

## Chapter one

### Introduction and Literature Review

#### 1.1 Introduction:

Normal homeostasis is an efficient and rapid mechanism for stopping bleeding from sites of blood vessel injury is an essential for survival . never the less such a response need to be tightly controlled to prevent extensive cause developing and to break down such clots when damage is repaired. The haemostatic system thus represents a delicate balance between pro coagulant and anticoagulant mechanisms lead to process for fibrinolysis (Hoffbrand V. *et al.*, 2006).

The five major components involved are platelets, coagulation factor, coagulation inhibitors, fibrinolysis and blood vessels (Hoffbrand V. *et al.*, 2006).

Tobacco is the single greatest cause of preventable death globally. Tobacco use leads most commonly to diseases affecting the heart and lungs, with smoking being a major risk factor for heart attacks, strokes, chronic obstructive pulmonary disease (COPD) (including emphysema and chronic bronchitis), and cancer (particularly lung cancer, cancers of the larynx and mouth, and pancreatic cancer) (WHO, 2008).

Epidemiologic studies clearly link cigarette smoking with vasocclusive cardiovascular diseases. Postmortem studies provide evidence of accelerated atherogenesis in asymptomatic smokers. However, the rapid regression of cardiovascular risk within the first year of quitting smoking is difficult to explain solely in terms of vascular diseases. Recent evidence indicates that plasma fibrinogen, which has been prospectively associated with the risk of ischemic heart disease, is elevated in urine confirm those involving radiolabeled platelet turnover, suggesting that platelets are activated in the circulation of chronic smokers. Altered hemostatic function, either as a direct result of smoking or caused by smoking-induced vascular damage, may account for the

more rapidly reversible component of cardiovascular risk observed in chronic smokers. (Garret A. *et al.*, 1998)

## **1.2 Literature review**

### **1.2.1 Haemostasis and haemostatic components:**

Hemostasis, the cessation of bleeding, occurs within the intravascular compartment lined with endothelium. Normal hemostasis and thrombosis (the occlusion of a blood vessel) is the sum of activity of two components:

(i) a cellular part and (ii) a protein portion from the blood plasma or cells in the intravascular compartment. Some patients have normal blood coagulation proteins but abnormal hemostasis due to a platelet defect (e.g., Bernard – Soulier syndrome). Alternatively, other patients have normal platelet function, but abnormal hemostasis due to a blood coagulation protein defect (e.g., Hemophilia A). (Schmaier A. *et al.*, 2012)

The cellular and protein components comprise the system and closely interact. The cellular components mostly consist of platelets and endothelium, but polymorphonuclear leukocytes and monocytes also contribute activating and regulating agents. Platelets, an anucleated cell fragment, provide an initial locus upon which hemostatic reactions occur. (Schmaier A. *et al.*, 2012)

Vessel walls constitutively are anticoagulant surfaces which when injured become procoagulant. Monocytes and neutrophils contribute tissue factor initiating hemostasis and have potent clot lysing system. The protein components that contribute to hemostasis and thrombosis include 3 protein systems: the blood coagulation (clot forming), the fibrinolytic (clot lysing) and anticoagulant (regulating) protein systems. Each of the proteins in these three systems balances the activities of the others. (Schmaier A. *et al.*, 2012)

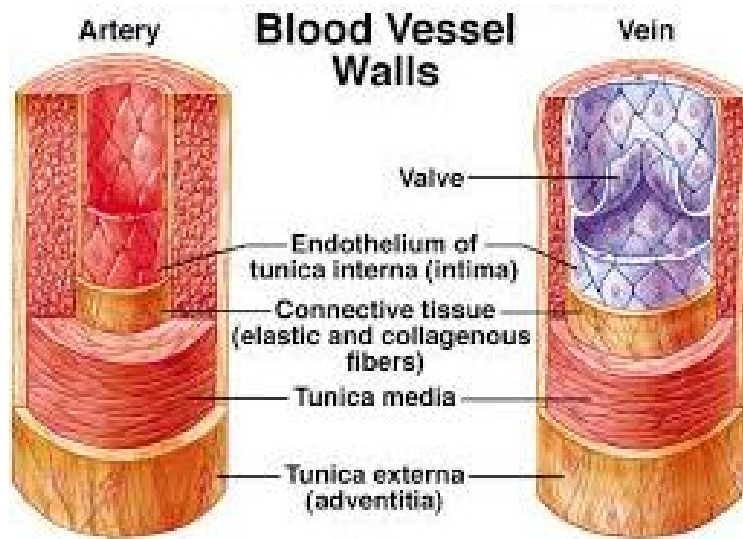
Regulation. Physiologic hemostasis is a tightly regulated balance between the formation and dissolution of hemostatic plugs by the coagulation and fibrinolytic systems. Blood coagulation proteins circulate as zymogens or proenzymes and are unactivated. When a stimulus injury occurs, the proenzymes

of the system are activated to enzymes initiating a series of proteolytic reactions leading to thrombin formation, the main clotting enzyme. The blood coagulation proteins become activated in a cascade. The anticoagulation proteins regulate the coagulation and fibrinolytic systems. The proteins of the anticoagulation system join those of the fibrinolytic system to prevent or counterbalance coagulation reactions. Thus, the hemostatic system is tightly modulated by a series of serine proteases (enzymes), their cofactors for activity, and serine protease inhibitors that regulate their function (Schmaier . *et al.*, 2012)

#### **1.2.1.1The Blood Vessel**

##### **1.2.1.1.1 General Structure of the Blood Vessel**

The blood vessel wall has three layers: intima, media and adventitia. The intima consists of endothelium and sub-endothelial connective tissue and is separated from the media by the elastic lamina interna. Endothelial cells form a continuous monolayer lining all blood vessels. The structure and the function of the endothelial cells vary according to their location in the vascular tree, but in their resting state they all share three important characteristics: they are ‘non thrombogenic’ (i.e. they promote maintenance of blood in its fluid state); they play an active role in supplying nutrients to the subendothelial structures; and they act as a barrier to cells, macromolecules and particulate matter circulating in the bloodstream. The permeability of the endothelium may vary under different conditions to allow various molecules and cells to pass (Barbara. *et al.*, 2011)



**Figure (1-1): Blood vessel wall structure**

#### **1.2.1.1.2 Endothelial Cell Function**

The luminal surface of the endothelial cell is covered by the glycocalyx, a proteoglycan coat. It contains heparan sulphate and other glycosaminoglycans, which are capable of activating antithrombin, an important inhibitor of coagulation enzymes. Tissue factor pathway inhibitor (TFPI) is present on endothelial cell surfaces bound to these heparans but also tethered to a glycosphosphoinositol (GPI) anchor. The relative importance of these two TFPI pools is not known. (Barbara. *et al.*, 2011).

Endothelial cells express a number of coagulation active proteins that play an important regulatory role such as thrombomodulin and the endothelial protein C (PC) receptor. Thrombin generated at the site of injury is rapidly bound to a specific product of the endothelial cell, thrombomodulin. When bound to this protein, thrombin can activate PC (which degrades factors Va and VIIIa) and a calcium-dependent peptidase which inhibits fibrinolysis. Thrombin also stimulates the endothelial cell to produce tissue plasminogen activator (tPA). The endothelium can also synthesize protein S, the cofactor for PC. Finally, endothelium produces von Willebrand factor (VWF), which is essential for platelet adhesion to the subendothelium and stabilizes factor VIII within the circulation. VWF is both stored in specific granules called Weibel Palade bodies and secreted

constitutively, partly into the circulation and partly toward the subendothelium where it binds directly to collagen and other matrix proteins. The expression of these and other important molecules such as adhesion molecules and their receptors are modulated by inflammatory cytokines. The lipid bilayer membrane also contains adenosine diphosphatase (ADPase), an enzyme that degrades adenosine diphosphate (ADP), which is a potent platelet agonist. Many of the surface proteins are found localized in the specialized lipid rafts and invaginations called 'caveolae', which may provide an important level of regulation. The endothelial cell participates in vasoregulation by producing and metabolizing numerous vasoactive sub-stances. On the one hand, it metabolizes and inactivates vasoactive peptides such as bradykinin; on the other hand, it can also generate angiotensin II, a local vasoconstrictor, from circulating angiotensin-I. Under appropriate stimulation the endothelial cell can produce vasodilators such as nitric oxide (NO) and prostacyclin or vasoconstrictors such as endothelin and thromboxane. These sub-stances have their principal vasoregulatory effect via the smooth muscle but also have some effect on platelets. The subendothelium consists of connective tissues composed of collagen (principally types I, III and VI), elastic tissues, proteoglycans and non-collagenous glycoproteins, including fibronectin and VWF. After vessel wall damage has occurred, these components are exposed and are then responsible for platelet adherence. This appears to be mediated by VWF binding to collagen. VWF then undergoes a conformational change and platelets are captured via their surface membrane glycoprotein Ib binding to VWF. Platelet activation follows and a conformational change in glycoprotein IIb/IIIa allows further, more secure, binding to VWF via this receptor as well as to fibrinogen. (Barbara. *et al.*, 2011).

#### **1.2.1.1.3 Vasoconstriction**

Vessels with muscular coats contract following injury, thus helping to arrest blood loss. Although not all coagulation reactions are enhanced by reduced flow, this probably assists in the formation of a stable fibrin plug by allowing activated factors to accumulate to critical concentrations. Vasoconstriction also occurs in the microcirculation in vessels without smooth muscle cells. Endothelial cells themselves can produce vasoconstrictors such as angiotensin II. In addition, activated platelets produce thromboxane A<sub>2</sub> (TXA<sub>2</sub>), which is a potent vasoconstrictor. (Barbara. *et al.*, 2011).

#### **1.2.1.2 Primary haemostasis:**

##### **1.2.1.2.1 Platelets:**

Platelets are small, anucleate cells that play a critical role in haemostasis and thrombosis. Platelets ordinarily circulate in the bloodstream in a quiescent state but undergo ‘explosive’ activation following damage to the vessel wall, leading to rapid formation of a platelet aggregate or vascular plug and occlusion of the site of damage. In simple terms, the more rapidly that platelets can achieve this, the lower the amount of blood that is lost.

Platelets are enriched in signalling proteins and surface receptors that enable them to achieve a rapid response, whereas major defects in platelet function or platelet number are associated with an excessive loss of blood. Significantly, disorders associated with vessel damage or a marked increase in platelet number or reactivity lead to thrombus formation in intact vessels and vascular occlusion and can cause arterial thrombotic disorders such as stroke and myocardial infarction, two of the major causes of morbidity and mortality in the Western world. (Hoffbrand V. *et al.*, 2006)

It is essential that platelets are able to achieve this interplay between quiescence and ‘explosive’ activation in high-pressure arteries and arterioles and in the low-

pressure venous system. To achieve this interplay, platelet reactivity is strictly controlled by a series of positive feedback signals, when needed, or inhibitory signals that counter activation in intact vessels. Key factors that support rapid activation at sites of vessel injury include thrombogenic components of the subendothelial matrix, the release of positive feedback signals from the platelet, the generation of thrombin by the coagulation cascade and activation of platelet integrins, enabling them to bind to integrins, enabling them to bind to their ligands within the bloodstream. Factors that oppose activation in the intact vasculature include the constitutive release of a gaseous transmitter, nitric oxide, from the endothelial cells that make up the vessel wall and the anti-platelet activity of the endothelial cell membrane itself. (Hoffbrand V. *et al.*, 2006)

The role of platelets in haemostasis is intimately linked to charged lipid surface that supports the generation of thrombin, which reinforces platelet activation. The major platelet integrin  $\alpha\text{IIb}\beta 3$  (also known as GPIIb–IIIa) also forms a bridge between the platelet cytoskeleton and the polymerized fibrin that is generated by the coagulation cascade. Outside-in signals through  $\alpha\text{IIb}\beta 3$  promote ‘clot retraction’, which serves to strengthen the thrombus and thereby prevent embolization, even in the presence of the very high shear forces that exist within small arterioles and capillaries. Thus, the processes of platelet activation and coagulation are part of an intertwined biological programme that gives rise to the orchestrated formation of a haemostatic plug. (Hoffbrand V. *et al.*, 2006)

The role of platelets is also far wider than that of simply supporting thrombus formation. Platelet dense and  $\alpha$ -granules are packed with a rich diversity of small molecules and proteins that play fundamental roles in other aspects of haemostasis and also in host defense. For example, platelets release agents that promote vessel constriction, vessel repair and leucocyte recruitment. In



particular, there is growing recognition of the role of platelets in inflammatory responses in the vasculature. Platelets have been proposed to play a key role in the onset and progression of vascular inflammatory disorders such as atherosclerosis and vascular infections such as endocarditis.(Hoffbrand V. *et al.*, 2006)

It is important to consider why platelets release such a diverse library of biological molecules. The cell most closely linked to platelet function in invertebrates, the haemocyte, had a far wider spectrum of actions than the platelet, supporting both inflammatory processes and aggregatory events. In higher organisms, two distinct sets of nucleated cells can be found, namely thrombocytes and leucocytes, whose primary roles are to support haemostasis and inflammatory responses respectively. Mammals, on the other hand, are the only animal to have a nucleated platelets. Given this route of evolution, it is not surprising that platelets have retained many of the features of inflammatory cells, especially given that they are the first and most abundant cell that accumulates at sites of vascular infection. The challenge is to establish the significance of the non-haemostatic molecules in platelets in health and disease. (Hoffbrand V. *et al.*, 2006)

Platelets are one of the major targets for the prevention and treatment of thrombotic and, to a lesser extent, vascular inflammatory disorders. Careful attention, however, should be given to the case as to whether a patient should receive anti-platelet therapy, and the nature that this therapy should take, bearing in mind that such treatment always carries a risk, albeit usually minor, of increased bleeding. For the majority of individuals receiving the major orally active anti-platelet drugs, aspirin or clopidogrel (a P2Y<sub>12</sub> ADP receptor antagonist), this risk is extremely low, unless otherwise contraindicated by conditions such as peptic ulcer. But, the risk is not zero. In this context, the

growing tendency of otherwise healthy passengers to take aspirin as an anti-platelet therapy in long-haul flights should be viewed with concern. The major risk in long-haul flights is venous rather than arterial thrombosis through stasis in peripheral veins. Venous thrombosis is minimally inhibited by treatment with aspirin, whereas this treatment does lead to a small increase in risk of bleeding, which exceeds the benefit. Heparin would be a far better alternative therapy, but has the drawback that it is only delivered by injection. (Hoffbrand V. *et al.*, 2006)

The decision on whether a patient should receive antiplatelet therapy is based on the net sum of risk factors for arterial thrombosis, such as age, weight, lifestyle, cholesterol, smoking and family history, and the potential of excessive bleeding. It is now accepted that individuals who are considered to be at a medium to high risk of thrombosis should receive some form of anti-platelet therapy (unless otherwise contraindicated), usually low-dose aspirin, in combination with other treatments such as statins or blood pressure-lowering drugs. Stronger inhibitors of platelet function, namely blockers of the major platelet integrin,  $\alpha\text{IIb}\beta 3$ , are used only in acute situations of thrombotic risk such as unstable angina or during surgical procedures such as angioplasty and stenting because of problems arising from excessive blood loss. (Hoffbrand V. *et al.*, 2006)

#### **1.2.1.2.1.1 Platelet Structure:**

The cell membrane lipid bilayer is partially or completely penetrated by a range of glycoprotein molecules. These function as receptors for a range of different agonists, adhesive proteins, coagulation factors, and other platelets. Specific membrane glycoproteins have been characterized with their associated functions. (Shinton N., 2006)

The most abundant glycoproteins on the platelet surface are glycoprotein IIb/IIIa (GpIIb/IIIa). These two glycoproteins form a heterodimer and carry receptors for fibrinogen, Von Willebrand Factor (VWF), and fibronectin (adhesive

proteins). The GpIIb/IIIa complex is a member of the integrin family of cellular adhesion molecules. Glycoprotein Ib is also important, as this contains a receptor for Von Willebrand Factor and thrombin. This receptor plays an essential part in the platelet–vessel wall interaction. A less well defined group of 7-transmembrane domain glycoproteins can be released in adenosine deaminase (ADA) adrenaline- and thrombin-mediated aggregation. Deficiencies of platelet membrane glycoproteins lead to disorders of platelet function. In addition, membrane glycoproteins act as specific alloantigenic sites. The cell membrane also contains phospholipid, associated with prostaglandin synthesis, calcium mobilization, and localization of coagulant activity to the platelet surface.(Shinton N., 2008)

The cell membrane lies the peripheral band of microtubules, which functions as the cell cytoskeleton. The microtubules maintain the discoid shape in the resting platelet but disassemble upon platelet aggregation and then reappear toward the center of the cell, entrapping granules. This is thought to help the release reaction.(Shinton N., 2008)

The surface-connected canalicular system is an extensive system of plasma membrane invaginations. It increases the surface area across which membrane transport can occur and through which platelet granules can discharge their contents during the secretory phase of platelet aggregation. Dilated canaliculi are the probable explanation for vacuoles seen in normal platelets. (Shinton N., 2006).

The dense tubular system is smooth endoplasmic reticulum, which is the site of prostaglandin synthesis and sequestration/release of calcium ions. (Shinton N., 2006).

Platelets also contain many organelles, including mitochondria, glycogen granules, lysosomes, peroxisomes, and two types of platelet-specific granules.

Platelet-specific granules are either dense osmophilic granules (dense bodies,  $\delta$ -granules) or  $\alpha$ -granules. Dense bodies contain 60% of the platelet storage pool of adenine nucleotides (such as adenosine diphosphate) and serotonin. Dense-body adenine nucleotides do not readily exchange with other adenine nucleotides in the platelet metabolic pool. The  $\alpha$ -granules contain a series of different proteins, some of which are platelet specific and some of which are found in the plasma or other cell types, such as coagulation factors. Major contents of  $\alpha$ - granules include VWF, platelet factor 4,  $\beta$ -thromboglobulin, thrombospondin, factor V, fibrinogen, fibronectin, platelet-derived growth factor, high-molecular-weight kininogen, and tissue plasminogen activator inhibitor-1. Contents of the platelet-specific granules are secreted in response to aggregating stimuli. (Shinton N., 2006)

Platelets have a number of specific antigens on their surface, many associated with platelet membrane glycoproteins Ia, Ib, Iib, IIIa, and possibly IV and V, such as HPA-1A associated with glycoprotein IIIa. These may be shared with other cells that have the adhesion receptors GP Ia and IIIa, which include vascular endothelium cells, smooth muscle cells, fibroblasts, and activated T-cells. There are no naturally occurring antibodies, these only arising from reaction to transfused platelets or placental transfer. Platelets also express human leukocyte antigen (HLA) class I antigens and ABO blood group antigens. These are of importance in immunological refractoriness to platelet transfusions. (Shinton N., 2006)

#### **1.2.1.2.1.2 Platelet production:**

Platelets are produced in the bone marrow by fragmentation of the cytoplasm of megakaryocytes, one of the largest cells in the body. The precursor of the megakaryocyte-the megakaryoblast-arises by a process of differentiation from the haemopoietic stem cell .The megakaryocyte matures by endomitotic

synchronous replication (i.e. DNA replication in the absence of nuclear or cytoplasmic division) enlarging the cytoplasmic volume as the number of nuclear lobes increase in multiples of two. A picture of mature polyploid megakaryocytes. Very early on invaginations of plasma membrane are seen, called the demarcation membrane, which evolves through the development of the megakaryocyte into a highly branched network. At a variable stage in development, most commonly at the eight nucleus stage, the cytoplasm becomes granular. Mature megakaryocytes are extremely large, with an eccentric placed single lobulated nucleus and a low nuclear to cytoplasmic ratio. Platelets form by fragmentation of megakaryocyte cytoplasm, approximately each megakaryocyte giving rise to 1000-5000 platelets. The time interval from differentiation of the human stem cell to the production of platelets averages approximately 10 days. (Hoffbrand V. *et al.*, 2006)

Thrombopoietin is the major regulator of platelet production and is constitutively produced by the liver and kidneys. Thrombopoietin increases the number and rate of maturation of megakaryocytes via c-Mpl receptor. Platelet levels start to rise 6 days after the start of therapy and remain high for 7-10 days. Unfortunately, thrombopoietin is not available for routine clinical practice. Platelets also have c-Mpl receptors for thrombopoietin and remove it from the circulation. Therefore, levels are high in thrombocytopenia as a result of marrow aplasia and vice versa. The normal platelet count is approximately  $250 \times 10^9/L$  (range  $150-400 \times 10^9/L$ ). (Hoffbrand V. *et al.*, 2006)

#### **1.2.1.2.1.3 platelets life span**

The normal platelet lifespan is 7-10 days. Up to one-third of the marrow output of platelets may be trapped at any one time in the normal spleen but this rises to 90% in cases of massive splenomegaly. (Hoffbrand V. *et al.*, 2006)

**1.2.1.2.1.4 platelets function:** The main function of platelets is the formation of mechanical plugs during the normal haemostatic response to vascular injury. In the absence of platelets, spontaneous leakage of blood through small vessels may occur. The immobilization of platelets at the sites of vascular injury requires specific platelet-vessel wall (adhesion) and platelet-platelet (aggregation) interactions. The blood flow conditions determine the specific receptor ligand interactions. (Hoffbrand V. *et al.*, 2006)

**1.2.1.2.1.4.1 Platelets Adhesion:**

The initiating event following vascular damage is platelet adhesion to exposed subendothelial matrix proteins. The platelet glycoprotein (GP) receptors which mediate adhesion are dependent on the rate of shear. Under the intermediate to high shear conditions found in arterioles, this event is strictly dependent on von Willebrand factor (vWF) and its receptor, the GPIb–IX–V complex. However, at the lower rates of shear found in the venous circulation and in the static conditions frequently used for experimental purposes, adhesion can occur directly to other subendothelial matrix proteins such as collagen and fibrinogen, although vWF also supports this event in these vessels. In both cases, adhesion is strengthened considerably through activation of platelet surface integrins, which leads to an increase in affinity for their adhesive ligands. (Hoffbrand. V. *et al.*, 2005)

Adhesion applies also to recruitment of circulating platelets into the thrombus vWF, exposed on the surface of the growing thrombus, also plays a fundamental role in this process, most notably at the high rates of shear that exist within arterioles and in diseased vessels. The platelet-bound vWF that supports these events is derived from plasma and via secretion from platelet  $\alpha$ -granules. Adhesion to the growing thrombus is supported by binding of fibrinogen to the

integrin  $\alpha\text{IIb}\beta 3$ , a process that is more correctly termed aggregation. (Hoffbrand, V. *et al.*, 2005 )

Von Willebrand factor VWF is involved in platelet adhesion to the vessel wall and to other platelets (aggregation). It also carries factor VIII and used to be referred to as factor VIII related antigen (VIII-Rag). It is a large cysteine-rich glycoprotein, with multimers made up on average of 2-50 subunits, with a molecular weight (MW) of  $0.8-20 \times 10^6$ . VWF is encoded by a gene on chromosome 12 and is synthesized both in endothelial cells and megakaryocytes, and stored in Weibel-Palade bodies and platelet granules respectively. Plasma VWF is almost entirely derived from endothelial cells, with two distinct pathways of secretion. The majority is continuously secreted and a minority is stored in Weibel-Palade bodies. The stored VWF can rise the plasma levels and it can be released under the influence of several secretagogues, like stress, exercise, adrenaline and infusion of desmopressin (1-deamino-8-D-arginine vasopressin, DDAVP). The VWF released from Weibel-Palade bodies is in the form of large and ultra large multimers, the most adhesive and reactive form of VWF. They are in turn cleaved in plasma to monomeric VWF and smaller multimers by the specific plasma metalloprotease, ADAMTS-13. (Hoffbrand, V. *et al.*, 2006).

#### **1.2.1.2.1.4.2 Platelets aggregation:**

Aggregation is used to describe cross-linking of platelets through binding of fibrinogen, or other bivalent or multivalent ligands such as vWF to the integrin  $\alpha\text{IIb}\beta 3$  on adjacent cells. In resting platelets, the integrin  $\alpha\text{IIb}\beta 3$  exists in a low-affinity conformation that is unable to bind to vWF or fibrinogen at the concentrations found within plasma (although it is able to bind to immobilized forms of these two ligands under static or low shear conditions). Upon platelet activation, so-termed ‘inside-out’ signals from other receptors cause  $\alpha\text{IIb}\beta 3$  to undergo a conformational change that increases its affinity for fibrinogen, vWF and other RGD (arginine–glycine–aspartate)-containing ligands, including fibronectin and CD40 ligand (CD40L). In turn, binding of fibrinogen and other ligands to  $\alpha\text{IIb}\beta 3$  promotes ‘outside-in’ signals that reinforce platelet activation. Although fibrinogen is considered to be the major ligand for  $\alpha\text{IIb}\beta 3$ , thrombus formation has been reported, albeit after a considerable delay, in mice deficient in both fibrinogen and vWF, bringing other RGD containing ligands into consideration as potential regulators of aggregation, most notably fibronectin, which is also present in plasma. (Hoffbrand. V. *et al.*, 2005)

The events that give rise to activation of  $\alpha\text{IIb}\beta 3$  remain poorly understood. The integrin can be activated through elevation of  $\text{Ca}^{2+}$  and by activation of protein kinase C, rap1b and phosphatidylinositol 3-kinase (PI3 kinase), although it is unclear whether these act through separate pathways or target a common regulatory protein. Elevation of  $\text{Ca}^{2+}$  is required for rapid activation of  $\alpha\text{IIb}\beta 3$  and plays an essential role in thrombus formation in vivo. Increasing evidence implicates the cytoskeleton-binding protein talin in the regulation of  $\alpha\text{IIb}\beta 3$ , although the molecular basis of its action remains to be determined. (Hoffbrand V. *et al.*, 2005 )



A deficiency or mutation in  $\alpha\text{IIb}\beta 3$  gives rise to the bleeding disorder Glanzmann's thrombasthenia. Although extremely rare, there are upwards of 300 patients who have been identified with this condition. Surprisingly, given the severity of the effect on platelet aggregation, these patients have relatively few bleeding problems, provided that they avoid unnecessary risk of trauma and are moved to a hospitalized environment following injury. (Hoffbrand. V. *et al.*, 2005)

#### **1.2.1.2.1.4.3 Platelet release reaction:**

Primary activation by various agonists induces intracellular signalling, leading to the release of  $\alpha$  and  $\beta$ - granules.  $\alpha$ -Granule contents play an important role in platelet aggregate formation and stabilization and, in addition, the ADP released from dense granules plays a major positive feedback role in promoting platelet activation. TXA<sub>2</sub> is the second of the two major platelet positive feedback loops important in secondary amplification of platelet activation to form a stable platelet aggregate. It is formed *de novo* upon activation of cytosolic phospholipase A<sub>2</sub> (PLA<sub>2</sub>) which is the rate limiting step. This liberates arachidonic acid from the membrane phospholipids, and is metabolized by cyclooxygenase to TXA<sub>2</sub>. It is a labile substance and lowers platelet cyclic adenosine monophosphate (cAMP) levels and initiates the release reaction. Thromboxane A<sub>2</sub> not only potentiates platelet aggregation, but also has powerful vasoconstrictive activity. The release reaction is inhibited by substances that increase the level of platelet cAMP. One such substance is the prostaglandin prostacyclin (PGI<sub>2</sub>) which is synthesized by vascular endothelial cells. It is a potent inhibitor of platelet aggregation and prevents their deposition on normal vascular endothelium. (Hoffbrand V. *et al.*, 2006)

#### **1.2.1.2.1.5 Clot formation and retraction**

The highly localized enhancement of ongoing platelet activation by ADP and TXA<sub>2</sub> results in a platelet plug large enough to plug the area of endothelial injury. In this platelet plug the platelets are completely degranulated and adherent to each other. This is followed by clot retraction which is mediated by GPIIb/IIIa receptors which link the cytoplasmic actin filaments to the surface bound fibrin polymers. (Hoffbrand V. *et al.*, 2006)

#### **1.2.1.3 Secondary Hemostasis:**

##### **1.2.1.3.1 Role of blood coagulation proteins in hemostasis:**

When a vessel wall is injured several events occur simultaneously. Von Willebrand factor helps flowing platelets to adhere to the vessel wall. Collagen in the vessel wall, now exposed, allows platelets to adhere by their collagen receptors leading to activation. Platelet activation leads to thrombin formation on or about the platelet surface. Independently or simultaneously depending upon the circumstance, vessel injury leads to exposure of subendothelial tissue factor (TF) along with factor VIIa activates factor IX to factor IXa. Factor IXa activates factor X to factor Xa which leads to thrombin formation. Thrombin proteolyzes fibrinogen to form fibrin, which is the protein basis of a clot. Thrombin also recruits more platelets to the site of injury. It is recognized that in vivo, several pathways lead to thrombin formation. Similarly, when injured, endothelium too becomes a procoagulant surface. The procoagulant nature of endothelial cells is due to increased expression of TF and factor VIIa to initiate thrombin formation, increased synthesis of factor V to serve as a co - factor for more thrombin formation, inactivation of thrombomodulin for protein C activation, and increased plasminogen activator inhibitor expression with reduced tissue plasminogen activator. (Schmaier A. *et al.*, 2012)

#### **1.2.1.3.2 The coagulation cascade**

Blood coagulation involves a biological amplification system in which relatively few initiation substances sequentially activate by proteolysis a cascade of circulating precursor proteins (the coagulation factor enzymes) which culminates in the generation of thrombin; this, in turn, converts soluble plasma fibrinogen into fibrin. (Hoffbrand V. *et al.*, 2006)

Fibrin enmeshes the platelet aggregates at the sites of vascular injury and converts the unstable primary platelet plugs to firm, definitive and stable Haemostatic plugs. The operation of this enzyme cascade requires local Concentration of circulating coagulation factors at the site of injury. (Hoffbrand V. *et al.*, 2006)

Surface-mediated reactions occur on exposed collagen, platelet phospholipid and tissue factor. With the exception of fibrinogen, which is the fibrin clot subunit, the coagulation factors are either Enzyme precursors or cofactors. All the enzymes, except factor XIII, are serine proteases (i.e. their ability to hydrolyse peptide bonds depends upon the amino acid serine at their active centre. The scale of amplification achieved in this System is dramatic,(e.g.1mol of activated factor XI through sequential activation of factors IX ,X and pro-thrombin may generate up to  $2 \times 10^8$ mol of fibrin). (Hoffbrand V. *et al.*, 2006)

##### **1.2.1.3.2.1 Plasma Protein Coagulation Factors**

They are present in the plasma in trace to small amounts and, although difficult to isolate in pure form, they can be identified and quantitated by characteristic behaviour in in vitro tests.

Factors II, VII, IX, X, XI, XII, and pre-kallikrein are inactive precursors of serine proteases, all retaining considerable homology with trypsin, which is one of the earliest evolutionary prototypes. The carboxy-terminal region

is similar in all the serine proteases, consists of about 250 amino-acid residues, and contains the active site region. The vitamin K-dependent factors (II, VII, IX; and X) show considerable identity of the amino terminal region, containing 10-12 carboxy glutamic acid residues essential for  $\text{Ca}^{++}$ -mediated phospholipid binding. The inactive zymogens are activated by limited proteolysis, often accompanied by the release of an activation peptide. Each of the active coagulation factors has a considerable degree of specificity for its substrate, this specificity being conferred by the 'binding pocket' and the conformation of the protein as a whole, which probably limits its substrate recognition. Factors V and VIII are larger glycoproteins; factor V has a molecular weight of approximately 330000, and factor VIII 360 000. Activation of these factors is also achieved by limited proteolytic cleavage, resulting in the formation of two non-covalently associated peptides derived from the amino-terminal and carboxy-terminal portions of the inactive molecule, in the case of factor Va being of molecular weight of 94.000 and 74.000 respectively. There is substantial homology between the heavy and light chains of factor Va and factor VIII. Von-Willebrand factor forms a non-covalent complex with factor VIII, thus preventing more rapid removal of the coagulation protein from the plasma. The other contributions of von Willebrand factor to haemostasis also relate to its adhesive properties, mediating adhesion of platelets to the subendothelial tissues and involvement in platelet aggregation. Von Willebrand factor circulates as heterogeneous collections of oligomers ranging from the dimer of 500 000 to species of greater than 10.000.000. Factor I or fibrinogen, is an asymmetrical protein consisting of three pairs of dissimilar polypeptide chains, A $\alpha$ 2, B $\beta$ 2, and Y2, linked by disulphide bonds. The molecular weight of fibrinogen is 340 000. The

amino-terminal segments of all six chains are probably clustered in a central domain with fibrinopeptides A and B protruding. The proteolytic cleavage of fibrinogen by thrombin, with the release of the fibrinopeptides, is responsible for the spontaneous polymerization of fibrin molecules to form an insoluble network. Factor XIII is an inactive pro-enzyme composed of two pairs of polypeptide chains ( $\alpha_2\beta_2$ ). A platelet factor XIII consists of only two  $\alpha$  chain subunits. Factor XIII is activated to a calcium-dependent transglutaminase by thrombin cleavage of a small peptide from the amino-terminal end of the  $\alpha$  subunits. The transglutaminase activity of factor XIII is primarily directed to forming isopeptide bonds between lysine and glutamine residues of the fibrin  $\gamma$  chains to form  $\gamma$ - $\gamma$  dimers, and the  $\alpha$  chains to form polymers. These reactions, termed cross-linking or stabilization, make fibrin resistant to plasmin attack and confer structural stability. The liver hepatocyte is the site of synthesis of most of the coagulation proteins. Vitamin K is required to convert the inactive forms of factors II, VII, IX, and X in the liver to their active forms by carboxylation of amino-terminal clusters of glutamic acid residues to gamma-carboxy glutamic acid. Factor VIII is probably synthesized in hepatic sinusoidal endothelial cells. Von Willebrand factor is synthesized in vascular endothelium and bone marrow megakaryocytes. Plasma factor V is derived from hepatic synthesis, while platelet factor V is probably synthesized in megakaryocytes. Factor XIII is similarly disposed. Synthesis of these proteins is controlled by autosomal genes, with the exception of factor VIII and factor IX where sex-linked genes operate. Inherited deficiency states usually result from the production of a defective molecule, although complete or partial failure of synthesis may occur in some cases.

Autosomal recessive inheritance probably explains the extreme rarity of most disorders; the disorders in which factor VIII or factor IX are inherited in the sex-linked manner (haemophilia A and haemophilia B) are relatively much more common. Fibrinogen concentration may be measured directly as clottable protein, but the other coagulation proteins are usually expressed in units of activity compared to that of a pool of normal plasma. Immunoreactive protein levels can also be measured. It is a general rule that the haemostatic efficiency of the coagulation system is not impaired until the activity of one or more clotting factors is less than 30 percent of normal (0.3 U fml). (Frankin F., 1989).

#### **1.2.1.3.2.2 The Vitamin K-Dependent Factors**

The vitamin K-dependent factors group includes coagulation factors II, VII, IX and X. However, it is important to remember that the anticoagulant proteins S, C and Z are also vitamin K-dependent. Each of these proteins contains a number of glutamic acid residues at its amino terminus that are g-carboxylated by a vitamin K-dependent mechanism. This results in a novel amino acid, g-carboxyglutamic acid, which by binding calcium is essential in promoting a conformational change in the protein and binding of the factor to negatively charged phospholipid. Because this binding is crucial for coordinating the interaction of the various factors, the proteins produced in the absence of vitamin K (PIVKAs) that are not g-carboxylated are essentially functionless. The vitamin K-dependent factors are proenzymes or zymogens, which require cleavage, sometimes with release of a small peptide (activation peptide), to become functional. Measurement of these activation peptides has been used as a means of assessing coagulation activation. (Barbara. *et al.*, 2011)

#### **1.2.1.3.2.3 Cofactors**

Factors VIII and V are the two most labile of the coagulation factors and they are rapidly lost from stored blood or heated plasma. They share considerable structural homology and are cofactors for the serine proteases FIX and FX, respectively; they both require proteolytic activation by factor IIa or Xa to function. Factor VIII circulates in combination with VWF, which is present in the form of large multimers of a basic 200 kDa monomer. One function of VWF is to stabilize factor VIII and protect it from degradation. In the absence of VWF, the survival of factor VIII in the circulation is extremely short (i.e. <2 h instead of the normal 8–12 h). VWF may also serve to deliver factor VIII to platelets adherent to a site of vascular injury. Once factor VIII has been cleaved and activated by thrombin it no longer binds to VWF. (Barbara. *et al.*, 2011)

#### **1.2.1.3.2. 4 The Contact Activation System**

The contact activation system comprises factor XII (Hageman factor), high molecular weight kininogen (HMWK) (Fitzgerald factor) and prekallikrein kallikrein (Fletcher factor). As mentioned earlier, these factors are not essential for haemostasis *in vivo*. Important activities are to activate the fibrinolytic system, to activate the complement system and to generate vasoactive peptides: in particular, bradykinin is released from HMWK by prekallikrein or FXIIa cleavage. Kallikrein and factor XIIa also function as chemoattractants for neutrophils. The contact activation system also has some inhibitory effect on thrombin activation of platelets and prevents cell binding to endothelium. Recent evidence implicates the contact system in thrombosis via activation by polyphosphate released from platelets. (Barbara. *et al.*, 2011)

When bound to a negatively charged surface *in vitro*, factor XII and prekallikrein are able to reciprocally activate one another by limited proteolysis, but the initiating event is not clear. It may be that a conformational change in

factor XII on binding results in limited autoactivation that triggers the process. HMWK acts as a (zinc-dependent) cofactor by facilitating the attachment of prekallikrein and factor XI, with which it circulates in a complex, to the negatively charged surface. It has been shown in in vitro studies that platelets or endothelial cells can provide the necessary negatively charged surface for this mechanism and also possess specific receptors for factor XI. The contact system can activate fibrinolysis by a number of mechanisms: plasminogen cleavage, urokinase plasminogen activator (uPA) activation and tissue plasminogen activator (tPA) release. Most importantly from the laboratory point of view, the contact activation system results in the generation of factor XIIa, which is able to activate factor XI, thus initiating the coagulation cascade of the intrinsic pathway. (Barbara. *et al.*, 2011)

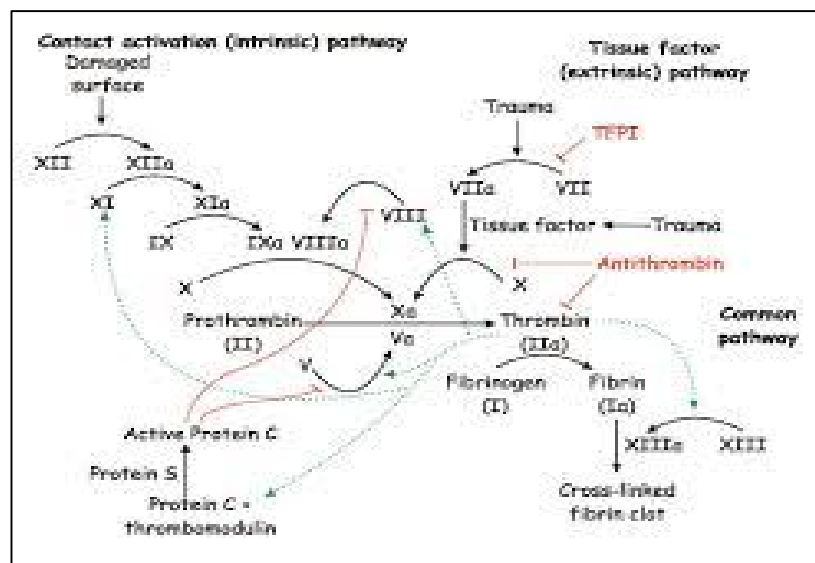
#### **1.2.1.3.2.5 Tissue Factor**

TF is the cofactor for the extrinsic pathway and the physiological initiator of coagulation. It is a transmembrane protein and constitutively present in many tissues outside the vasculature and on the surface of stimulated inflammatory cells such as monocytes and, under some conditions, endothelial cells. Factor VIIa binds to TF in the presence of calcium ions and then becomes enzymatically active. Small amounts of factor VIIa are present in the circulation but have virtually no enzymic activity unless bound to TF. The factor VIIa–TF complex can activate both factor X and factor IX and therefore two routes to thrombin production are stimulated. Factor Xa subsequently binds to TFPI and then to factor VIIa to form an inactive quaternary (Xa–TF–VIIa–TFPI) complex. This mechanism therefore functions to shut off the extrinsic pathway after an initial stimulus to coagulation has been provided. (Barbara. *et al.*, 2011)



#### 1.2.1.3.2.6 Final Common Pathway

Once factor X is activated to Xa, the extrinsic and intrinsic pathways enter a common pathway. Factor II, prothrombin, is activated to thrombin (factor IIa), which normally circulates in the blood as an inactive factor. Following the activation of factor Xa, it remains platelet bound and activates factor V. The complex of factors Xa and Va on the platelet surface is formed near platelet-bound factor II molecules. In turn, the platelet-bound Xa/Va complex cleaves factor II into thrombin, factor IIa. The stage is accelerated by factor V and ionized calcium.



**Figure (1-2): Coagulation cascade**

#### 1.2.1.3.2.7 Fibrin Formation

Clotting is the visible result of the conversion of plasma fibrinogen into a stable fibrin clot. Thrombin plays a major role in converting factor XIII to XIIIa and in converting fibrinogen to fibrin. Fibrin formation occurs in three phases: proteolysis, polymerization, and stabilization. Initially, thrombin, a protease enzyme, cleaves fibrinogen, which results in a fibrin monomer, fibrinopeptide A, and fibrinopeptide B fragments. In the second step, the fibrin monomers

spontaneously polymerize end-to-end due to hydrogen bonding. Finally, the fibrin monomers are linked covalently by factor XIIIa into fibrin polymers. These polymers form a meshy network, and the final fibrin solution is converted to a gel when more than 25% of the fibrinogen is converted to fibrin. Factor XIII is converted to the active form, factor XIIIa, in two steps. In the first step, thrombin cleaves a peptide from each of the two alpha chains of factor XIII with formation of an inactive intermediate form of factor XIII. In the second step, calcium ions cause factor XIII to dissociate, forming factor XIIIa. Fibrinogen is normally present in the plasma as a soluble molecule. Subsequent to the action of thrombin, fibrinogen is transformed into fibrin, an insoluble gel. This conversion of fibrinogen to a cross-linked gel occurs in several stages. Factor XIIIa introduces peptide bonds within the polymerized fibrin network. This cross-linking makes the fibrin more elastic and less susceptible to lysis by fibrinolytic agents. Fibrin forms a loose covering over the injured area, reinforces the platelet plug, and closes off the wound. After a short period, the clot begins to retract and becomes smaller and more dense. This retraction process is thought to be caused by the action of platelets trapped along with erythrocytes and leukocytes in the clot. As the fibrin filaments gather around the aggregated platelets, the platelets send out cytoplasmic processes that attach to the fibrin and pull the fibers closer together. When a clot forms in a test tube, clot retraction can be observed. (Turgeon M., 2012)

#### **1.2.1.4 Fibrinolysis**

It is widely acknowledged that the principal functions of the fibrinolytic system are to ensure that excess fibrin deposition is either prevented or rapidly removed (i.e. that a localized pro-coagulant response is achieved without compromising blood circulation generally) and, following re-establishment of haemostasis, the fibrin mesh is removed during wound healing. The system of

profibrinolytic and antifibrinolytic factors that has evolved to meet these requirements is closely coupled to that which results in fibrin clot formation. Fibrinolysis is essentially a localized, surface - bound phenomenon, with most events being catalysed by the presence of cross - linked fibrin itself, i.e. fibrin orchestrates its own destruction. For this reason, the assays of fibrinolytic factors carried out in the soluble phase, in particular in systemic blood, may be misleading and should be interpreted with great caution. Components of the fibrinolytic system. These include PLG and plasmin, several endogenous (tissue or plasma derived) or exogenous (e.g. bacterial or venom derived) PLG activators, and a number of inhibitors of plasmin or of the PLG activators and a simplified representation of their interaction. Both endogenous and exogenous fibrinolytic factors have been used clinically to treat venous and arterial thrombosis, with varying degrees of success. (Hoffbrand V. *et al.*, 2005)

#### **1.2.1.4.1 Plasminogen and plasmin**

Plasminogen is a single chain glycoprotein zymogen of the serine protease plasmin, which carries out the enzymatic degradation of cross-linked fibrin. Besides its active site serine, plasmin contains five kringle domains, four of which have a lysine - binding site, through which the molecule interacts with lysine residues in its substrates (e.g. fibrin), its activators (e.g. tissue PLG activator and urinary PLG activator) and its inhibitors (principally PLG activation inhibitor type 1). In its native form, it has a glutamic acid residue at its N - terminus and is known as Glu - PLG. Conversion of PLG to plasmin can proceed via two routes. Most PLG activators cleave the Arg561 - Val562 bond to form Glu - plasmin, a disulphide - linked two -chain molecule. The heavy chain is derived from the N - terminal region and bears the lysine - binding sites, whereas the C - terminal light chain contains the serine active centre. Glu - plasmin, despite being a serine protease, is functionally ineffective as its lysine -

binding sites remain masked. It is converted autocatalytically to Lys - plasmin by N - terminal cleavage, chiefly between Lys76 and Lys77, which exposes the lysine - binding sites and thus markedly enhances its interaction with fibrin. Both Glu - and Lys - plasmin also attack the same Lys76 – Lys77 bond in Glu - PLG to form the zymogen Lys - PLG. This binds to fibrin before activation to the protease and is thus brought into close proximity with the physiological PLG activators (which also bind to fibrin) that convert it to Lys - plasmin. As a consequence, the conversion of PLG to plasmin by tPA is enhanced by two to three orders of magnitude; this serves to localize the fibrinolytic response to the fibrin clot, where plasmin is to some extent protected from the effects of circulating antiplasmins, which (as indicated below) would otherwise neutralize plasmin extremely rapidly ( <50 ms). The fact that Lys - PLG is potentially a much more effective agent in fibrinolysis than Glu - PLG is reflected in its half - life, which is around 20 hours compared with 50 hours for the latter.

#### **1.2.1.4.2 Action of plasmin on fibrin and fibrinogen**

Plasmin can hydrolyse a variety of substrates including FV and FVIII, but its major physiological targets are fibrin and fibrinogen, which are split progressively into a heterogeneous mixture of small soluble peptides (plasmin attacks at least 50 cleavage sites in fibrinogen) known collectively as fibrin degradation products (FDPs). The first stage in the proteolysis of fibrinogen involves the removal of several small peptides (fragments A, B and C) from the C - terminus of the A  $\alpha$ - chains, each involving cleavage after a lysine residue. This is rapidly followed by removal of the first 42 amino acids from the N-terminal end of the B  $\beta$ - chain (the B  $\beta$ 1 – 42 fragment). The large residual portion, which is known as fragment X, and which still contains fibrinopeptide A, remains thrombin - clottable and will agglutinate some *Staphylococcus* spp. Assay of the B  $\beta$ 1 – 42 fragment released from fibrinogen by plasmin gives a

sensitive index of fibrinogenolytic activity. Asymmetrical digestion of all three pairs of chains of fibrin or fibrinogen then occurs with the release of the D fragment, in which the chains remained linked by disulphide bonds. The residue, known as fragment Y, is again attacked by plasmin, cleaving a second fragment D and leaving the disulphide - linked N - terminal ends of all six chains, which are referred to as fragment E. Fragments Y, D and E are not thrombin - clottable and do not agglutinate staphylococci. Their presence can be detected immunologically using an antibody - coated latex bead agglutination assay, which provides a simple test for most FDPs, although carefully prepared serum must be used to prevent cross - reactivity of the antibody with fibrinogen in plasma. These assays detect the degradation products of both fibrin and fibrinogen indiscriminately. Following thrombin generation and consequent activation of FXIII, intermolecular or intramolecular transamidation of the  $\alpha$ - or  $\beta$ - chains by FXIIIa occurs and then the action of plasmin yields characteristic D - dimer, D - dimer - E fragments and oligomers of fragments X and Y (collectively known as cross - linked FDP or XDP), in addition to X, Y, D and E. These XDPs can be detected very simply using monoclonal antibody - coated latex beads. Because the monoclonal antibodies to XDPs do not cross - react with fibrinogen, they can be detected directly in citrated plasma. The presence of D - dimers in blood samples can be used in a clinical algorithm that predicts the likelihood of the presence of venous thrombosis. Furthermore, plasmin induced cleavage of the N - terminal end of the  $\beta$ - chain of fibrin (the B  $\beta$ 1 - 14 fibrinopeptide B fragment having been removed by thrombin) produces a  $\beta$ 15 - 42 fragment, the detection of which indicates fibrin (as opposed to fibrinogen) degradation. Consequently, assays for the B  $\beta$ 1 - 42 and  $\beta$ 15 - 42 fragments used in combination may be clinically useful by indicating whether fibrinogen or fibrin has been degraded, and thus whether fibrinolytic activity is primary or

secondary to fibrin formation. However, clinically, FDP assays are used to detect DIC, when mixed fibrin/fibrinogen degradation products appear in the circulation. (Hoffbrand V. *et al.*, 2005)

## **1.2.2 Tobacco**

### **1.2.2.1 Tobacco smoking:**

Tobacco smoking is the practice of burning tobacco and inhaling the smoke (consisting of particle and gaseous phases). (A more broad definition may include simply taking tobacco smoke into the mouth, and then releasing it, as is done by some with tobacco pipes and cigars.) The practice may have begun as early as 5000-3000 BC. Tobacco was introduced to Eurasia in the late 17th century where it followed common trade routes. The practice encountered criticism from its first import into the Western world onwards, but embedded itself in certain strata of a number of societies before becoming widespread upon the introduction of automated cigarette-rolling apparatus. (Parrot et al., 1989)

German scientists identified a link between smoking and lung cancer in the late 1920s, leading to the first anti-smoking campaign in modern history, albeit one truncated by the collapse of the Third Reich at the end of the Second World War. In 1950, British researchers demonstrated a clear relationship between smoking and cancer. Evidence continued to mount in the 1980s, which prompted political action against the practice. Rates of consumption since 1965 in the developed world have either peaked or declined. However, they continue to climb in the developing world. (Parkin C. *et al.*, 1998)

Smoking is the most common method of consuming tobacco, and tobacco is the most common substance smoked. This agricultural product is often mixed with additives and then combusted. The resulting smoke is then inhaled and the active substances absorbed through the alveoli in the lungs. Combustion was traditionally enhanced by addition of potassium or other nitrates. Many

substances in cigarette smoke trigger chemical reactions in nerve endings, which heighten heart rate, alertness, and reaction time, among other things. Dopamine and endorphins are released, which are often associated with pleasure. As of 2008 to 2010, tobacco is used by about 3 billion people (about 49% of men and 11% of women) with about 80% of this usage in the form of smoking. The gender gap tends to be less pronounced in lower age groups. Many smokers begin during adolescence or early adulthood. During the early stages, a combination of perceived pleasure acting as positive reinforcement and desire to respond to social peer pressure may offset the unpleasant symptoms of initial use, which typically include nausea and coughing. After an individual has smoked for some years, the avoidance of withdrawal symptoms and negative reinforcement become the key motivations to continue. (Richard P. *et al*, 2000)

In a study done by Jennifer O' Loughlin and her colleagues, the first smoking experiences of seventh-grade students were studied. They found out that the most common factor leading students to smoke is cigarette advertisements. Smoking by parents, siblings and friends also encourage students to smoke. (Richard P. *et al.*, 2000)

#### **1.2.2.2 Cigarette Components:**

Is a broad-leafed plant of the nightshade family, indigenous to North and South America, whose dried and cured leaves are often smoked in the form of a cigar or cigarette, or in a smoking pipe, or in a water pipe or a hookah. Tobacco is also chewed, "dipped" (placed between the cheek and gum), and consumed as finely powdered snuff tobacco, which is sniffed into the nose. The word "tobacco" is an Anglicization of the Spanish word "tabaco", whose roots are unclear; it is thought to derive from the Native American word "tabago," for a Y-shaped pipe used in sniffing tobacco powder. (Pakhale. S. *et al.*, 1998)

Tobacco contains nicotine, an organic alkaloid and powerful neurotoxin, particularly to insects. All means of consuming tobacco result in the absorption of nicotine in varying amounts into the user's bloodstream, and over time the development of a tolerance and dependence. Absorption quantity, frequency, and speed seem to have a direct relationship with how strong a dependence and tolerance, if any, might be created. A lethal dose of nicotine is contained in as little as one half of a cigar or three cigarettes; however, only a fraction of the nicotine contained in these products is actually released into the smoke, and most clinically significant cases of nicotine poisoning are the result of concentrated forms of the compound used as insecticides (Pakhale S.*et al.*, 1998)

#### **1.2.2.3 Effect of smoking on blood:**

Blood is a vehicle for delivering oxygen and nutrients to the body's tissues and organs without die. The blood vessels (our circulatory system) are blood piping highways. The inside of each healthy blood vessel is coated with a thin Teflon like layer of cells that ensure smooth blood flow. Carbon monoxide from smoking or second-hand smoke damages this important layer of cells, allowing fats and plaque to stick to vessel walls. Nicotine then performs a double whammy of sorts. (Elian .2002)

First, each time new nicotine arrives into brain it causes the body to activate its fight or flight stress defenses. This in turn causes the immediate release of stored fats into the bloodstream, fats intended to be used to provide the instant energy needed to either fight or flee the saber tooth tiger.

The extra food we consumed during a big meals each day was converted to fat and stored. It was then pumped back into our bloodstream with each new puff of nicotine. It's how we were able to skip meals and what causes many of us to experience wild blood sugar swings when trying to quit. In fact, many of the



symptoms of withdrawal - like an inability to concentrate - are due to nicotine no longer feeding us while we continue to skip meals. (Elian .2002)

They need to eat more food in order to avoid extreme blood sugar fluctuations when quitting. We need to learn to spread our normal daily calorie intake out more evenly over the entire day. We need to learn to once again feed ourselves. (Pakhale S.*et al.*, 1998)

The heavy blasts of stored fats released by nicotine stick to vessel walls damaged by toxic carbon monoxide. Recently learned that nicotine itself, inside the vessels, somehow causes the growth of new blood vessels (vascularization) that then provides a rich supply of oxygen and nutrients to the fats and plaques that have stuck to damaged vessel walls. This internal nicotine vascularization (vessels within vessels) hardens a smoker's arteries and veins and further accelerates their narrowing and clogging.) (Elian .2002)

A rough sense of the damage in the lungs but degree of clogging has already occurred inside the blood vessels How long do we have before our coronary arteries that supply life giving oxygen and nutrients to our heart muscle become 100% clogged When it happens it's called a heart attack and the portion of the heart muscle that receives oxygen from a particular coronary artery will quickly suffocate and die. How long do we have before our carotid arteries supplying life giving oxygen and nutrients to our brain become 100% clogged when it happens it's called a stroke and the portion of the brain serviced by the artery suffocates and dies. (Elian .2002)

The damage being done isn't just to the vessels supplying blood to our heart and brain. It's occurring, to one degree or another, inside every vessel in a smoker's body. It affects everything from blood vessels associated with hearing, to the skin's blood supply that shows itself in wrinkles, early aging, hair loss and tooth loss. Below are links to images showing varying degrees of artery clogging

followed by links that aid in understanding our body's circulatory system. (Elian .2002)

### **1.3 Rationale**

Smoking is a major health problem that results insignificant morbidity and mortality. Scanty information on haemostatic studies smokers was observed in Sudan, That is why this study was conducted .The main concept is to assess the Prothrombin Time and Partial Thromboplastin Time test values of cigarette smokers and to observe if there is any significant effect of cigarette smoking on these parameters. Smoking resulting in increase heart rate and inhalation of foreign substances that may affect the respiratory system

## **1.4 Objectives**

### **1.4.1 General objective:**

To measure some of the Haemostatic parameters in Sudanese Tobacco smoker in Khartoum state.(PT , APTT)

### **1.4.2 Specific objectives:**

- To measure Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT) in some Sudanese Tobacco smokers.
- To compare the PT and APTT of tobacco smoker group with normal individuals group .
- To measure the PT and APTT values according to the duration of smoking and the number of cigarette smoked per day

## **Chapter two**

### **2.Material and method**

#### **2.1 Study Design**

This is a case control study conducted in tobacco smoker in Khartoum state from April to September 2014 to determine PT and APTT .

#### **2.2 Study population and sample size**

Males with different ages range from 20 – 60 years were included in this study. One hundred samples were collected randomly from a tobacco cigarette smokers, and 50 samples were collected from healthy individuals as a control group.

#### **2.3 Data collection**

The data was collected by, a designed questionnaire which included (number of cigarette smoked per day, duration of smoking )

#### **2.4 Inclusion criteria**

Tobacco consumers not under anticoagulant therapy or any condition can affect the result were included in this study .

#### **2.5 Exclusion criteria**

Individuals who has chronic diseases which may affect in coagulation process or using any anticoagulant therapy were excluded .

#### **2.6 Sampling**

Randomized system was used to collect samples from area of study .

#### **2.7 Sample collection**

Under aseptic conditions 2.5 ml of venous blood was collected in container with tri-sodium citrate as anticoagulant, the blood was centrifuged for 15 minutes at 4500 rpm to obtain platelets poor plasma (PPP).

**2.8 Ethical consideration** All participants were informed about this study and its benefits on their health, and verbal consent were obtained.

## **2.9 Data analysis**

The data was analyzed using SPSS computer program. (Frequencies, means, cross tabulation and P. values) were calculated.

## **2.10 Methods:**

### **2.10.1 Prothrombin time (PT) (using automated Bio Bas coagulometry):**

#### **2.10.1.1 Principle of coagulometer:**

The coagulometer clot has an optical measurement system which detects a sudden variation in optical density when a clot is formed. The chronometer and the stirring system are activated by a sudden change of the optical density, This permits the initiation of the time measurement. When the reagent and the plasma are in contact an O.D. variation is produced, that automatically activates the digital chronometer and the magnetic mixer. When the clot starts to be formed, an O.D. variation is produced and stops the chronometer and the mixer. The clotting time appears on the display.

#### **2.10.1.2 Principle of PT:**

The PT was performed by automated testing measure the clotting time of plasma in the presence of an optimal concentration of tissue extract (thromboplastin) with calcium chloride ( $\text{CaCl}_2$ ) which indicates over all the efficiency of the extrinsic clotting system .

#### **2.10.1.3 Reagents and materials:**

- 1) Pooled normal plasma control.
- 2) Prothrombin time kits.
- 3) Kit contents: PT reagent contains an extract of rabbit brain with buffer, stabilizer and calcium chloride.
- 4) Coagulation analyzer
- 5) Small cuvettes.
- 6) Magnetic.

7) Pipette tips.

8) Calibrated pipettes.

#### **2.10.1.4 Assay procedure:**

- Cuvettes were placed in an incubation area for prewarming at 37°C for at least 3 minutes.
- A magnetic was dispensed to each cuvette ,In the incubation area 100  $\mu\text{l}$  of PPP of patient (which were thawing in water bath at 37 °C or control was dispensed in each cuvette.
- After warming the cuvette was transferred to test column area,100 $\mu\text{l}$  of the mixture  $\text{Ca}^{+}$  /thromboplastin was dispensed into cuvette with the test column area.
- The timer was started immediately pipette key.
- Then after clot produce the instrument automatically stopped the timer and the result of PT appear at the display of the instrument per seconds.

#### **2.10.1.5 Normal value:**

11 – 17 seconds (depend on PT reagent).

#### **2.10.2 Activated Partial Thromboplastin time (APTT) (using Automated bio bas coagulometry):**

##### **2.11.2.1 Principle of APTT:**

The APTT was performed by automated testing in the batch or state mode. in the APTT an aliquot of undiluted, platelet poor plasma was incubated at 37°C with a particulate factor XII activator( i.e. ,silica, celite, kaolin ,ellagicacid ,etc).A reagent containing phospholipid (partial thromboplastin) was added, followed by  $\text{CaCl}_2$  .the time required for clot formation after the addition of  $\text{CaCl}_2$  .it measure over all activity of in intrinsic pathway.

##### **2.11.2.2 Reagents and Materials:**

- Pooled normal plasma control.

- Activated partial thromboplastin time kits.

Kit contents:\* APTT reagent contains kaolin cephalin with phospholipid, buffer and preservatives.

\*CaCl<sub>2</sub> (.025M) contain sodium azide.

- Coagulation analyzer
- Small cuvettes
- Magnetic.
- Pipette tips.
- Calibrated pipettes.

#### **2.11.2.3 Assay procedure:**

- Cuvettes were placed in incubation area for prewarming at 37°C for at least 3 minutes
- A magnetic was dispensed to each cuvette
- In the incubation area 100μl of PPP of patient (which were thawing in water bath at 37 °C ) or control was dispensed in each cuvette
- 100μl of cephalin /kaolin mixture was add to each cuvette
- After incubation for 3 minutes the cuvette transform to test column area
- Then 100μl of CaCl<sub>2</sub> .025M was dispensed into cuvette within test column area.
- The timer was started immediately by pressing the pipette key
- Then after clot produce the instrument automatically stopped the timer and the result of APTT appear at the display of the instrument per seconds.

#### **2.11.2.4 Normal value:**

27 – 42 seconds (depend on APTT reagent).



## **Chapter three**

### **3.Results**

This is case control study, was done in Khartoum state during the period from April to September 2014. The aim of the study is to determine the affect of tobacco cigarette smoking on Prothrombin Time and Activated Partial Thromboplastin Time. A total of 150 samples were collected from Sudanese healthy males, 100 samples as a case group (smokers), and 50 samples as a control group (non-smokers).

#### **3.1: Demographic data :**

The results showed that all of study population were males, 66.70% were case group (smokers), and 33.40% were control (non-smokers) healthy individuals (fig. 3.1).

Distribution of age group were 49% between ( 20 – 30) years old, 15% between (31 – 40) years, 16% between (41 – 50) years, and 20% between ( 51 – 60 )years old in the case group (fig. 3.2). While, in the control group 74% between( 20 – 30 )years, 4% between ( 31 – 40) years, 6% between (41 – 50) years, and 16% between (51 – 60) years (fig. 3.3)

The results showed the distribution of study population according to duration of smoking, in which (1- 5) years ,( 6 – 10)years , (11 – 15) years ,(16 – 20 )years, and (21 – 25 )years were 38%, 24%, 22% ,9% ,and 7% respectively . (Table 3.1)

### **3.2.Laboratory data:**

Figure (3.4) illustrated that the means of the PT was  $18.2 \pm 3.2$  second , and  $13.1 \pm 1.5$  second for the case and control group, respectively. The results revealed significant increase difference between the case and control group ( $P < 0.05$ ).

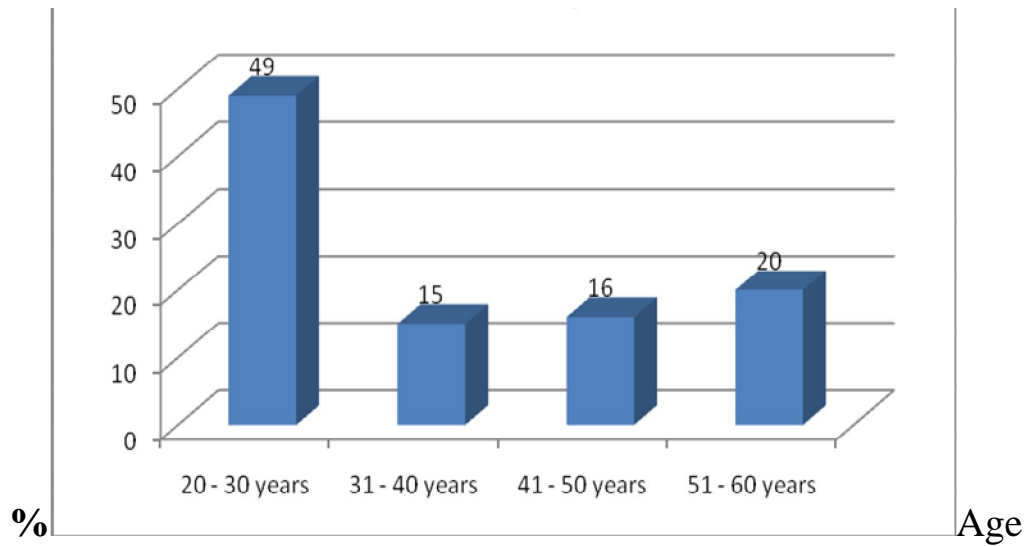
The effect of smoking on the value of Activated Partial Thrombolastin Time in the case and control group shows that the mean of the APTT were  $33.4 \pm 3.5$  , and  $30.8 \pm 2.1$  or the case and control group, respectively. The result revealed no significant difference between the case and control group ( $P > 0.05$ ). Figure (3.5)

The frequency of the case group were divided into five group according to the duration of smoking as follow (1-5 )year ,(6-10),(11-15),(16-20) and (21-25) year ,in which the means of PT were 17.6 , 18.4,18.5,19.4 and 18.5 respectively and the mean of APTT were 33.2 ,33.4 , 34.2 ,32.3 ,and 33.4 respectively. Statistically, the results revealed insignificant difference according to the duration of smoking groups for the PT and APTT when compare with control ( $P > 0.05$ ) (fig. 3.6 and fig. 3.7).

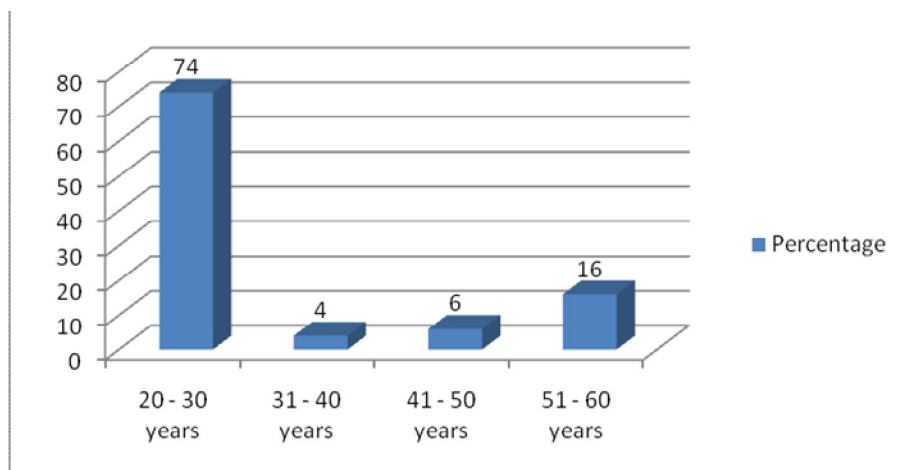
According to number of cigarette smoked per day the smokers were grouped into three categories. The result shows that the mean of PT and APTT was 16.2, 33.4 respectively for the group using less than five cigarettes per day, and 18.2, 33.1 for the smokers smoked more than 5 cigarettes and less than ten cigarettes per day, and for the smokers using more than 10 cigarette per day the PT and APTT were 18.7, 34.3 respectively. Statistically, the result revealed insignificant variation for the PT and APTT according to the number of cigarette ( $P > 0.05$ ) (fig. 3.6, fig. 3.7).

**Table (3.1):Distribution of study population in case and control group**

<b>Group</b>	<b>%</b>
case	66.70%
Control	33,40%



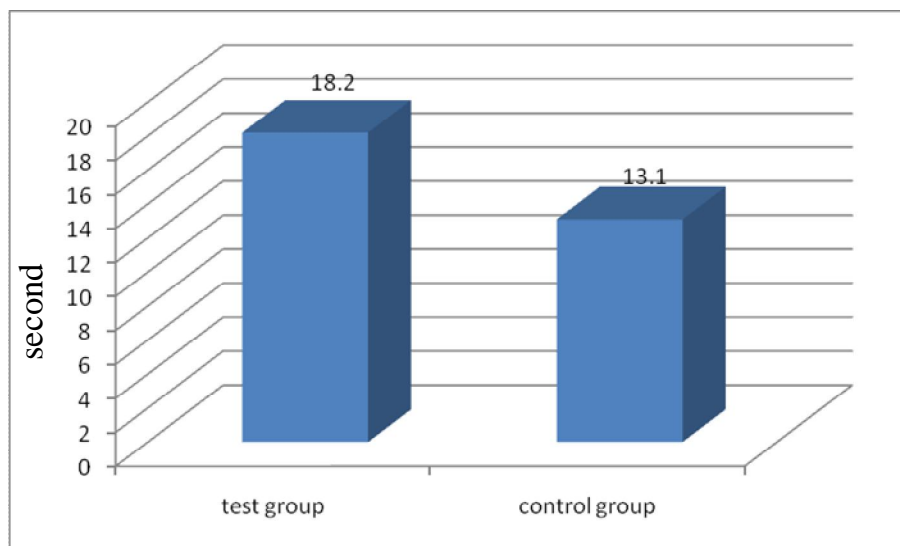
**Fig (3.2): Distribution of study population according to age group of the case group**



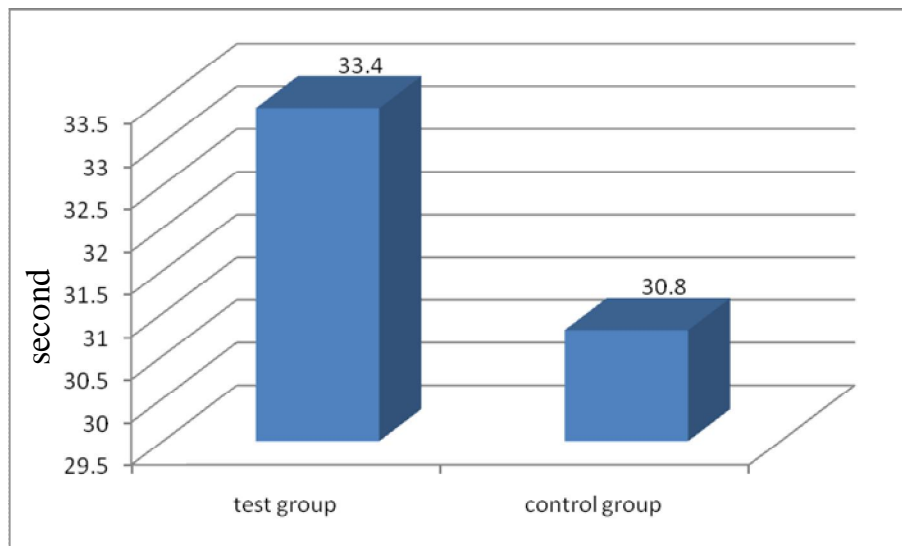
**Fig (3.3) :Percentage of age groups of the control group**

**Table (3.1): Distribution of case group according to duration of smoking**

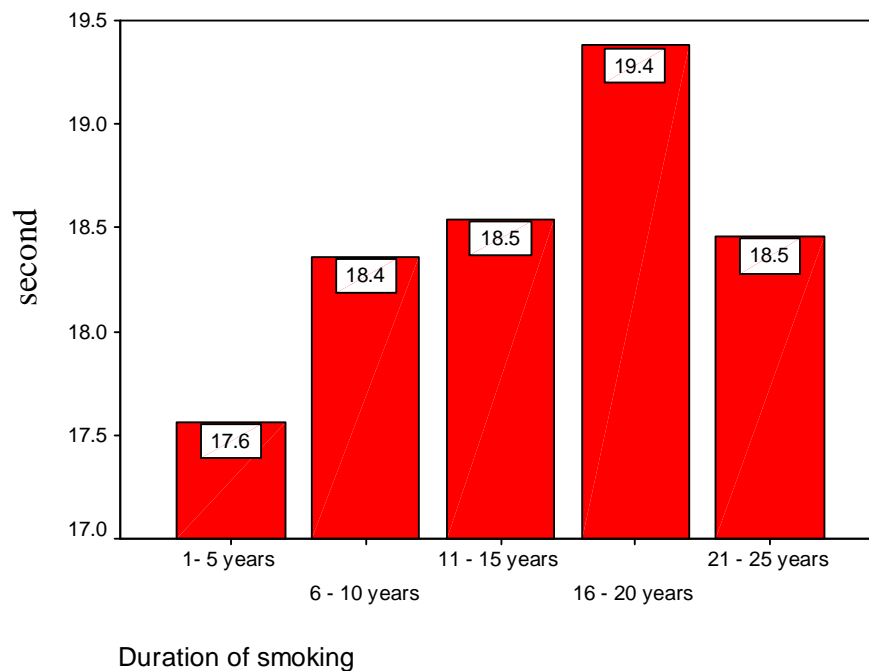
<b>Duration of smoking (years)</b>	<b>Percentage</b>
1 - 5 years	38 %
6 - 10 years	24 %
11 - 15 years	22 %
16 - 20 years	9 %
21 - 25 years	7 %
Total	100 %



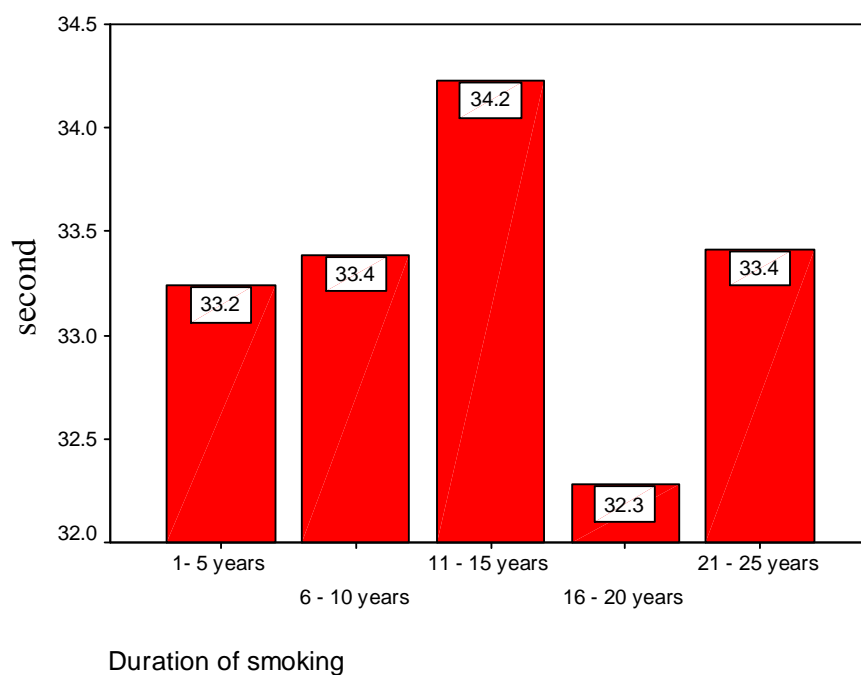
**Fig (3.4):The mean values of Prothrombin Time for the case and control Groups (P. value: 0.000)**



**Fig (3.5): The mean values of Activated Partial Thromboplastin Time for the case and control groups(P. value: 0.713)**

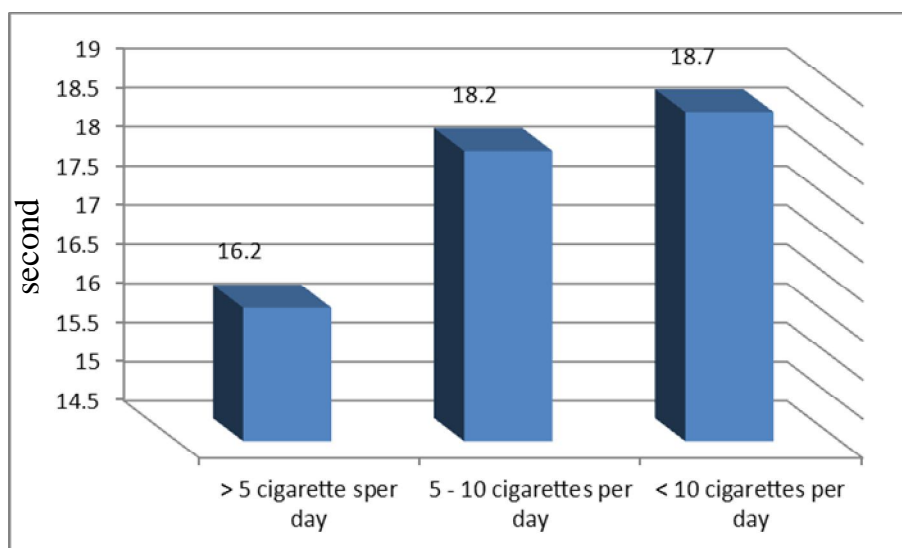


**Fig (3.6): The mean values of Prothrombin Time according to the duration of smoking (P.value :0.517)**

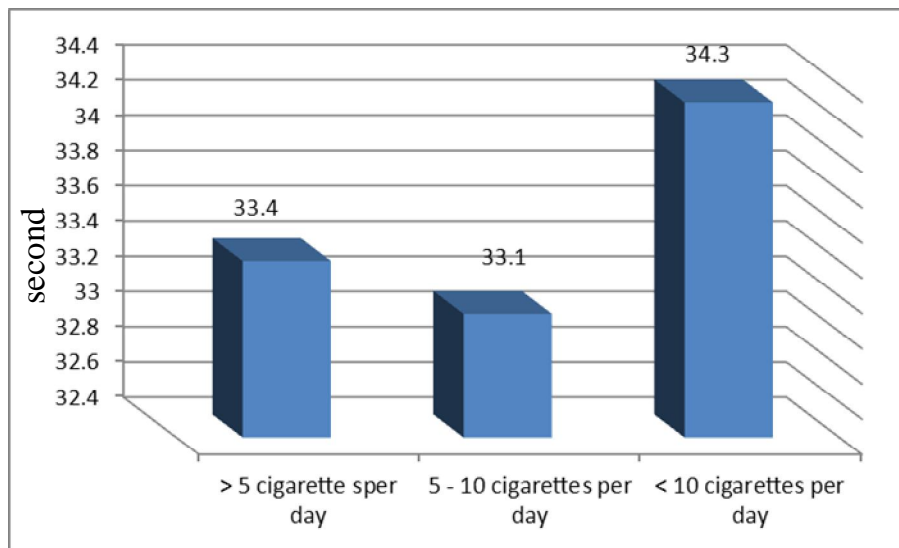


**Fig (3.7): The mean values of APTT according to the duration of smoking**

**P.value :0.832**



**Fig (3.8): The mean values of PT according to the number of cigarette per day (P. value: 0.817)**



**Fig (3.9): Mean values of APTT according to the number of cigarette per day( P. value: 0.823)**



## Chapter four

### Discussion, conclusion and Recommendations

#### 4.1 Discussion:

This is case control study was conducted in Khartoum State during the period of April to September 2014. The study included 100 smoker males ,age was ranged between (20 – 60 years) and the duration of smoking of each patient was taken, as well as, the number of cigarette smoked per day to determine the affection of tobacco cigarette smoking on Prothrombin Time and Activated Partial Thromboplastin time.

The study showed that the mean of PT value for test group (smokers) was significantly increased when compared with PT of the control group (non-smokers) ( $P < 0.05$ ) which disagree with the study of Akpotuzor *et al.*, (2009) in which PT was significantly decreased ( $11.4 \pm 1.7$ ) seconds with  $P\text{-value} < 0.05$  . These differences could, possibly, be due to the fact that the previous investigators used large sample size (240 subjects) in their study and from both gender males and females . While the mean of APTT showed insignificant difference ( $P > 0.05$ ) that disagree with the study of Akpotuzor. *et al.*, (2009) that showed significant decrease in the mean ( $24.6 \pm 4.3$ ) seconds .

Furthermore, this study revealed no difference in PT and APTT according to the number of cigarettes smoked per day ( $P > 0.05$ ). No recorded data in the literature review concerning the effect of number of cigarette per day on PT and APTT.

According to the Duration of smoking the value of PT and APTT was statistically insignificant ( $P\text{-values} > 0.05$ ), while in Akpotuzor. *et al.*, (2009) ,revealed significantly decrease in the mean values of PT and APTT.

## **4.2 Conclusion:**

- The study showed that a significantly prolongation of PT value in smokers group and APTT was normal.
- APTT values obtained was insignificant regarding to cigarette smoking.
- There was no significant relationships with duration of smoking and No. of cigarette per day with the PT and APTT values.

### **4.3 Recommendations:**

- This study was exclusively for male smokers, and it's better if it was done for both genders.
- Passive smoking may affect the blood coagulation so it is better to do research on it to show if there is effect.
- Increase the awareness of people and especially the families about health problems associated with tobacco using health education through various forms such as educational institutes, non-governmental organizations and community leaders.
- Further such studies covering wide area are needed to elucidate the full dimension of the problem so that the health policy makers can plan and decide for the problem control and refute at this study.

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**Appendix (1)**  
**Sudan University of science and technology**  
**Collage of medical laboratory sciences**  
**Hematology department**  
**Questionnaire about (Cigarette Smokers)**

SAMPLE NO {     }

Name.....

- Age:.....
- Sex                      Male :
- No of cigarette per day .....
- Duration of smoking: .....

\_ Others .....

Lab investigations :

PT ..... sec

APTT.....sec

Signature : .....

Date: .....