Kit



Instruction Manual, Jan. 2012, B7-015-GT104 (Rev. 2012.01)



### PROTOCOL LIST

Table 1. Protocols according to the sample groups (8 Protocols)

Samples	Protocol Type
Blood, Body Fluids	Type A Protocol
Tissues, Rodent tail	Type B Protocol
Cell, Buffy coat	Type C Protocol
Dried Blood Spots	Type D Protocol
Fixed Tissues	Type E Protocol
Bacteria	Type F Protocol
Biological Swabs	Type G Protocol
Animal Hair	Type H Protocol

### SAMPLE PREPARATION

#### Amounts of starting material

Use the amounts of starting material indicated in Table 2.

Table 2. Amounts of starting material for G-spin™ Total Kit procedures

Sample	Amount
Blood, plasma, serum	200 µl
Buffy coat	200 µl
Tissue	25 mg*
Cultured cells	1 x 10 <sup>6</sup> cells
Bacterial culture (Liquid Culture)	3.00 (OD <sub>600</sub> )

\*When isolating DNA from spores, 10 mg samples should be used.

Small samples should be adjusted to 200 µl with PBS before loading. For samples larger than 200 µl, the amount of Buffer CL and other reagents added to the sample before loading must be increased proportionally. Application of the lysed sample to the spin column will require more than one loading step if the initial sample volume is increased. The amounts of Buffer WA and Buffer WB used in the wash steps do not need to be increased. Scaling up the tissue protocol is possible in principle. The user should determine the maximum amount of tissue used. It is important not to overload the column, as this can lead to significantly lower yields than expected.



#### Preparation of buffy coat

Buffy coat is a leukocyte-enriched fraction of whole blood. Preparing a buffy-coat fraction from whole blood is simple and yields approximately 5–10 times more DNA than an equivalent volume of whole blood. Prepare buffy coat by centrifuging whole blood at 2,500 x g for 10 minutes at room temperature. After centrifugation, 3 different fractions are distinguishable: the upper clear layer is plasma; the intermediate layer is buffy coat, containing concentrated leukocytes; and the bottom layer contains concentrated erythrocytes.

#### Copurification of RNA

G-spin™ Total Kit spin columns are able to copurify DNA and RNA when both are present in the sample (see Table 3). RNA may inhibit some downstream enzymatic reactions but will not inhibit PCR. If RNA contained genomic DNA is required, the treatment of RNase A should be bypassed to the sample.

Table 3. Amounts of starting material for G-spin™ Total Kit procedures

Sample	DNA composition	RNA composition
Blood, plasma, serum, Buffy coat	95–100 %	0–5%
Tissues (Liver, Heart / Others)	15–20% / 40–50%	80–85%/50–60%
Cultured cells, Bacterial culture	30–50%	50–80%

### COLUMN INFORMATION

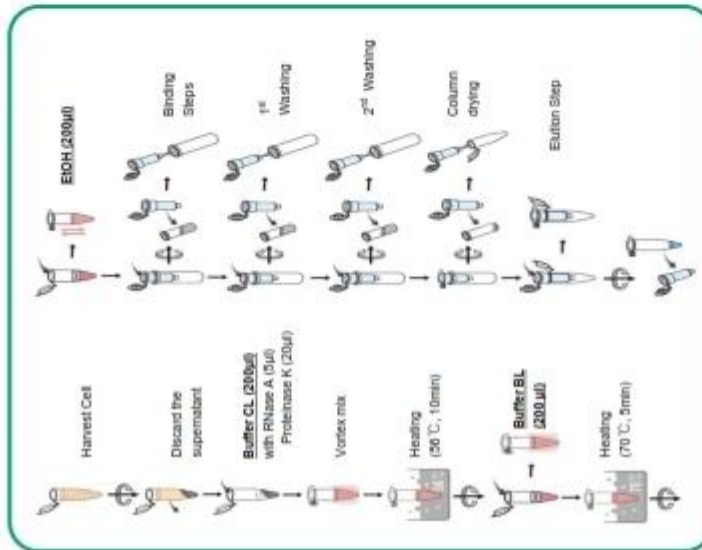
- The G-spin™ Total DNA Extraction Kit Spin Column

Column membrane <sup>1</sup>	Silica-based membrane
Spin Column <sup>1</sup>	Individually, in inserted in a 2.0 ml Collection Tube
Loading Volume	Maximum 800 µl
DNA Binding Capacity	Maximum 45 µg
Recovery	85 - 95% depending on the elution volume
Elution Volume	Generally, eluted with 30 - 200 µl of elution buffer

<sup>1</sup> Do not store the Column packs under completely dried conditions. It may be affected to DNA binding capacity. The Spin Columns are stable for over 2 year under these conditions



### Quick Guide – Cell gDNA Extraction



### PROTOCOL A (for Blood, body fluids)

1. Pipet 200 µl of whole blood or body fluids into a 1.5 ml microcentrifuge tube (not provided).

Note : If the volume of sample is less than 200 µl, use Buffer CL or PBS Buffer

2. Add 20 µl of Proteinase K and 5 µl of RNase A Solution into sample tube and gently mix.

Note : It is possible to add Proteinase K to blood sample that have already been measured into 1.5 ml tube. It is important to assure proper mixing after adding the Proteinase K and RNase A solution.

3. Add 200 µl of Buffer BL into upper sample tube and mix thoroughly.

Note : Avoid any vigorous vortexing because doing so may induce genomic DNA leakage. In order to assure efficient lysis, it is important that the blood sample and Buffer BL are mixed thoroughly to yield a lysis solution.

4. Incubate the lysate at 56 °C for 10 min.

Note : For complete lysis, mix 3 or 4 times during incubation by inverting tube. If it lysis perfectly, the red color of lysate becomes the dark green.

5. Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the rtd.

6. Add 200 µl of absolute ethanol into the lysate, and mix well by gently inverting 5 - 6 times or by pipetting. DO NOT vortex. After mixing, briefly centrifuge the 1.5 ml tube to remove drops from inside of the lid.

Note : This step is an equilibration step for binding genomic DNA to column membrane. It is important to assure proper mixing after adding the ethanol, until not showing 2-phase which is not mixed. Also, this step conduces to gDNA efficiently cell lysate through a column.

7. Carefully apply the mixture from step 6 to the Spin Column (in a 2 ml Collection Tube) without wetting the rim, close the cap, and centrifuge at 13,000 rpm for 1 min. Discard the filtrate and place the Spin Column in a 2 ml Collection Tube (reuse).

Note : Close each Spin Column in order to avoid aerosol formation during centrifugation. Do not transfer any solid materials.

8. Add 700 µl of Buffer WA to the Spin Column without wetting the rim, and centrifuge for 1 min at 13,000 rpm. Discard the flow-through and reuse the Collection Tube.

# Appendix 3. GoTaq® Green Master Mix Protocol

## Certificate of Analysis

### GoTaq® Green Master Mix

Cat. #	Size
M7121	10 reactions
M7122	100 reactions
M7123	1,000 reactions
M712B-C	1 × 1,250µl

Cat.# M7121, M7122 and M7123 include GoTaq® Green Master Mix, 2X, and Nuclease-Free Water.

Cat.# M712B-C does not include Nuclease-Free Water.

**Description:** GoTaq® Green Master Mix<sup>(A2)</sup> is a premixed ready-to-use solution containing bacterially derived Taq DNA polymerase, dNTPs, MgCl<sub>2</sub> and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. GoTaq® Green Master Mix contains two dyes (blue and yellow) that allow monitoring of progress during electrophoresis. Reactions assembled with GoTaq® Green Master Mix have sufficient density for direct loading onto agarose gels.

GoTaq® Green Master Mix is recommended for any amplification reaction that will be visualized by agarose gel electrophoresis and ethidium bromide staining. The master mix is not recommended if any downstream applications use absorbance or fluorescence excitation, as the yellow and blue dyes in the reaction buffer may interfere with these applications. The dyes absorb between 225–300nm, making standard A<sub>260</sub> readings to determine DNA concentration unreliable. The dyes have excitation peaks at 488nm and between 600–700nm that correspond to the excitation wavelengths commonly used in fluorescence detection instrumentation. However, for some instrumentation, such as a fluorescent gel scanner that uses a 488nm excitation wavelength, there will be minimal interference since it is the yellow dye that absorbs at this wavelength. Gels scanned by this method will have a light grey dye front (corresponding to the yellow dye front) below the primers.

**GoTaq® Green Master Mix, 2X:** GoTaq® DNA Polymerase is supplied in 2X Green GoTaq® Reaction Buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 3mM MgCl<sub>2</sub>. Green GoTaq® Reaction Buffer is a proprietary buffer containing a compound that increases sample density, and yellow and blue dyes, which function as loading dyes when reaction products are analyzed by agarose gel electrophoresis. The blue dye migrates at the same rate as 3–5kb DNA fragments, and the yellow dye migrates at a rate faster than primers (<50bp), in a 1% agarose gel.

**Storage Conditions:** See the Product Information Label for storage recommendations.



AP91M712 1016M712



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## Quality Control Assays

**Functional Assay:** GoTaq® Green Master Mix is tested for performance in the polymerase chain reaction (PCR). GoTaq® Green Master Mix, 1X, is used to amplify a 360bp region of the α-1-antitrypsin gene from 100 molecules of human genomic DNA. The resulting PCR product is visualized on an ethidium bromide-stained agarose gel.

**Nuclease Assays:** No contaminating endonuclease or exonuclease activity detected.

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Signed by:

*Ron Wheeler*  
R. Wheeler, Quality Assurance

## I. Standard Application

### Reagents to be Supplied by the User

template DNA                      downstream primer  
upstream primer                    mineral oil (optional)

1. Thaw the GoTaq® Green Master Mix at room temperature. Vortex the Master Mix, then spin it briefly in a microcentrifuge to collect the material at the bottom of the tube.
2. Prepare one of the following reaction mixes on ice:

#### For a 25µl reaction volume:

Component	Volume	Final Conc.
GoTaq® Green Master Mix, 2X	12.5µl	1X
upstream primer, 10µM	0.25–2.5µl	0.1–1.0µM
downstream primer, 10µM	0.25–2.5µl	0.1–1.0µM
DNA template	1–5µl	<250ng
Nuclease-Free Water to	25µl	N.A.

#### For a 50µl reaction volume:

Component	Volume	Final Conc.
GoTaq® Green Master Mix, 2X	25µl	1X
upstream primer, 10µM	0.5–5.0µl	0.1–1.0µM
downstream primer, 10µM	0.5–5.0µl	0.1–1.0µM
DNA template	1–5µl	<250ng
Nuclease-Free Water to	50µl	N.A.

#### For a 100µl reaction volume:

Component	Volume	Final Conc.
GoTaq® Green Master Mix, 2X	50µl	1X
upstream primer, 10µM	1.0–10.0µl	0.1–1.0µM
downstream primer, 10µM	1.0–10.0µl	0.1–1.0µM
DNA template	1–5µl	<250ng
Nuclease-Free Water to	100µl	N.A.

3. If using a thermal cycler without a heated lid, overlay the reaction mix with 1–2 drops (approximately 50µl) of mineral oil to prevent evaporation during thermal cycling. Centrifuge the reactions in a microcentrifuge for 5 seconds.
4. Place the reactions in a thermal cycler that has been preheated to 95°C. Perform PCR using your standard parameters.

## II. General Guidelines for Amplification by PCR

### A. Denaturation

- Generally, a 2-minute initial denaturation step at 95°C is sufficient.
- Subsequent denaturation steps will be between 30 seconds and 1 minute.

### B. Annealing

- Optimize the annealing conditions by performing the reaction starting approximately 5°C below the calculated melting temperature of the primers and increasing the temperature in increments of 1°C to the annealing temperature.
- The annealing step is typically 30 seconds to 1 minute.

### C. Extension

- The extension reaction is typically performed at the optimal temperature for Taq DNA polymerase, which is 72–74°C.
- Allow approximately 1 minute for every 1kb of DNA to be amplified.
- A final extension of 5 minutes at 72–74°C is recommended.

### D. Refrigeration

- If the thermal cycler has a refrigeration or “soak” cycle, the cycling reaction can be programmed to end by holding the tubes at 4°C for several hours.
- This cycle can minimize any polymerase activity that might occur at higher temperatures, although this is not usually a problem.

### E. Cycle Number

- Generally, 25–30 cycles result in optimal amplification of desired products.
- Occasionally, up to 40 cycles may be performed, especially for detection of low-copy targets.

## III. General Considerations

### A. GoTaq® Green Master Mix Compatibility

GoTaq® Green Master Mix is compatible with common PCR additives such as DMSO and betaine. These additives neither change the color of GoTaq® Green Master Mix nor affect dye migration.

If both agarose gel analysis and further downstream applications involving absorbance or fluorescence will be used, the two dyes can be removed from reactions using standard PCR clean-up systems such as the Wizard® SV Gel and PCR Clean-Up System (Cat# A0281).

### B. Primer Design

PCR primers generally range in length from 15–30 bases and are designed to flank the region of interest. Primers should contain 40–60% (G + C), and care should be taken to avoid sequences that might produce internal secondary structure. The 3'-ends of the primers should not be complementary to avoid the production of primer-dimers. Primer-dimers unnecessarily deplete primers from the reaction and result in an unwanted polymerase reaction that competes with the desired reaction. Avoid three G or C nucleotides in a row near the 3'-end of the primer, as this may result in nonspecific primer annealing, increasing the synthesis of undesirable reaction products. Ideally, both primers should have nearly identical melting temperatures ( $T_m$ ); in this manner, the two primers should anneal roughly at the same temperature. The annealing temperature of the reaction is dependent upon the primer with the lowest  $T_m$ . For assistance with calculating the  $T_m$  of any primer, a  $T_m$  Calculator is provided on the BioMath page of the Promega web site at: [www.promega.com/biomath/](http://www.promega.com/biomath/)

### C. Amplification Troubleshooting

To overcome low yield or no yield in amplifications (e.g., mouse tail genotyping applications), we recommend the following suggestions:

- Adjust annealing temperature. The reaction buffer composition affects the melting properties of DNA. See BioMath Calculator to calculate the melting temperature for primers in the GoTaq® reaction ([www.promega.com/biomath/](http://www.promega.com/biomath/)).
- Minimize the effect of amplification inhibitors. Some DNA isolation procedures, particularly genomic DNA isolation, can result in the copurification of amplification inhibitors. Reduce the volume of template DNA in reaction or dilute template DNA prior to adding to reaction. Diluting samples even 1:10,000 has been shown to be effective in improving results, depending on initial DNA concentration.
- Increase template DNA purity. Include an ethanol precipitation and wash step prior to amplification to remove inhibitors that copurify with the DNA.
- Add PCR additives. Adding PCR-enhancing agents (e.g., DMSO or betaine) may improve yields. General stabilizing agents such as BSA (Sigma Cat# A7030; final concentration 0.16mg/ml) also may help to overcome amplification failure.

### D. More information on Amplification

More information on amplification is available online at the Promega web site: [www.promega.com/products/pcr/](http://www.promega.com/products/pcr/)

## Appendix 4. HindIII restriction enzyme digestion protocol

**Thermo**  
SCIENTIFIC

### PRODUCT INFORMATION

**HindIII**

**#ER0501** 5000 U

**Lot:** \_\_\_ **Expiry Date:** \_\_\_

5'...**A**↓**A** **G C T T**...3'

3'...**T T C G A**↑**A**...5'

Concentration: 10 U/μL

Source: *Haemophilus influenzae* Rd

Supplied with: 1 mL of 10X Buffer R

1 mL of 10X Buffer Tango

**Store at -20°C**



In total 3 vials.

BSA included

[www.thermoscientific.com/onebio](http://www.thermoscientific.com/onebio)

### RECOMMENDATIONS

**1X Buffer R** (for 100% HindIII digestion)

10 mM Tris-HCl (pH 8.5), 10 mM MgCl<sub>2</sub>, 100 mM KCl,  
0.1 mg/mL BSA.

**Incubate at 37°C.**

### Unit Definition

One unit is defined as the amount of HindIII required to digest 1 μg lambda DNA in 1 hour at 37°C in 50 μL of recommended reaction buffer.

### Dilution

Dilute with Dilution Buffer (#B19): 10 mM Tris-HCl (pH 7.4 at 25°C), 100 mM KCl, 1 mM EDTA, 1 mM DTT, 0.2 mg/mL BSA and 50% glycerol.

### Double Digests

Thermo Scientific Tango Buffer is provided to simplify buffer selection for double digests. 98% of Thermo Scientific restriction enzymes are active in a 1X or 2X concentration of Tango™ Buffer. Please refer to [www.thermoscientific.com/doubledigest](http://www.thermoscientific.com/doubledigest) to choose the best buffer for your experiments.

1X Tango Buffer: 33 mM Tris-acetate (pH 7.9 at 37°C), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/mL BSA.

### Storage Buffer

HindIII is supplied in: 10 mM Tris-HCl (pH 7.5 at 25°C), 250 mM KCl, 1 mM DTT, 0.1 mM EDTA, 0.2 mg/mL BSA and 50% glycerol.

### Recommended Protocol for Digestion

- Add:  
nuclease-free water 16  $\mu$ L  
10X Buffer R 2  $\mu$ L  
DNA (0.5-1  $\mu$ g/ $\mu$ L) 1  $\mu$ L  
HindIII 0.5-2  $\mu$ L\*
  - Mix gently and spin down for a few seconds.
  - Incubate at 37°C for 1-16 hours.
- The digestion reaction may be scaled either up or down.

### Recommended Protocol for Digestion of PCR Products Directly after Amplification

- Add:  
PCR reaction mixture 10  $\mu$ L (~0.1-0.5  $\mu$ g of DNA)  
nuclease-free water 18  $\mu$ L  
10X Buffer R 2  $\mu$ L  
HindIII 1-2  $\mu$ L\*
- Mix gently and spin down for a few seconds.
- Incubate at 37°C for 1-16 hours.

\* This volume of the enzyme is recommended for preparations of standard concentrations (10 U/ $\mu$ L), whereas HC enzymes (50 U/ $\mu$ L) should be diluted with Dilution Buffer to obtain 10 U/ $\mu$ L concentration.

### Thermal Inactivation

HindIII is inactivated by incubation at 80°C for 20 min.

## ENZYME PROPERTIES

### Enzyme Activity in Thermo Scientific REase Buffers, %

B	G	O	R	Tango	2X Tango
0-20	20-50	0-20	100	50-100	50-100

### Methylation Effects on Digestion

- Dam: never overlaps – no effect.
- Dcm: never overlaps – no effect.
- CpG: never overlaps – no effect.
- EcoKI: never overlaps – no effect.
- EcoBI: may overlap – cleavage impaired.

### Stability during Prolonged Incubation

A minimum of 0.1 units of the enzyme is required for complete digestion of 1  $\mu$ g of lambda DNA in 16 hours at 37°C.

### Digestion of Agarose-embedded DNA

A minimum 5 units of the enzyme is required for complete digestion of 1  $\mu$ g of agarose-embedded lambda DNA in 16 hours.

### Number of Recognition Sites in DNA

$\lambda$	$\Phi$ X174	pBR322	pUC57	pUC18/19	pTZ19R/U	M13mp18/19
6	0	1	1	1	1	1

For **CERTIFICATE OF ANALYSIS** see back page

Rev.9

# Appendix 5. Primers

No.	Oligo Name	Sequence (5' → 3')	Yield [OD]	Yield [µg]	Yield [nmol]	Concentration [µmol/µl]	Vol. for 100pmol/µl	Tm [°C]	MW [g/mol]	GC-Content	Synthesis Scale	Purification	Modification	Barcode
1	Nadir1 (F)	TCTAGAAACACAGTTGCCTG GC (20)	10.1	282	46.1	-	461	57.3	6116	50 %	0.01 µmol	HPSF	-	00194
2	Nadir1 (R)	ATAGCACTGGGAGCATT GAAGC (22)	14.2	377	55.4	-	554	60.3	6808	50 %	0.01 µmol	HPSF	-	00194
3	Nadir2 (F)	TGCCAGTGTAAACAAG ACCA (22)	13.4	367	54.9	-	549	60.3	6688	50 %	0.01 µmol	HPSF	-	00194
4	Nadir2 (R)	TGTTATCACACTGGTGCT AA (20)	12.3	344	56.4	-	564	53.2	6106	40 %	0.01 µmol	HPSF	-	00194
5	TB I	CCTCGAGCGTAGGCGT CGG (20)	10.8	320	51.7	-	517	67.6	6175	75 %	0.01 µmol	HPSF	-	00194
6	TB II	CTCGTCCAGCCCGCTT CGG (20)	11.0	350	57.9	-	579	67.6	6045	75 %	0.01 µmol	HPSF	-	00194

-PII {  
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## Appendix 6. Coagulation tests

SIEMENS

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### INNOVANCE® D-Dimer

Revision bar indicates update to previous version.

#### Intended Use

INNOVANCE® D-Dimer is a particle-enhanced, immunoturbidimetric assay for the quantitative determination of cross-linked fibrin degradation products (D-dimers) in human plasma for use on coagulation analyzers. The INNOVANCE® D-Dimer assay is indicated for use in conjunction with a clinical pretest probability (PTP) assessment model to exclude deep vein thrombosis (DVT) and pulmonary embolism (PE) disease in outpatients suspected of DVT or PE. INNOVANCE® D-Dimer can be used for the monitoring of the relative change in D-dimer concentration.

#### Summary and Explanation

Coagulation activation results in the cleavage of fibrinogen to fibrin monomer. The fibrin monomers spontaneously aggregate to fibrin and are cross-linked by factor XIII; this produces a fibrin clot. In response to the coagulation process the fibrinolytic system is activated resulting in the conversion of plasminogen into plasmin, which cleaves fibrin (and fibrinogen) into the fragments D and E. Due to cross-linkage between D-domains in the fibrin clot, the action of plasmin releases fibrin degradation products with cross-linked D-domains. The smallest unit is D-dimer. Detection of D-dimers, which specifies cross-linked fibrin degradation products generated by reactive fibrinolysis, is an indicator of coagulation activity. Fibrin degradation products are not consistently "D-dimer" but are a mixture of fragments and complexes of different molecular mass (e.g. DD 195 kD, DD/E 228 kD DXXD 693 kD, YXD/DXY 850 kD) containing the D and E domain<sup>1</sup>. An association between a certain mixture or molecular mass and the clinical condition has not been demonstrated. The in-vivo half life of D-dimer is approximately 8 hours<sup>2</sup>.

Elevated D-dimer levels are observed in all diseases and conditions with increased coagulation activation, e.g. thromboembolic disease, disseminated intravascular coagulopathy (DIC), acute aortic dissection, myocardial infarction, malignant diseases, obstetric complications, third trimester of pregnancy, surgery or polytrauma<sup>3,4</sup>.

The major diagnostic application of D-dimer testing is in the exclusion of thromboembolic events, such as deep vein thrombosis or pulmonary embolism. If D-dimer results are below the decision threshold, a thromboembolic event can be excluded with a test-specific negative predictive value (NPV). The use of D-dimer testing in combination with a well-validated clinical pretest probability score represents an efficient and safe screening tool for the exclusion of thromboembolic events<sup>3,4</sup>. However, symptoms being present since a certain period of time, e.g. longer than a week, may produce normal D-dimer values<sup>3</sup>.

For the diagnosis of DIC a scoring system has been suggested, in which elevated D-Dimer levels represent the major indicator of DIC<sup>5</sup>.

Furthermore, increased D-dimer levels have been shown to be associated with the risk for recurrent thromboembolic events after discontinuation of oral anticoagulant therapy<sup>6,10,11</sup>, and a poor prognosis in cardiac diseases, such as ischemic heart disease, chronic atrial fibrillation or heart failure<sup>7</sup>. It has been demonstrated that ETP and D-dimer with INNOVANCE® D-Dimer were independently associated with the risk of recurrence of VTE: Patients with high ETP and/or high INNOVANCE® D-Dimer values may benefit from prolonged anticoagulation<sup>10</sup>.



For exclusion of venous thrombosis or pulmonary embolism the analyte D-dimer should not be used as an aid in patients with<sup>9</sup>:

- Therapeutic dose anticoagulant therapy for > 24 hours
- Fibrinolytic therapy within previous 7 days
- Trauma or surgery within previous 4 weeks
- Disseminated malignancies
- Aortic aneurysm
- Sepsis, severe infections, pneumonia, severe skin infections
- Liver cirrhosis
- Pregnancy, or only with specific reference ranges.

Trimester-specific reference ranges during pregnancy have been suggested for INNOVANCE® D-Dimer<sup>12</sup>.

Thrombosis superimposed on a ruptured or unstable atherosclerotic plaque is the leading cause of most ischemic cardiovascular events. Elevated INNOVANCE® D-Dimer levels have been demonstrated to be significant prognostic markers in patients with angiographically confirmed coronary artery disease<sup>13</sup>.

Patients with acute coronary syndrome mostly show normal levels of D-dimer. In contrast, patients with aortic dissection or aortic aneurysm mostly have highly elevated levels of D-dimer. Therefore, the detection of D-dimer in patients with acute chest pain may help to differentiate between both clinical conditions<sup>8</sup>.

## Principle of the Procedure

Polystyrene particles covalently coated with a monoclonal antibody (8D3)<sup>14</sup> are aggregated when mixed with samples containing D-dimer. The D-dimer cross-linkage region has a stereosymmetrical structure, i.e. the epitope for the monoclonal antibody occurs twice. Consequently, one antibody suffices in order to trigger an aggregation reaction, which is then detected turbidimetrically via the increase in turbidity.

## Reagents

### Note:

INNOVANCE® D-Dimer can be used with many automatic coagulation analyzers. Siemens Healthcare Diagnostics provides Reference Guides (Application Sheets) for several coagulation analyzers. The Reference Guides (Application Sheets) contain analyzer/assay specific handling information which may differ from that provided in these Instructions for Use. In this case, the information contained in the Reference Guides (Application Sheets) supersedes the information in these Instructions for Use. Please also consult the instruction manual of the instrument manufacturer.

## Composition

INNOVANCE® D-Dimer	Form	Ingredient	Concentration	Source
<b>REAGENT</b>	Lyophilized	Polystyrene particles coated with monoclonal antibodies to D-dimer <sup>14</sup> Human serum albumin Preservatives: amphotericin B, gentamicin	0.1 g/L 0.5 g/L	Mouse Human

INNOVANCE® D-Dimer	Form	Ingredient	Concentration	Source
<b>BUFFER</b>	Liquid	Saline buffer Dextrane Imidazole Preservative: sodium azide	13 g/L   < 1 g/L	
<b>SUPPLEMENT</b>	Liquid	Saline buffer Heterophilic blocking reagent Preservative: sodium azide	0.63 g/L  < 1 g/L	Mouse
<b>DILUENT</b>	Liquid	Saline buffer Imidazole Preservative: sodium azide	6.8 g/L  < 1 g/L	
<b>CALIBRATOR</b>	Lyophilized	Human plasma, D-dimer preparation <sup>h)</sup> Preservatives: 5-chloro-2-methyl-4-isothiazole-3-one and 2-methyl-4-isothiazole-3-one sodium azide	5.0 mg/L (FEU)  < 1.0 mg/L  < 1 g/L	Human

<sup>a)</sup> antibody concentration may vary from lot to lot

<sup>b)</sup> nominal value per vial

### Precautions

For *in-vitro* diagnostic use.

Contains sodium azide (< 1 g/L) as a preservative. Sodium azide can react with copper or lead pipes in drain lines to form explosive compounds. Dispose of properly in accordance with local regulations.



#### CAUTION! POTENTIAL BIOHAZARD

INNOVANCE® D-Dimer **REAGENT** and INNOVANCE® D-Dimer **CALIBRATOR** contain human source material. Each donor or donor unit was tested and found to be negative for human immunodeficiency virus (HIV) 1 and 2, hepatitis B virus (HBV) and hepatitis C virus (HCV) using either tests found to be in conformance with the In Vitro Diagnostic Directive in the EU or FDA approved tests. Because no known test can offer complete assurance of the absence of infectious agents, all human derived products should be handled with appropriate caution.

### Reagent Preparation

All kit components are lot-specific except INNOVANCE® D-Dimer **DILUENT**. The combination of lots other than those specified for the particular kit lot may lead to incorrect results.

Follow the preparation instructions prior to use according to the table below. Storage instructions are detailed in section "Storage and Stability".

#### Instructions for the preparation of the kit components

Instructions	INNOVANCE® D-Dimer REAGENT	INNOVANCE® D-Dimer BUFFER / SUPPLEMENT / DILUENT	INNOVANCE® D-Dimer CALIBRATOR
Reconstitution	1. Dissolve with 4.0 mL distilled water 2. Invert 3 times 3. Leave the vial for at least 15 minutes at 15–25 °C	Ready to use	1. Dissolve with 1.0 mL distilled water 2. Mix carefully without foam formation 3. Leave the vial for at least 15 minutes at 15–25 °C
Prior to placing on the system	1. Mix well (again) by inverting 3 times 2. Avoid foam formation 3. Remove bubbles	1. Avoid foam formation 2. <b>BUFFER</b> only: resuspend potential precipitates by gently swirling. Any residual precipitates after resuspension do not impact test results 3. Remove bubbles	1. Mix (again) carefully 2. Do not use if the vial contains a visible clot
Aliquoting	1. Mix well (again) by inverting 3 times 2. Aliquot into an empty vial provided with the same kit 3. Discard empty vials if unused until complete consumption of the kit	1. Aliquot into an empty vial provided with the kit 2. Discard empty vials if unused until complete consumption of the kit	n.a.
Freeze and thaw	1. Use the original container or the empty vial provided with the kit 2. Follow storage instructions in section "Storage and Stability" 3. Thaw at 37 °C within 10 minutes and mix carefully. Thereafter the vial may no longer be stored at 2–8 °C 4. Do not freeze again after thawing		Refer to section "Storage and Stability" Mix carefully after thaw
Placing on the system	Use position indicated in the respective Reference Guides (Application Sheets)		
Note	The reconstitution, opening or freezing date may be documented on the vial label using the framed free space		

### Storage and Stability

The kit may be used up to the expiry date indicated on the label if stored unopened at 2 to 8 °C.

#### Stability after reconstitution or first opening (closed vial)

Temperature	INNOVANCE® D-Dimer REAGENT	INNOVANCE® D-Dimer BUFFER	INNOVANCE® D-Dimer SUPPLEMENT	INNOVANCE® D-Dimer DILUENT	INNOVANCE® D-Dimer CALIBRATOR
2–8 °C	4 weeks	4 weeks	4 weeks	4 weeks	-
≤ -18 °C*	4 weeks	4 weeks	4 weeks	4 weeks	-
15–25 °C	-	-	-	-	4 hours

\* Do not refreeze after thawing. Follow the freeze and thaw instructions in section "Preparation of the Reagents".

Information about on-board stability is specified in the Reference Guides (Application Sheets) for the different analyzers.

### Specimen Collection and Handling

- Use citrated platelet poor plasma for testing.
- Obtain the plasma by carefully mixing 1 part sodium citrate solution (0.11 mol/L or 3.2 %) with 9 parts venous blood. Avoid foam formation.
- An evacuated tube system or syringe may be used.
- Centrifuge the blood tube directly after blood collection for 15 minutes at 1500 x g to 2500 x g. Please refer to CLSI guideline H21-A5<sup>13</sup> for further details. The manufacturer's instructions for the sampling equipment must also be observed.
- Clarify turbid plasma once more by centrifugation at approx. 15,000 x g for 10 minutes.

### Stability of the Plasma Samples

15 to 25 °C	4 hours
2 to 8 °C	24 hours
≤ -18 °C	4 weeks**

\*\* If frozen within 4 hours of blood collection.

### Preparation of Frozen Samples

- Freeze plasma within 4 hours of blood collection at ≤ -18 °C.
- Thaw frozen plasma within 10 minutes at 37 °C, homogenize by carefully mixing without foam formation and centrifuge at approx. 15,000 x g for 10 minutes. Then carry out the D-dimer determination within 2 hours. Do not freeze more than two times.
- For specific handling information on the various analyzers, please consult the respective Reference Guides (Application Sheets).

## Procedure

### Materials Provided

INNOVANCE® D-Dimer Kit, [REF] OPBP 03 with

3 x → 4.0 mL	INNOVANCE® D-Dimer [REAGENT], reagent
3 x 5.0 mL	INNOVANCE® D-Dimer [BUFFER], buffer
3 x 2.6 mL	INNOVANCE® D-Dimer [SUPPLEMENT], supplementary reagent
3 x 5.0 mL	INNOVANCE® D-Dimer [DILUENT], sample diluent
2 x → 1.0 mL	INNOVANCE® D-Dimer [CALIBRATOR], calibrator
12 x	[EMPTY VIAL], empty vials 3 x each for INNOVANCE® D-Dimer [REAGENT], INNOVANCE® D-Dimer [BUFFER], INNOVANCE® D-Dimer [SUPPLEMENT], and INNOVANCE® D-Dimer [DILUENT]

INNOVANCE® D-Dimer Kit, [REF] OPBP 07 with

6 x → 4.0 mL	INNOVANCE® D-Dimer [REAGENT], reagent
6 x 5.0 mL	INNOVANCE® D-Dimer [BUFFER], buffer
6 x 2.6 mL	INNOVANCE® D-Dimer [SUPPLEMENT], supplementary reagent
6 x 5.0 mL	INNOVANCE® D-Dimer [DILUENT], sample diluent
2 x → 1.0 mL	INNOVANCE® D-Dimer [CALIBRATOR], calibrator

### Additional materials required but not provided

INNOVANCE® D-Dimer Controls, [REF] OPDY  
 INNOVANCE® D-Dimer Sample Diluent, [REF] OPBR  
 Coagulation analyzer  
 Distilled water

## Multifibren® U

Revision bar indicates update to previous version.

### Intended Use

Quantitative determination of fibrinogen in plasma.

### Summary and Explanation

Depressed levels of fibrinogen are observed in:

- a. acquired hypo- or afibrinogenemia. Acquired fibrinogen deficiency states occur especially as a result of intravascular proteolysis of fibrinogen by thrombin (disseminated intravascular coagulation, e.g. in obstetrics, after surgery), snake venoms or plasmin (primary hyperfibrinolysis after streptokinase, urokinase or tPA therapy). Furthermore, moderate hypofibrinogenemias may occur in cases of diminished production (in acute or chronic liver diseases), loss into the intravascular space (e.g. in ascites or acute hemorrhage and burns) or increased degradation (in shock or carcinoma).
- b. congenital hypo- and afibrinogenemias.

Temporarily elevated levels of fibrinogen are observed as a result of the behavior of fibrinogen as an "acute-phase protein":

- a. Transitory hyperfibrinogenemias may occur after operations, traumas, myocardial infarction and infections<sup>1</sup>.
- b. Persistent hyperfibrinogenemias may be seen in patients with neoplasias and chronic inflammatory diseases.

Levels of fibrinogen increase slightly with age. Elevated fibrinogen levels are a risk factor for cardiovascular disease<sup>2</sup>.

### Principle of the Method

Modification of the Clauss method.

Citrated plasma is brought to coagulation by a large excess of thrombin. Here the coagulation time depends largely on the fibrinogen content of the specimen.

### Reagents

#### Materials provided

Multifibren® U, [REF] OWZG 23

10 x → 5 mL or

Multifibren® U, [REF] OWZG 19

10 x → 2 mL

Each pack of Multifibren® U contains a lot- and method-specific evaluation table.

#### Composition

Multifibren® U: bovine thrombin (50 IU/mL), fibrin-aggregation retarding peptide (gly-pro-arg-proala-amide, 0.15 g/L), calcium chloride (1.5 g/L), hexadimethrine bromide, polyethylene glycol 6000, sodium chloride, Tris, bovine albumin; Preservative: sodium azide (< 1 g/L).

### Warnings and Precautions

For *in-vitro* diagnostic use only.

#### Multifibren® U

**H412:** Harmful to aquatic life with long lasting effects.

**P273, P501:** Avoid release to the environment. Dispose of contents and container in accordance with all local, regional, and national regulations.

Safety data sheets (MSDS/SDS) available on [www.siemens.com/diagnostics](http://www.siemens.com/diagnostics)

Contains sodium azide (< 0.1 %) as a preservative. Sodium azide can react with copper or lead pipes in drain lines to form explosive compounds. Dispose of properly in accordance with local regulations.

### Preparations of the Reagents

Dissolve the Multifibren® U with the amount of distilled or deionized water indicated on the label. Mix carefully once more before using.

Bring the reagents to 37 °C before measuring (not required for automated coagulation analyzers with heated reagent probes).

### Storage and Stability

Stored unopened at 2 to 8 °C Multifibren® U may be used by the expiry date given on the label.

Stability after reconstitution:

37 °C	8 hours
15 to 25 °C	1 day
2 to 8 °C	5 days
≤ -20 °C	2 months

Information about on-board stability is specified in the Reference Guides (Application Sheets) for the different coagulation analyzers.

### Materials required, but not provided

Fibrinogen Calibrator Kit  
Kaolin Suspension for Fibrintimer (BFT® II)  
Control Plasma N  
Control Plasma P  
Ci-Trol® Level 1  
Coagulation analyzer (see chapter "Equipment")

### Equipment

Multifibren® U can be used on automated coagulation analyzers. Siemens Healthcare Diagnostics provides Reference Guides (Application Sheets) for several coagulation analyzers. The Reference Guides (Application Sheets) contain analyzer/assay specific handling and performance information which may differ from that provided in these Instructions for Use. In this case, the information contained in the Reference Guides (Application Sheets) supersedes the information in these Instructions for Use. Please also consult the instruction manual of the instrument manufacturer!

### Specimens

Mix 1 part sodium citrate solution (0.11 mol/L) with 9 parts venous blood. Centrifuge the blood specimen at 1500 x g for no less than 15 minutes at room temperature. Remove the supernatant plasma and store at 15 to 25 °C until required for the test.

Stability of the samples:

15 to 25 °C	8 hours
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## Procedure

### Manual:

Bring Multifibren® U to 37 °C before using.	
Pipette into a test tube warmed to 37 °C:	
<b>Sample</b>	<b>100 µL</b>
Incubate for 60 seconds at 37 °C	
<b>Multifibren® U (37 °C)</b>	<b>200 µL</b>
Determine coagulation time	

### Automatic:

See chapter "Equipment".

### Calculating the reference curve

Calculating the reference curve is done with the Fibrinogen Calibrator Kit.

These calibrators are tested as samples in the particular coagulation analyzer. The graphic representation of the values obtained is best plotted in a log-log format.

A new reference curve must be calculated each time there is a change in the device used or a change in the lot of Multifibren® U.

### Internal Quality Control

Normal range: Control Plasma N,  
Ci-Trol® Level 1

Pathological range: Control Plasma P

Two levels of quality control material (normal and pathologic range) have to be measured at start of the test run, with each calibration, upon reagent vial changes and at least every eight hours on each day of testing. The controls should be processed just like the samples. Each laboratory should determine its own quality control range, either by means of the target values and ranges provided by the manufacturer of the controls or by means of its own range established in the laboratory. If the control values lie outside the range determined beforehand, then the reagent, calibration curve and coagulation analyzer should be checked. Do not release patient results until the cause of deviation has been identified and corrected.

### Calculating the Analytical Results

The results can be evaluated either with the enclosed table or with a reference curve calculated in the laboratory.

Fibrinogen concentration is given in g/L. The data provided in the evaluation table are only valid for reagents with matching lot numbers and the indicated coagulation analyzers.

A separate reference curve must be calculated for each coagulation analyzer, automated or not.

The reference curves from the evaluation table can be verified with the help of Control Plasma N, Ci-Trol® Level 1 or with Control Plasma P. If the measured results lie outside of the indicated range, then a reference curve specific to the laboratory should be calculated.

### Limitations of the Procedure

Degradation products of fibrin(ogen) lead to prolonged coagulation times and therefore to diminished recovery of fibrinogen. Heparin (up to 2 U/mL) does not affect the test. Therapy with direct thrombin inhibitors, e.g. hirudin, may contribute to diminished recovery<sup>3</sup>.

Some analyzers in the KC 4/10/40 series may yield false results when running the test.

# Stago STA<sup>®</sup> Staclot<sup>®</sup> APCR kit

We are pleased to announce the resumed availability of the Stago STA<sup>®</sup> Staclot<sup>®</sup> APCR kit.

The principle of the assessment of activated protein C resistance (APC-R) is based on an unusually small prolongation of the clotting time of the tested plasma in the presence of PCa and in calcified medium.

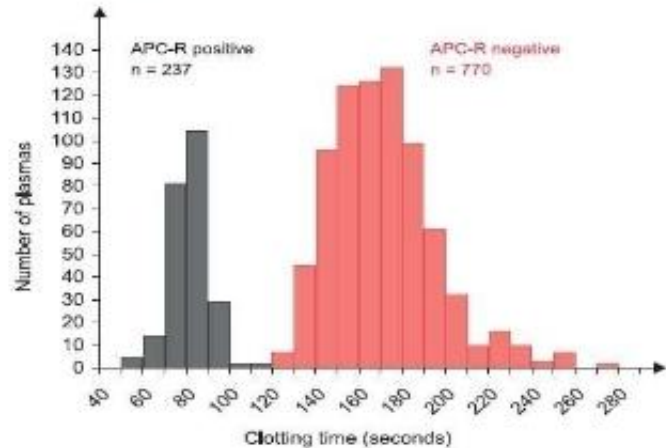
In the STA<sup>®</sup> Staclot<sup>®</sup> APC-R system, coagulation of the diluted test plasma is achieved in the presence of factor V deficient plasma and *Crotalus viridis halleri* venom. The venom acts as an activator of factor X and therefore triggers the coagulation cascade downstream from factor X, thus eliminating the influence of all coagulation factors acting upstream.

The prolongation of the clotting time of a normal plasma in the presence of PCa results from the capacity of the PCa (Reagent 3), to inactivate the factor Va of the tested plasma.

Plasmas whose clotting times obtained with the STA<sup>®</sup> Staclot<sup>®</sup> APC-R procedure are equal to or greater than 120 seconds are considered to be APC-R negative. On the other hand, plasmas whose clotting times are less than 120 seconds are considered APC-R positive.

1,007 plasmas that had been characterised by molecular biology for the factor V Leiden (of which 236 were APC-R positive by molecular biology) were tested with the STA<sup>®</sup> Staclot<sup>®</sup> APC-R procedure

As an illustration, the distribution of the clotting times measured with the STA<sup>®</sup> - Staclot<sup>®</sup> APC-R reagents for these 1,007 plasmas is indicated in the diagram below:



Compared with molecular biology for factor V Leiden, the STA<sup>®</sup> Staclot<sup>®</sup> APC-R procedure demonstrated the following performance characteristics:

- Sensitivity: 99.6%
- Specificity: 99.7%
- Positive predictive value: 99.2%
- Negative predictive value: 99.9%

STA<sup>®</sup> Staclot<sup>®</sup> APC-R is in stock now.

Order via:

Email [orders@stago.com](mailto:orders@stago.com)

fax +61 3 9855 8999 or

phone 1800 4 78246 Australia

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## Human prothrombin fragment 1+2, F1+2 ELISA kit



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**FOR RESEARCH USE ONLY; NOT FOR THERAPEUTIC OR DIAGNOSTIC APPLICATIONS!  
PLEASE READ THROUGH ENTIRE PROCEDURE BEFORE BEGINNING!**

### Intended use

This immunoassay kit allows for the specific measurement of human prothrombin fragment 1+2, F1+2 concentrations in cell culture supernates, serum and plasma.

### Introduction

Prothrombin is a carbohydrate-protein compound in plasma essential to coagulation. In response to bleeding, a complex series of clotting-factor interactions leads to its conversion by thromboplastin to thrombin, which transforms fibrinogen in plasma into fibrin. Fibrin and platelets combine to form a clot. Hemophilia is caused by a hereditary lack of one of the clotting factors. Vitamin K is needed to synthesize prothrombin, so conditions that impair the vitamin's absorption result in prothrombin deficiency and a tendency to prolonged bleeding.

Prothrombin and prothrombin fragment F1+2 (F1+2) were demonstrated in the tumor stroma on cancer cells and on small blood vessels in areas of neoangiogenesis at the host-tumor interface (gastric and pancreatic cancer tissues). F1+2 is an indicator of local activation of blood coagulation in cancer tissue.

Thrombin itself is impossible to quantitate and so the use of surrogate markers is necessary. The measurement of F1+2 would be an excellent marker of thrombin generation. This is helped by the fact that F1+2 is not generated in vivo by any other mechanism. Fragment 1+2 has a half life of about 1 hour and is cleared from the bloodstream by the liver.

### Test principle

This assay employs the quantitative sandwich enzyme immunoassay technique. A antibody specific for F1+2 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any F1+2 present is bound by the immobilized antibody. An enzyme-linked antibody specific for F1+2 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of F1+2 bound in the initial step. The color development is stopped and the intensity of the color is measured.

### Materials and components

Reagent	Quantity
Assay plate	1

1

Standard	2
Sample Diluent	1 x 20ml
Assay Diluent A	1 x 10ml
Assay Diluent B	1 x 10ml
Detection Reagent A	1 x 120ul
Detection Reagent B	1 x 120ul
Wash Buffer	1 x 30ml
(25 x concentrate)	
Substrate	1 x 10ml
Stop Solution	1 x 10ml

### Sample collection and storage

**Cell culture supernates** - Remove particulates by centrifugation and assay immediately or aliquot and store samples at  $\leq -20^{\circ}$  C. Avoid repeated freeze-thaw cycles.

**Serum** - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at approximately 1000 x g. Remove serum and assay immediately or aliquot and store samples at  $-20^{\circ}$  C.

**Plasma** - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1000 x g at  $2 - 8^{\circ}$  C within 30 minutes of collection. Store samples at  $\leq -20^{\circ}$  C. Avoid repeated freeze-thaw cycles.

**Note:** Citrate plasma has not been validated for use in this assay.

### Limitations of the procedure

#### **FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.**

1. The kit should not be used beyond the expiration date on the kit label.
2. Do not mix or substitute reagents with those from other lots or sources.
3. If samples generate values higher than the highest standard, further dilute the samples with the Assay Diluent and repeat the assay. Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
4. This assay is designed to eliminate interference by soluble receptors, ligands, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

### Reagent preparation

**Bring all reagents to room temperature before use.**

**Wash Buffer** - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 ml of Wash Buffer.

**Standard** - Reconstitute the **Standard** with 1.0 mL of **Sample Diluent**. This reconstitution produces a stock solution of 2,000 pmol/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making serial dilutions. The undiluted standard serves as the high standard (2,000 pmol/mL). The **Sample Diluent** serves as the zero standard (0 pmol/mL).

**Detection Reagent A and B** - Dilute to the working concentration specified on the vial label using

**Assay Diluent A and B** (1:100), respectively.

#### Assay procedure

Allow all reagents to reach room temperature. Arrange and label required number of strips.

1. Prepare all reagents, working standards and samples as directed in the previous sections.
2. Add 100  $\mu$ L of **Standard**, Control, or sample\* per well. Cover with the adhesive strip. Incubate for 2 hours at 37° C.
3. Remove the liquid of each well, don't wash.
4. Add 100  $\mu$ L of **Detection Reagent A** to each well. Incubate for 1 hour at 37°C. **Detection Reagent A** may appear cloudy. Warm to room temperature and mix gently until solution appears uniform.
5. Aspirate each well and wash, repeating the process three times for a total of three washes. Wash by filling each well with Wash Buffer (350  $\mu$ L) using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 100  $\mu$ L of **Detection Reagent B** to each well. Cover with a new adhesive strip. Incubate for 1 hours at 37° C.
7. Repeat the aspiration/wash as in step 5.
8. Add 90  $\mu$ L of **Substrate Solution** to each well. Incubate for 30 minutes at room temperature. Protect from light.
9. Add 50  $\mu$ L of **Stop Solution** to each well. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm.

#### Specificity

This assay recognizes recombinant and natural human F1+2. No significant cross-reactivity or interference was observed.

#### Sensitivity

The minimum detectable dose of human F1+2 is typically less than 7.8 pmol/mL.

The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest detectable concentration that could be differentiated from zero.

#### Detection Range

31.2-2,000 pmol/mL. The assay range was estimated by calculating the coefficient of variation (CV) of each standard constructing five independent standard curves. The standard curve concentrations used for the ELISA's were 2,000 pmol/mL, 1,000 pmol/mL, 500 pmol/mL, 250 pmol/mL, 125 pmol/mL, 62.5 pmol/mL, 31.2 pmol/mL.

#### Important Note:

1. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

2. It is recommended that no more than 32 wells be used for each assay run if manual pipetting is used since pipetting of all standards, specimens and controls should be completed within 5 minutes. A full plate of 96 wells may be used if automated pipetting is available.
3. Duplication of all standards and specimens, although not required, is recommended.
4. When mixing or reconstituting protein solutions, always avoid foaming.
5. To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
6. To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.

#### Calculation of results

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the F1+2 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

#### Storage of test kits and instrumentation

1. Unopened test kits should be stored at 2-8°C upon receipt and the microtiter plate should be kept in a sealed bag with desiccants to minimize exposure to damp air. The test kit may be used throughout the expiration date of the kit (six months from the date of manufacture). Refer to the package label for the expiration date.
2. Opened test kits will remain stable until the expiring date shown, provided it is stored as prescribed above.
3. A microtiter plate reader with a bandwidth of 10nm or less and an optical density range of 0-3 OD or greater at 450nm wavelength is acceptable for use in absorbance measurement.

#### Precaution

The Stop Solution suggested for use with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.