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Investigating the Ultrastructure of Fibrin Networks, Platelets and Coagulation Profiles in Healthy Individuals

BY

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Abstract

Normal blood flow in healthy individuals is achieved by the maintenance of a balance between the procoagulant and anticoagulant (fibrinolytic) pathways in the blood. A number of changes occur during aging with regards to fibrin network structure, platelet morphology and the overall coagulation profile of individuals. The aim of this study was to determine the characteristics necessary for fibrin networks and platelets to be classified as healthy as well as the effect of aging and sex on the coagulation potential of healthy individuals.

36 Voluntary individuals (separated into six different age groups) were selected and their platelets, fibrin networks and coagulation potential were studied using Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) and Fibrinogen Levels and Thromboelastography® (TEG®). For SEM and TEM thrombi were formed by the addition of human thrombin to the freshly prepared platelet-rich plasma of the participants. The thrombi were fixed and prepared according to electron microscopy guidelines before being viewed. Fibrinogen Levels and TEG® were performed by trained technicians in a Haematology Laboratory. Statistical analysis was performed on the fibrin fibres of the fibrin networks to determine the average diameter of the fibres and to compare the fibre thickness of different individuals in different age groups. Statistical analysis was also performed on the TEG® results in order to compare the TEG® variables of each age group.

With regards to the coagulation profiles of the participants the Fibrinogen Levels were found to increase with age, but was not statistically significant. The same was true for the TEG® variables, Alpha Angle and Maximum Amplitude. The Reaction Time and K-Time, however, decreased with an advance in age, but not with statistical significance. The TEG® Index however was found to increase with statistical significance with advancing age (P=0.0065). All the variables were found to be within the normal set medical ranges provided by the manufacturers. The ultrastructure of fibrin networks were found to consist mostly of major fibres with minor fibres sparsely arranged in between, especially in younger individuals. The fibrin networks become denser with an advance in age and seem to be due to an increase in the amount of minor fibres. Statistically the obtained histograms did not have the expected bimodal distribution pattern, but rather appeared “merged” with no clear distinction between the major and minor fibres. With advancing age the thin fibres form “net-like” structures that cover sections of the thrombus. Fibre bundles could be observed after the age of 50. Platelet surface morphology was found to be similar in all the age groups and the same was true for the platelet interior. The overall amount of granules within the granulomere of the platelets was found to decrease after the age of 40. Slight spreading of the platelets could be observed in ages over 50 years.

The TEG® Index was found to be directly proportional to an advance in age. The TEG® variables, however, did not differ significantly between the six assessed age groups. No statistically significant difference could be observed when comparing the Fibrinogen Levels of the participants. Changes in the fibrin network morphology, related to aging, systematically increase due to aging and are most prominent after the age of 60. Intermediate fibre diameters were found to be present in addition to the major and minor fibres. Major fibres appear to decrease in diameter with advancing age, rather than decreasing in number. Platelet morphology showed no real differences with an increase in age when comparing the different age groups. Sex-related differences could not be found with regard to platelet morphology and coagulation profiles; however, the fibrin network structure appears to be influenced by sex. Sex-related differences in the fibrin network morphology were found to appear after the age of 30 and persisted up to the age of 60 after which the differences seem to disappear. From the results obtained in the current study, it can therefore be concluded that age-related changes occur in all individuals, but are most prominent in the SEM images of the fibrin network ultrastructure. Sex-related differences could only be observed in fibrin networks. Electron microscopy can thus be assumed to be more sensitive to alterations in coagulation than blood assays such as TEG®.

Declaration

I, Mia-Jeanne Engelbrecht, hereby declare that this research dissertation is my own work and has not been presented by me or anyone else for any degree at this or at any other university.

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"...no matter where you live, the biggest defect we human beings have is our shortsightedness. We don't see what we could be. We should be looking at our potential, stretching ourselves into everything we can become."

-Mitch Albom ("Tuesdays with Morrie")

I would like to thank the following people for helping me realize my potential during the past year and my Lord and Savior for guiding me every step of the way and for introducing such wonderful people into my life.

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Table of Abbreviations, Symbols and Chemical Formulae

µm	micrometer
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
Cyclic-AMP	Cyclic Adenosine Monophosphate
kDa	kiloDalton
nM	nano Molar
nm	nano meter
α	Alpha
β	Beta
%	Percentage
γ	Gamma

glu	glutamic acid
lys	lysine
t-PA	Tissue Plasminogen Activator
DNA	Deoxyribonucleic Acid
TAFI	Thrombin Activable Fibrinolysis Inhibitor
PAI-1	Plasminogen Activation Inhibitor-1
EPCR	Endothelial Cell Protein C Receptor
IL-6	Interleukin-6
TNF-α	Tumor Necrosis Factor α
TGF-β	Tissue Growth Factor β
β-TBG	β-thromboglobulin
DAG	1,2-diacylglycerol

IP3	1,4,5-triphosphate
PIP2	phosphatidylionsitol-4,5-bisphosphate
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
TEG®	Thromboelastography®
®	Registered
PT	Prothorombin Time
PPT	Partial Thrombin Time
ml	Milliliter
INR	International Normalized Ration
aPPT	Activated Partial Thromboplastin Time
R-Time	Reaction Time

mm	Millimeter
K-Time	Time necessary for a fixed degree of vasoelasticity to be reached
MA	Maximum Amplitude
SEC	Student Ethics Committee
HRT	Hormone Replacement Therapy
$CaCl_2$	Calcium chloride
°	Degree
P	Probability
FPRP	Freshly Prepared Human Platelet-Rich Plasma
HIV	Human Immunodeficiency Virus
HCV	Hepatitis C Virus
U/ml	Units per milliliter

µl	Microlitre
PTY (LTD)	Propriety Limited
PBS	Phosphate Buffer Solution
ºC	Degrees Celsius
OsO_4	Osmium Tetraoxide
OCS	Open Canalicular System
PDGF	Platelet Derived Growth Factor

Chapter 1

Introduction

Haemostasis in the human body plays a fundamental role in the general well-being of individuals. Two of the most important components of this process are platelets and the fibrin network. In order for haemostasis to be successful, all the components need to be functioning optimally. Research has shown that conditions like inflammation, viral infections and genetic predisposition, change the homeostatic profile and this cause disease. Also, research has shown that lifestyle (e.g. smoking) may change haemostasis in such a way that will result in the dysfunction of the coagulation system. Even the use of prescribed medication like hormone replacement therapy or oral contraception may change the coagulability profile of a healthy individual. Importantly also, natural occurring changes due to age may have an important effect on haemostasis.

Currently, there are many possible causes for a changed coagulation system, and we are gaining an increased pool of information with regards to how fibrin and platelets change in different conditions. In order to better understand fibrin and platelet ultrastructure and coagulation profiles in relation to disease, there is a need to understand how these coagulation profiles in relation to ultrastructure may vary in a control population - who do not smoke, suffer from disease or use hormone replacement. Importantly, this thesis will therefore focus on age groups, and try to determine what characteristics a coagulation profile, in relation to the morphology of fibrin networks and platelets need to consist of, to be classified as a healthy normal fibrin network; and to link this to sex.

The following paragraphs will provide an introduction to the rationale of the thesis and place the research aims in perspective.

Blood forms part of the extracellular fluid which acts as a buffer between the internal and external environment and surrounds cells in the human body. Blood is responsible for the transportation of substances from one area in the body to another and represents the circulating portion of extracellular fluid. Blood is made up of plasma and contains a variety of blood cells. Cellular elements and plasma proteins of blood are suspended in the plasma. The presence of plasma proteins in blood is the only distinguishing factor between plasma and extracellular fluid (Silverthorn 2004).

Plasma proteins are secreted into the blood after the production thereof in the liver. A variety of plasma proteins (such as albumins, globulins and fibrinogen) are present in blood. Fibrinogen plays a major role in blood coagulation. Cellular elements are also present in blood and include red blood cells (erythrocytes), white blood cells (leukocytes) and platelets (also known as thrombocytes). Platelets are pinched off of large megakaryocytes and are essential in the clotting of blood (Silverthorn 2004).

In order for normal blood flow to be maintained a balance must exist between haemostasis and fibrinolysis. The maintenance of this balance is dependent on a complex network of physiological processes that follow the proteolytic reactions. Basically haemostasis involves the interaction of three factors: vasoconstriction, thrombocyte aggregation and blood clotting. The coagulation cascade’s primary function is to enforce the primary platelet plug formed by aggregating platelets. The cascade involves a series of enzymatic reactions that concludes in the formation of a protein fibre mesh or fibrin network (Meyer *et al*. 2004; Rau *et al*. 2007).

The fibrin network in conjunction with platelets play an essential role in the blood coagulation cascade and haemostasis. These factors are therefore important in the maintenance of the balance between the procoagulant and anticoagulant pathways. The final product of the entire coagulation process is the formation of a blood clot (thrombus) and the ultimate repair of the damaged area (Smith & Morrissey 2008).

The overall coagulability of an individual’s blood has, in recent years, been monitored by routine plasma-based tests; however, these tests only reflect a very small amount of thrombin present at the initiation of coagulation. It is important to include the entire amount of thrombin present in order to have an accurate representation of the entire coagulation profile of the individual (Johannson *et al*. 2009).
Thromboelastography ® (TEG®) provides a complete review of the entire coagulation process and is not limited to certain discreet portions. TEG® can be used to analyze

coagulation factors and cellular elements in a single test. TEG® therefore represents an accurate depiction of the global, real-time coagulation potential of blood. An evaluation of the entire coagulation cascade is therefore possible (Scarpelini *et al*. 2009).

Fibrin clot structure is dependent on a number of fluctuating variables. Normal fibrin networks consist of ordered, uniformly packed fibres. The fibre networks can be morphologically divided into two types: major thick fibre networks and minor thin fibre networks. Thick fibres are predominant in normal fibrin networks with thin fibres sparsely arranged between the thick fibres. Typically the fibrin fibre diameters possess a bimodal distribution pattern (Pretorius *et al*. 2010).

Platelets are small, colourless, disk-like bodies with no nucleus. They have a diameter of two to four µm and can survive for approximately 10 days. The cell membranes of platelets are highly permeable and extensively invaginated. Inert platelets normally circulate in blood, close to the endothelial lining of the blood vessel. When vascular damage occurs, the circulating thrombocytes are activated and adhere to the subendothelial structures of the damaged area. At the site of injury the platelets attract other thrombocytes to the same location, and a thrombocyte aggregate is formed. The coagulation pathway reactions take place on the aggregated and adhered thrombocytes which serve as the substrate for the cascade (Meyer *et al*. 2004).

A number of changes have been found in relation to coagulation profiles, thrombocyte structure and the ultrastructure of fibrin networks with regards to an advance in age. The changes that occur during old age have been studied in recent years and various reasons for these changes have been hypothesized and include, but are not restricted to, a widespread increase in fibrinogen and other coagulation factors.

The possibility of age-related changes has been studied as far back as 1961 when Hume suggested that the fibrin content present in blood increases with age. It has been found that not only fibrinogen but also other factors also increase with age. These factors includes fibrinogen, factors V, VII, VIII and IX, Von Willebrand factor, kininogen, pre-kallikrein, D-dimer levels, plasma-antiplasmin complex and plasminogen activator inhibitor-1. In previous research it was found that there is an increase in fibrinogen levels between the ages of 18 and 85 (Hume 1961).

The ultrastructure of thrombocytes, in contrast to the fibrin networks, appears to remain constant in all age groups. The alterations in morphology that have been found are most likely due to impaired aggregation. The major differences relating to age found between individuals of different age groups are found in the fibrin network structure. Pretorius and co-workers (2010) found that the major fibres become thinner with advancing age. Minor fibres, on the other hand was found to be predominant in older individuals. In older individuals the thin fibres form a “net-like” structure that is common throughout the fibrin network (Pretorius *et al*. 2010).

The possibility of sex-related differences have also been studied in recent years but resulted in ambiguous findings. Hume’s results indicated that there is an increase in the fibrin content within the blood of older individuals, but he found no difference in the fibrin content when comparing males and females. He concluded that there is no difference between the sexes in old age individuals, but that a difference exists between males and females in the younger individuals (Hume 1961). Tracy and co-workers (1992) also studies sex-related differences and concluded that even though fibrinogen levels steadily increase with age, there is no significant difference between the sexes (Tracy & Bovill 1992).

Accordingly, in this thesis, as mentioned in the introductory paragraph, the possible role of sex on the alteration of coagulation profiles is also investigated. The overall aim of this study was to investigate the ultrastructure of platelets and fibrin networks, as well as coagulation profiles of healthy individuals in different age groups.

Due to contradicting results, the following research questions will be addressed in this thesis:

- What characteristics does a fibrin network need to consist of to be classified as a healthy normal fibrin network?
- When studying platelets, what characteristics need to be present to classify a platelet as normal and healthy?
- It is known that age-related changes occur in normal healthy individuals, but what are these changes and in what age group can these changes be observed, with regard to the fibrin networks, thrombocytes and coagulation profiles?

- The role of sex in the alterations in the fibrin networks and thrombocytes has been briefly described, but can it be concluded that sex plays a role in the changes that occur with advancing age?

In order to answer the abovementioned research questions, comparative studies of different age groups was performed using Thromboelastography®, Scanning Electron Microscopy and Transmission Electron Microscopy.

This thesis concludes with a summary of the results obtained in all the above-mentioned research questions as well as the possible future application thereof.

Chapter 2

Literature Review

2.1 Introduction

In this thesis the ultrastructure of platelets and fibrin networks as well as coagulation profiles of healthy individuals in different age groups are being investigated. The current chapter includes an overview of thrombocyte structure, activation and function as well as a discussion on the balance between haemostasis and fibrinolysis. Furthermore, the coagulation and fibrinolytic pathways are described as well as the factors involved in these processes. This chapter includes a section on the role of ageing in coagulation and ends off with a comparison between males and females.

2.2 Blood and its Composition

Blood is part of the extracellular fluid that bathes cells and acts as a buffer against the external environment. Blood carries material from one area in the body to another and is the circulating portion of the extracellular fluid (Silverthorn 2004). Blood consists of a plasma portion and contains a variety of blood cells. The plasma consists of fluid in which the cellular elements and plasma proteins of blood are suspended. Plasma and extracellular fluid is identical except for the presence of plasma proteins in blood (Silverthorn 2004).

Plasma proteins are produced by the liver and are secreted into the blood. Blood contains numerous plasma proteins such as albumins, globulins and fibrinogen. Fibrinogen plays a major role in blood clotting (Silverthorn 2004). Together with the plasma proteins, blood also contains cellular elements comprising of erythrocytes (responsible for the transportation of oxygen), leukocytes (play a role in immunity) and thrombocytes (or platelets). Platelets are formed when small fragments break off of megakaryocytes and are a necessity in blood clotting and specifically in the initiation of the coagulation cascade (Silverthorn 2004).

2.3 Thrombocytes

Thrombocytes originate from large cells named megakaryocytes. Megakaryocytes are polyploidy cells with a lobed nucleus. Their immense size is reached due to a large number of mitotic divisions without the division of the cytoplasm or the nucleus (Silverthorn 2004). Megakaryocytes extend their outer edges through the endothelium into the blood stream and from these projections fragments are pinched off and thrombocytes are formed (Silverthorn 2004).

2.3.1 Structure of Thrombocytes

The hormone thrombopoietin is responsible for the production of thrombocytes and is produced in the liver (Silverthorn 2004). Thrombopoietin is also responsible for the production of megakaryocytes and is thus essential in all the steps necessary for thrombocyte production (Meyer *et al*. 2004).

Thrombocytes are small, colourless, disk-like bodies with no nucleus (Meyer *et al*. 2004). Thrombocytes have a diameter of two to four µm and have a survival time of approximately 10 days in the bloodstream (Meyer *et al*. 2004; Silverthorn 2004). Thrombocytes' cell membranes are highly permeable and extensively invaginated (Meyer *et al*. 2004).

Thrombocyte cytoplasm can be divided into two distinct regions, firstly the outer hyalomere which contains a cytoskeleton formed by microtubules and actin filaments, both of which support the disk-like shape of the thrombocyte (Pretorius *et al*. 2007). The second region is the inner granulomere that contains various granules responsible for the granular appearance (Pretorius *et al*. 2007). The two most common types of granules are alpha granules and dense granules (Meyer *et al*. 2004). The alpha granules contain amongst others, fibrinogen, Von Willebrand factor and thrombospodin. Dense granules contain energy sources such as ADP and ATP as well as calcium. In addition to the granules the cytoplasm also contains mitochondria, Golgi apparatus and an endoplasmic reticulum (Meyer *et al*. 2004).

Due to the well known internal characteristics described above, it can be concluded that the presence of alterations in the ultrastructure of thrombocytes or unnecessary activation can be indicative of underlying medical conditions when thrombocytes are viewed under an electron microscope (Pretorius *et al*. 2007).

2.3.2 Activation of Thrombocytes

Although thrombocytes are always present in blood, they are usually inert, until activating factors are encountered (Silverthorn 2004). The most important activating factors are the exposure of tissue factor when a vessel wall is damaged and when the endothelium is activated to initiate a blood coagulation cascade (Pretorius *et al*. 2007). Under normal conditions, when thrombocytes are quiescent and circulate freely, only a small amount of inactive receptors are present, however, once activated these receptors increase to approximately 80, 000 receptors per thrombocyte (Meyer *et al*. 2004).

When thrombocytes are activated their discoidal shape changes to a sphere and the surface receptors become activated. The release of the cytoplasmic granules also occurs during this time (Meyer *et al*. 2004). Simultaneously the release leads to the production of ADP from ATP and cyclic-AMP as well as the release of calcium from internal stores in the dense bodies (Meyer *et al*. 2004). The production and use of these substances result in a cascade of reactions that involve various chemical substances such as clotting factors, thromboxane, serotonin and growth factors (Meyer *et al*. 2004).

The ultimate effect is the establishment of a thrombocyte aggregate that is adhered to the injured area. Other circulating thrombocytes will adhere to the initial layer promoting and initiating the coagulation cascade (Meyer *et al*. 2004).

2.3.3 Thrombocyte Functions

Thrombocytes have a number of widespread functions of which the most important functions are related to blood clotting. Thrombocytes adhere to an injured area as well as other thrombocytes which result in the formation of a platelet plug that helps maintain the integrity of the vessel. In addition to the platelet plug, thrombocytes also act as procoagulant factors. Once the thrombocytes are activated, vesicles are released from their surface, exposing binding sites for coagulation proteins. Thrombocytes release serotonin and thromboxane A2 that is responsible for local vasoconstriction and initiates blood clotting respectively (Meyer *et al*. 2004). Growth factors are released at the same time to induce angiogenesis and consequently the repair of lesions in vessel walls (Meyer *et al*. 2004). Thrombocytes also play a role in phagocytosis (Meyer *et al*. 2004).

2.4 Haemostasis

Normal blood flow is maintained by the presence of a balance between haemostasis and fibrinolysis. Maintaining this balance involves a mutually dependent network of physiological processes that follow the proteolytic reactions. In short, haemostasis involves the interaction of three factors: vasoconstriction, thrombocyte aggregation and blood clotting (Rau *et al*. 2007).

When a blood vessel wall is damaged, repair must take place as quickly as possible to prevent the loss of blood into the surrounding environment (Silverthorn 2004). The general repair processes can be simplified into three steps:

- firstly pressure within the vessel must be decreased long enough to create a mechanical seal (blood clot)
- secondly the blood vessel must be repaired
- thirdly the attraction of lymphocytes that are responsible for the removal of debris that may have been left behind by the clot (Silverthorn 2004)

However this process is complicated by the pressure that is exerted within the blood vessel and this is where haemostasis plays a pivotal role.

Haemostasis involves the cessation of bleeding and involves both blood coagulation and the contraction of the damaged blood vessel (Meyer *et al*. 2004). The main aim of haemostasis is to keep blood within the blood vessel (Silverthorn 2004). Haemostasis mainly involves the cleavage of inactive fibrinogen to form fibrin. The conversion process is thrombin-mediated (Wolberg & Campbell 2008).

The procoagulant part of the haemostatic process is divided into three steps that can be discussed in further detail:

The first step involves the vasoconstriction of the damaged vessel. The vasoconstriction is caused by paracrines that are released by the endothelium of the damaged area. The release results in a temporary decrease in the pressure within the blood vessel and subsequently the blood flow in the vessel. The decrease in the pressure and the blood flow greatly contributes to the formation of a platelet plug (Silverthorn 2004).

The second step involves the formation of a mechanical seal that closes off the damaged area. This seal is referred to as a platelet plug. The formation of the platelet plug is started off by the adhesion of thrombocytes to the exposed collagen in the vessel walls. The collagen releases cytokines which act as attractants for thrombocyte adhesion and activation. The activated platelets also initiate the aggregation of thrombocytes to form a loose platelet plug over the injured area (Silverthorn 2004).

The third step occurs simultaneously with the second step and is characterized by the coagulation cascade. In this cascade, inactive plasma proteins (proenzymes) that are present in the blood plasma are activated to form active enzymes. Thrombin converts fibrinogen into fibrin which acts as reinforcement to the platelet plug. The reinforced platelet plug is referred to as a blood clot (Silverthorn 2004). Thus, the end result of the procoagulant part of haemostasis is the formation of cross-linked fibrin polymers and the subsequent deposition of these polymers (Rau *et al*. 2007). Once the cell wall has been repaired by cell division and growth, the clot is slowly dissolved by plasmin (Silverthorn 2004).

2.5 The Coagulation Cascade

The competence of the coagulation system is dependent on the maintenance of a balance between the procoagulant and the anticoagulant pathways (haemostasis). The balance is largely maintained by the action of thrombin (Bates & Weitz 2006; Di Cera 2008).

The main function of the cascade is to stabilize the primary platelet plug to form a stable haemostatic plug (Meyer *et al*. 2004). The cascade consists of a series of enzymatic reactions that ends in the formation of a protein fibre mesh. Another function of the cascade is to promote thrombocyte adhesion and aggregation. The coagulation cascade (illustrated in Figure 2.1) occurs during the third step of haemostasis and can be divided into two pathways: the intrinsic and extrinsic pathways (Silverthorn 2004).

The Intrinsic Pathway is activated by the exposure of collagen and other activating factors present in the damaged area. The proteins necessary for this pathway are already present in the blood plasma before damage occurs. The exposure of collagen is the activator of the first enzyme, namely factor XII which in turn is the initiator of the coagulation cascade (Silverthorn 2004).

The Extrinsic Pathway is activated when tissue thromboplastin or tissue factor (a mixture of proteinphospholipids) is exposed in the damaged region. Factor VII is activated by tissue factor which subsequently activates the extrinsic pathway (Silverthorn 2004).

The Intrinsic and Extrinsic pathways converge to follow the Common Pathway. In the course of the Common Pathway fibrinogen is activated by thrombin to form fibrin. Fibrin is insoluble and forms the fibre mesh that reinforces the platelet plug (Silverthorn 2004).

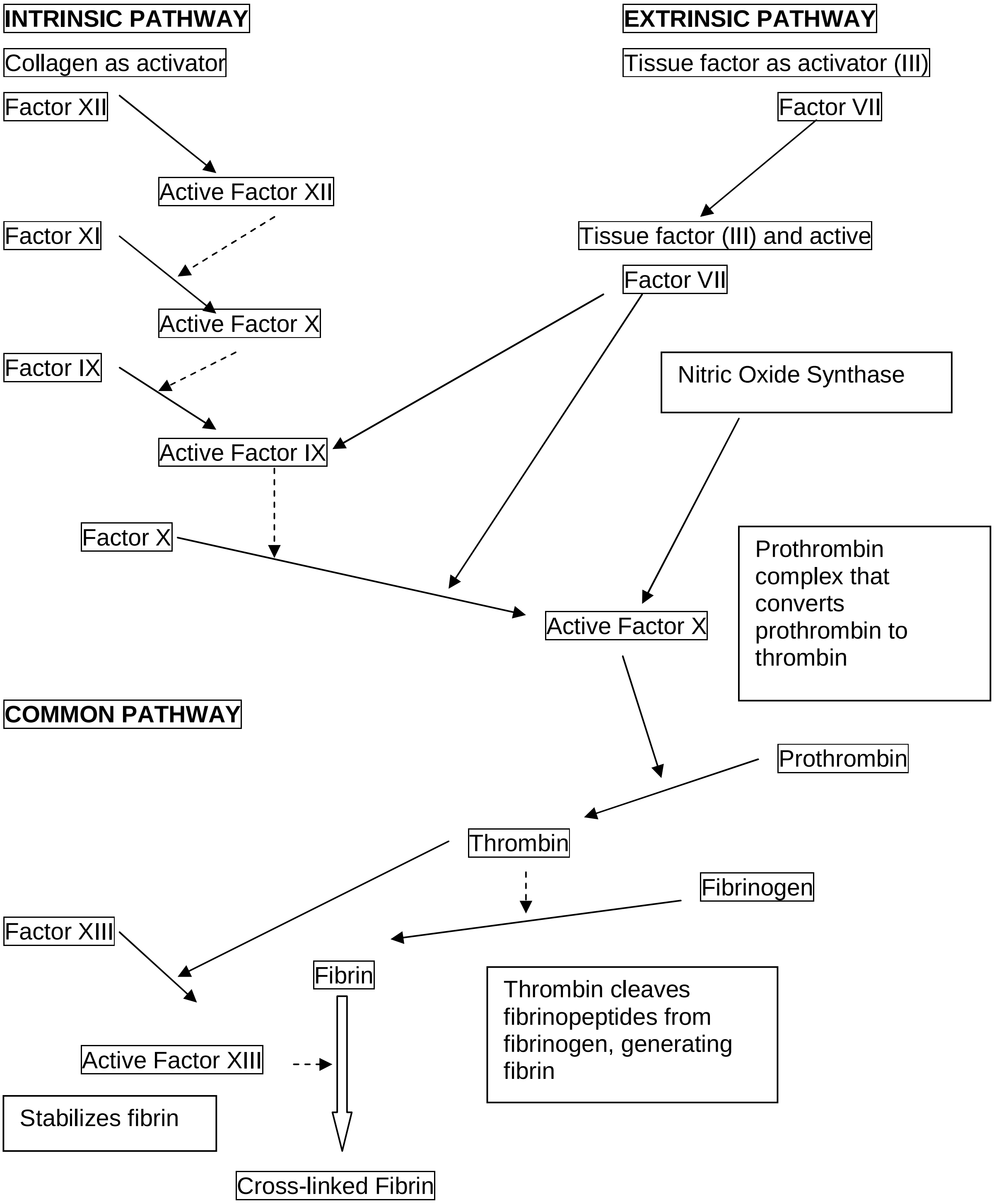


Figure 2.1: The Coagulation Cascade (adapted from Wilkerson & Sane 2002 and Silverthorn 2004)

The prothrombinase complex is responsible for the conversion of prothrombin to thrombin. The complex consists of factor V, factor X (a serine protease), calcium and a membrane containing phospholipids (Majumder *et al*. 2008). The prothrombinase complex is formed on the phospolipid containing membrane (Majumder *et al*. 2008).

The end result of the coagulation cascade is the formation of fibrin that is converted by active factor XII to form a cross-linked fibrin polymer. The fibrin polymer reinforces the primary platelet plug at the site of vascular injury (Silverthorn 2004; Meyer *et al*. 2004). The fibrin meshes, together with platelets, trap erythrocytes which are used for further reinforcement. The fibrin network also incorporates plasmin molecules that are essential for the dissolving of the clot once repair has been completed (Silverthorn 2004).

The process is usually referred to as a cascade, but rather involves a network of interactions between the intrinsic and extrinsic pathway factors. The factors interact with one another and also involve positive feedback loops that sustain the cascade until a specific plasma protein has been completely consumed (Silverthorn 2004). The proteins that play an important role in the cascade are mostly serine protease protoenzymes and pro-cofactors, except for factor XIII (Meyer *et al*. 2004). All coagulation factors, excluding fibrinogen, are either cofactors or enzyme precursors (Meyer *et al*. 2004).

2.6 Fibrinogen and Fibrin

Fibrinogen is the major plasma glycoprotein that is present in the blood of all vertebrates. It plays an essential role in the final step of blood coagulation. Fibrinogen has a molecular weight of 340 kDa and has a trinodular conformation (Sugo *et al*. 2006; Wolberg & Campbell 2008).

Fibrinogen is produced in the liver (Silverthorn 2004) and is polymerized by the enzyme thrombin to form insoluble fibrin. Thrombin itself is activated by a cascade of enzymatic reactions that is activated by injury or damage to a blood vessel (Sugo *et al*. 2006). Fibrin is formed by a series of ordered molecular reactions and interactions (Sugo *et al*. 2006). It has been concluded in previous studies that the thrombin concentration that is present during the formation of the clot, ultimately determines the strength of the fibrin structure as well as the integrity of the formed thrombus (Wolberg & Campbell 2008).

The fibrin structure, when viewed by means of light microscopy, presents the microstructure of the network, rather than the ultrastructure as with an electron microscope (Whittaker & Przyklenk 2009). With light microscopy these fibres are observed as fibre bundles and not as individual fibres. The fibre bundles are similar to collagen fibres and consequently similar to the difference between collagen fibrils and fibres (groups of fibrils) (Whittaker & Przyklenk 2009).

2.6.1 Thrombin and Thrombin Concentration

Thrombin is an allosteric protease and is dependent on sodium for activation (Di Cera 2008). Thrombin plays opposing roles in the coagulation cascade and is responsible for the conversion of fibrinogen to fibrin (procoagulant function) as well as the activation of the protein C pathway that results in fibrinolysis (anticoagulant function) (Di Cera 2008). The binding of sodium to thrombin is responsible for the procoagulation, prothrombotic and signaling functions, but this binding is not essential for the activation of protein C (Di Cera 2008). Throughout fibrinolysis, thrombin is controlled by thrombomodulin (Di Cera 2008).

The thrombin concentration present at the site of clot formation has a great influence on the thickness of the fibres as well as the density of the fibrin clot that is formed. A low thrombin concentration results in very turbid and highly permeable blood clots (Wolberg & Campbell 2008). The highly permeable clots consist of thick, loosely arranged fibrin strands. In the presence of high concentrations of thrombin, the clots are relatively non-turbid with less permeability and consist of a dense network of thinner fibrin strands (Shah *et al*. 1985; Weisel & Nagaswami 1992; Ryan *et al*. 1999; Wolberg & Campbell 2008).

The variations in the structure of the blood clot influence both the vasoelasticity and the anti-fibrinolytic resistance of the clot (Wolberg & Campbell 2008), furthermore, recent studies have indicated that the generation of thrombin correlates directly with the biochemical characteristics of the clot, such as the rate of clot formation, the structure of the clot and the mechanical stability of the formed thrombus (Wolberg & Campbell 2008).

The thrombin concentration varies throughout the coagulation process. In the course of the initial and amplification phases of thrombus formation, the concentration of thrombin is low. The low concentration is necessary for the activation of factors V and VIII as well as the activation of thrombocytes (Wolberg & Campbell 2008). In the propagation phase, however, the concentration of thrombin increases dramatically. This thrombin burst is responsible for the conversion of fibrinogen to fibrin (Wolberg & Campbell 2008). During coagulation the concentration of thrombin can vary between one nM and 500 nM (Wolberg & Campbell 2008).

2.6.2 A Healthy Fibrin Network

Normal fibrin networks consist of ordered, uniformly packed fibres (Sugo *et al*. 2006). Fibres can be morphologically divided into two types of fibres namely major thick fibres and minor thin fibres. Thick fibres are found more commonly in normal fibrin networks with thin fibres distributed sparsely between the thick fibres (Pretorius & Oberholzer 2009). The thick fibres have an average width of 75-120 nm and are usually more than 10 µm in length (Sugo *et al*. 2006). Few branches can be observed when studying a healthy fibrin network and clot turbidity is usually in the range of 0.33 to 0.35 (Sugo *et al*. 2006).

A typical fibre bundle has a width of approximately 2.5 µm and thus consists of 10 to 25 individual fibres (Whittaker & Przyklenk 2009). Nodules can also be found within the fibrin network. Each nodule generally has at least three fibers originating from a single node (Sugo *et al*. 2006). Fibres can typically be observed as structures forming a relatively straight line (Pretorius & Oberholzer 2009).

The structure of fibrin clots is not exclusively dependent on the reactions between normal fibrinogen and thrombin or their concentrations, but also on a variety of other factors such as plasma proteins (especially albumin), pH of the environment, negatively charged substances and other ions. A small alteration in the concentration or presence of these factors could result in the formation of an abnormal fibrin clot (Sugo *et al*. 2006).

It has been found that thrombin cleaves off the N-terminal peptides from both the α- and β-fibrinogen chains (Ferry & Morisson 1947). The cleavage results in the formation of double-stranded protofibrils. The protofibrils ultimately thicken to form protofibril chains. The turbidity of the clot increases with an increase in the thickness of the protofibrils (Carr *et al*. 1977; Wolberg *et al*. 2002; Wolberg & Campbell 2008).

In the normal arrangement of fibrin networks, the protofibrils associate laterally with each other and link together at the αC-domain (which contains a charged cluster in conjunction with the peptide backbone) to form thick fibres. Fibre branching rarely occurs, and therefore these fibres adhere to each other to form fibre bundles (Ferry & Morisson 1947; Sugo *et al*. 2006).

Within the fibrin networks, thrombocytes also form an integral part of the thrombus and in normal healthy networks these thrombocytes have a smooth membrane with pseudopodia present. The pseudopodia originate from the body of the thrombocyte (Pretorius & Oberholzer 2009).

Excessive bleeding and the unnecessary formation of thrombi can be a manifestation of an abnormal formation of haemostatic thrombi and the improper digestion of intravascular clots, respectively (Sugo *et al*. 2006). In the presence of inflammation fibrinogen can be used as a marker since it is an acute phase protein (Pretorius & Oberholzer 2009).

2.6.3 Fibrin Assembly

The organization of fibrin plays a very important role in the structural integrity of blood clots (Wolberg 2007; Whittaker & Przyklenk 2009) and therefore, fibrin assembly needs to proceed in a highly ordered fashion. The organization of fibrin is necessary, as it involves a variety of molecular interactions (within the coagulation pathway) and also interactions with thrombocytes (Pretorius *et al*. 2006).

The molecular interactions necessary for fibrin assembly can be divided into three distinct steps:

(i) Fibrin is converted from fibrinogen to fibrin by fibrinogen-linked thrombin that is responsible for the cleavage of fibrinogen A and B proteins
(ii) The formation of an imbricated double-stranded protofibrils
(iii) The arrangement of protofibrils into thick fibres, the fibres into bundles and the bundles into networks through lateral association between the protofibrils (Sugo *et al*. 2006)

Fibrinogen is produced in the liver and is secreted by hepatocytes into the blood (Collen 1972; Pretorius *et al*. 2006). Fibrinogen is a homodimer (consisting of two identical parts) with a half-life of four days and has a catabolic rate of 25% of the plasma pool per day (Collen 1972; Pretorius *et al*. 2006). Each part of the homodimer consists of three different polypeptide chains that can be found on the long arm of chromosome four (Henry *et al*. 1984; Matsuda & Sugo 2001; Roberts *et al*. 2001). These chains are referred to as the A or α, B or β and the γ chains (Pretorius *et al*. 2006). The amino terminals are arranged into a disulfide knot to form an E-terminal in the centre and two D-domains on both sides of the E-domain. Together it forms a trinodular structure (Henschen 1983; Everse *et al*. 1998; Matsuda & Sugo 2001; Roberts *et al*. 2001; Pretorius *et al*. 2006).

Under normal physiological conditions, thrombin cleaves off the A and B fibrinogen proteins, by hydrolysis. The hydrolysis converts fibrinogen to fibrin and exposes a binding site on the central domain. The binding sites comprise of a Glycine-Proline-Arginine arrangement of amino acids. These binding sites interact with complementary binding sites on the ends of other fibrin molecules (Ryan *et al*. 1999; Pretorius *et al*. 2006).

The Aα group of Aα-Glycine-1 is located between γ-Aspartic Acid-364 and γ-Aspartic Acid-330. The guanidine group of Aα-Arginine can be found between the carboxyl group of γ-Aspartic Acid-364 and γ-Gluamine-329 in this site (Matsuda & Sugo 2001; Pretorius *et al*. 2006).

The interactions are non-covalent and results in the formation of two protofibrils. The protofibrils grow in length and aggregate to form fibres that interact with other fibres to form a larger network of fibres (Pretorius *et al*. 2006).

The Aα and C domains, located on fibrinogen and fibrin, interact intramolecularly and it is likely that these regions interact with the central portion of the molecule. During the activation of fibrinogen to fibrin, fibrinogen undergoes a conformational change. The Aα C-linked domain dissociates from the molecule's central region and is thus available for interactions that occur intermolecularly. The interactions that occur between the Aα and C domains are necessary for lateral aggregation during the polymerization of fibrin (Weisel & Medved 2001; Pretorius *et al*. 2006).

Clot formation can be further described in detail when studying the biochemical reactions. In the first step clot formation is initiated by the activation of thrombin which results in the polymerization of fibrinogen to fibrin. This is followed by the organization of the fibrin network. In the second step cleavage of glutamic acid (glu)-plasminogen takes place at lysine-77, this result in the formation of lysine (lys)-plasminogen. The polymerization together with the activation of the thrombin-induced fibrin clot enhances the network and reorganizes it as well. The alterations in the network produce thicker fibres as well as globular complexes containing fibrin and lys-plasminogen. The plasminogen is the seed of its own destruction necessary in step three when the clot is dissolved. The third step involves the dissolution of the clot and is due to a secondary rise in turbidity which is initiated by the effects of the lys-plasminogen and the clot reorganization (Meh *et al*. 2001; Pretorius *et al*. 2006).

Recent studies have focused on the influence of not only genetic but also environmental factors that might affect fibrin's mechanical properties (Liu *et al*. 2006; Whittaker & Przyklenk 2009). An interesting factor involves the location of clot formation within the body. The location can be observed in the alignment of fibre bundle orientation. *In vitro* clots appeared to have a random fibre orientation and clots originating in coronary arteries appeared to have greater alignment (Whittaker & Przyklenk 2009).

2.7 Fibrinolysis

Fibrinolysis is brought on by the activation of plasminogen by thrombomodulin and thrombin complex that results in the dissolution of the blood clot (Meyer *et al*. 2004). The fibrinolytic pathway which also involves Protein C is activated by the coagulation cascade. The main objective of fibrinolysis is to prevent excessive clot formation which could result in thrombosis (Rau *et al*. 2007).

Clots formed within blood vessels are removed by the process of fibrinolysis. Fibrinolysis involves the action of a proteolytic enzyme named plasmin (or fibrinolysin) that dissolves the clot (Meyer *et al*. 2004). The degradation is mainly due to the action of a serine protease (plasmin) which is present in the circulatory system as an inactive zymogen, called plasminogen (Rau *et al*. 2007).

Plasmin is not present unbound in the blood, but plasminogen, the inactive precursor enzyme of plasmin, is present in its unbound form (Meyer *et al*. 2004). Plasminogen can be activated by an array of factors which include both extrinsic and intrinsic compounds. Extrinsic factors consist of tissue plasminogen activator (t-PA) which is released during the fibrinolytic process. The intrinsic factors consist of factor XII, XI and kallikrein (Meyer *et al*. 2004).

2.7.1 The Fibrinolytic Pathway

Plasmin has a larger number of actions than thrombin in other substrates, since it can hydrolyze arginine and lysine peptide bonds in these substrates. The cleavage of certain peptide bonds in fibrinogen and fibrin results in the formation of an assortment of degradation products. The degradation products retain their thrombin-susceptible sites and act as thrombin inhibitors in a competitive manner. The degradation products are known as Fragment X (Meyer *et al*. 2004). Smaller fragments (known as Fragment Y) are also formed and act as competitive inhibitors of fibrin polymerization (Meyer *et al*. 2004). Plasmin is also able to digest both Factors V and VIII of the coagulation cascade (Meyer *et al*. 2004).

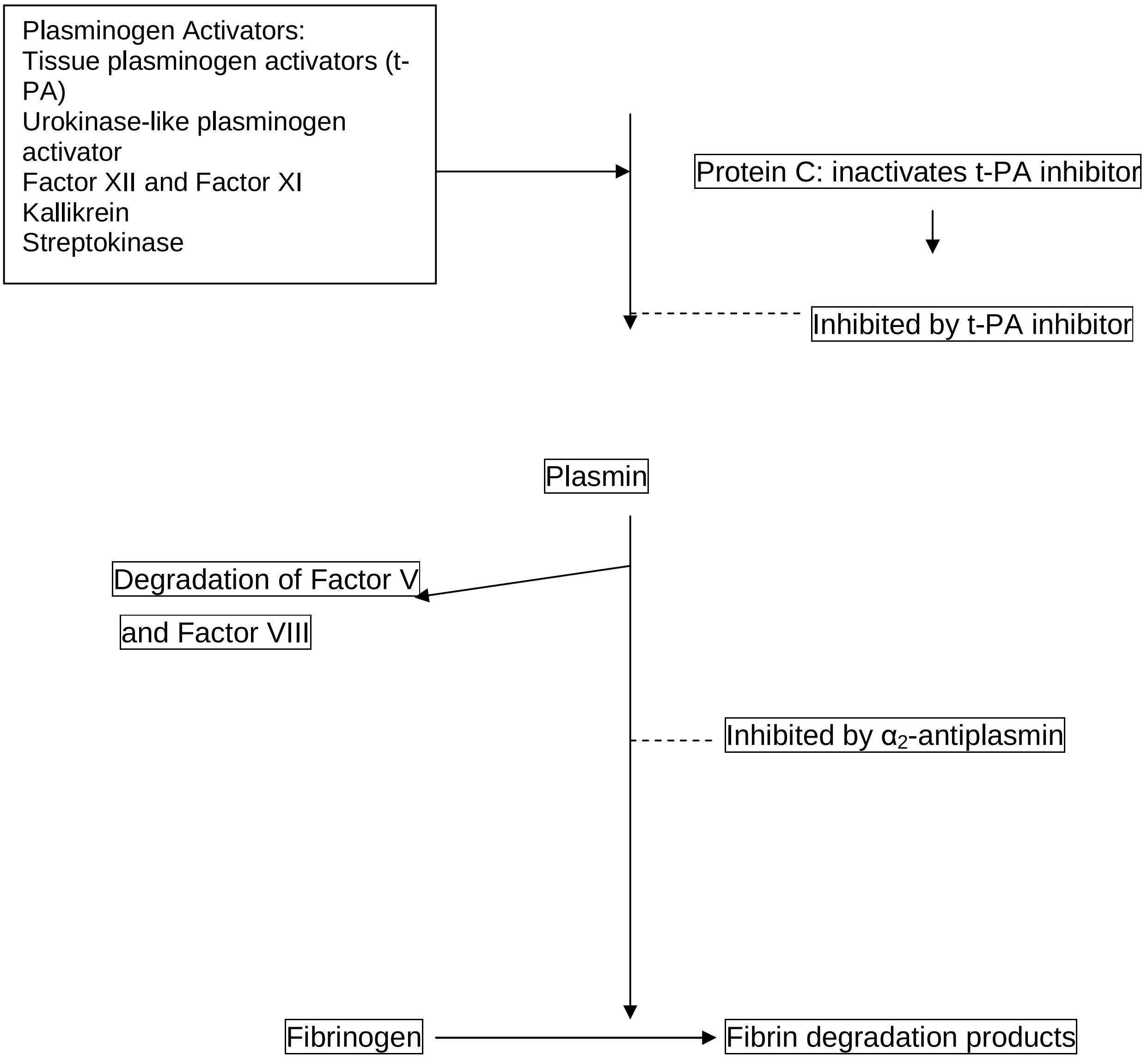


Figure 2.2: The Fibrinolytic System (redrawn from Meyer *et al*. 2004)

Fibrin acts in an autoregulatory manner where it serves as a cofactor for the activation of plasminogen as well as the substrate for plasmin. In the presence of fibrin (after the formation of the blood clot), tissue plasminogen activator (t-PA) is responsible for the cleavage of plasminogen to plasmin which in turn proteolyses the fibrin (Rau *et al*. 2007). In this autoregulation fibrin is necessary as a cofactor for the reaction, and therefore the degradation of plasmin limits further activation of plasminogen (Wiman & Collen 1978; Loskutoff & Quigley 2000; Levi *et al*. 2004; Rau *et al*. 2007).

Urokinase is another powerful plasminogen activator that plays a role in fibrinolysis. Urokinase is produced in renal epithelial cells and lines channels that must be kept clear of fibrin (Meyer *et al*. 2004). Urokinase can be used as treatment in myocardial infarction and both t-PA and urokinase can be produced through recombinant DNA technology (Meyer *et al*. 2004).

It was once thought that vascular endothelium was purely a lining for arterial walls, but recently it has also come to light that the endothelium plays a key role in the control of haemostasis, in particular in the control of fibrinolysis (Smith *et al*. 2003).The serine protease t-PA is synthesized in, and released by, the endothelial cells and t-PA is the main activator of the fibrinolytic pathway (Lijnen & Collen 1997; Smith *et al*. 2003; Miles *et al*. 2005; Rau *et al*. 2007). The ability of the vascular endothelium to release t-PA is essential for the effective fibrinolysis endogenously (Smith *et al*. 2003). It has also been concluded by various researchers that the presence of active t-PA is more effective for endogenous thrombolysis, because it preferentially binds plasminogen bound to fibrin, thereby increasing the fibrinolytic activity on the surface of the formed thrombus (Brommer 1984; Fox *et al*. 1984; Smith *et al*. 2003).

t-PA, once bound to fibrin, enhances the conversion of thrombus-bound plasminogen to plasmin (Meyer *et al*. 2004). t-PA binds to fibrin as well as Annexin II and other receptors on the surfaces of both the endothelium and the thrombocytes. Since fibrin is dependent on t-PA (Meyer *et al*. 2004) the generation of plasmin as well as fibrinolysis can be restricted solely to the location of thrombus formation (Rau *et al*. 2007). Protein C also stimulates fibrinolysis, but this is due to the destruction of the t-PA inhibitor (Meyer *et al*. 2004).

Fibrinolysis is mainly under the control of t-PA, α_2-antiplasmin, and thrombin activatable fibrinolysis inhibitor (TAFI) and PAI-1 (Rau *et al*. 2007). These factors affect different compounds in the coagulation cascade.

Thrombin activatable fibrinolysis inhibitor (TAFI) is present in the plasma and is activated by the thrombin-thrombomodulin complex. This is the same complex that activates the protein C pathway (Esmon 2003; Dahlbäck & Villoutreix 2005). TAFI is present in the plasma as a proenzyme, but after activation it functions as a carboxypeptidase.

Activated TAFI is responsible for the removal of the C-terminal lysine residues of fibrin (Bouma & Meijers 2003; Weiler *et al*. 2004; Dahlbäck & Villoutreix 2005).

α_2-Antiplasmin is synthesized by the liver and has a molecular weight of 63 kDa. It consists of 452 amino acids and has a half-life of 2.5 days (Aoki 1984; Collen & Wiman 1979; Coughlin 2000; Rau *et al*. 2007). α_2-Antiplasmin is the major inhibitor of plasmin and therefore the rate of fibrinolysis is directly proportional to the α_2-Antiplasmin concentration at the site of thrombus formation (Rau *et al*. 2007).

PAI-1 (a glycoprotein) is produced in the endothelial cells, thrombocytes and mesenchymal cells that surround the vasculature. PAI-1 has a molecular weight of 50 kDa and has a half-life of one to two hours (Van Mourik *et al*. 1984; Sprengers & Kluft 1987; Fay 2004; Rau *et al*. 2007). PAI-1 is responsible for the regulation of t-PA and is therefore considered the main physiological inhibitor of the activation of plasminogen (Wiman & Collen 1978; Heimark *et al*. 1980; Vaughan 2001; Cesarman-Maus & Hajjar 2005; Rau *et al*. 2007). Thrombocytes initially release PAI-1 after activation to prevent early fibrinolysis of the thrombus that has formed. However, later in the coagulation process, t-PA and plasminogen or plasmin is bound to the formed fibrin within the thrombus. The location protects the t-PA from being inhibited by PAI-1. Since t-PA is not inhibited, this results in plasmin generation and consequent fibrinolysis (Wiman & Collen 1978; Heimark *et al*. 1980; Vaughan 2001; Cesarman-Maus & Hajjar 2005; Rau *et al*. 2007).

2.8 The Protein C Pathway

The process of blood coagulation is regulated by a number of anticoagulant factors. Under normal physiological conditions the anticoagulant factors outweigh the coagulation promoters (Nicolaes & Dahlbäck 2002; Esmon 2003; Dahlbäck 2005; Dahlbäck & Villoutreix 2005). A very important factor in the regulation of this pathway is vitamin K-dependent Protein C (Nicolaes & Dahlbäck 2002; Esmon 2003; Dahlbäck 2005; Dahlbäck & Villoutreix 2005).

The protein C pathway is responsible for the regulation of thrombin formation in the area that surrounds the formed thrombus (Esmon 2003; Rau *et al*. 2007). Protein C is a zymogen (a proenzyme) that is localized to the endothelium (Blom *et al*. 2004). The protein C pathway is a typical zymogen pathway that consists of two steps. Firstly, the activation of the zymogen (protein C) and secondly, the inactivation of proteases by inhibitors found in the plasma (Walker & Fay 1992). The localization of protein C is due to the binding of protein C to endothelial cell protein C receptor (EPCR) (Blom *et al*. 2004; Rau *et al*. 2007). Thrombin is also localized to the site of injury by its binding to thrombomodulin, an integral membrane protein (Rau *et al*. 2007). Protein C activation regulates the coagulation process by the proteolytic inactivation of factor V and factor VIII of the coagulation cascade (Walker & Fay 1992). Factor V is found in circulation in its inactive form and consists of a large single-chain glycoprotein and has little intrinsic procoagulant activity (Majumder *et al*. 2008). The inactivation results in the inhibition of the coagulation cascade at two important steps (Walker & Fay 1992).

The protein C pathway consists of a large number of diverse proteins that exert their effect at different stages of the pathway. The proteins can be grouped together by their actions e.g. proteins that are responsible for the activation of protein C, proteins that involve the regulation of protein C activity and the proteins that inhibit the effect of protein C (Dahlbäck & Villoutreix 2005a). Protein C also stimulates fibrinolysis, further preventing coagulation (Walker & Fay 1992).

2.8.1 The Activation of Protein C

Thrombin, as previously mentioned, plays a major role in the coagulation system; it is therefore interesting that thrombin is also a key regulator of the protein C pathway that is responsible for anticoagulant activity (Walker & Fay 1992). The activation of protein C takes place on the endothelial cell surface (Esmon 2003; Dahlbäck & Villoutreix 2005). Thrombomodulin is a protein that enhances the rate of protein C activation by altering the substrate specificity of thrombin. The alteration of the substrate specificity results in the conversion of thrombin with procoagulant effects to an enzyme with anticoagulant functions (Walker & Fay 1992). Thrombomodulin binds to thrombin to form a thrombin-thrombomodulin complex, which is responsible for the activation of protein C. However, together with the thrombin-thrombomodulin complex a number of divalent cations must also be present for the activation of this pathway. Calcium is the most important cation that is essential for the activation of protein C (Esmon & Owen 1981; Walker & Fay 1992). The endothelial cell surface bound thrombin-thrombomodulin complex cleaves protein C to form a serine protease, known as activated protein C (Weiler *et al*. 2004; Rau *et al*. 2007).

2.8.2 The Effect of Thrombomodulin on Thrombin

Thrombomodulin acts as a cofactor to thrombin which amplifies the rate of activation of protein C (Dahlbäck & Villoutreix 2005). Thrombomodulin binds to a specific site on thrombin, exosite I. During the procoagulant process this site is occupied by fibrinogen and then fibrin after its activation. Thus, by binding to this site, thrombin's procoagulant activities are decreased (Fuentes-Prior *et al*. 2000; Rau *et al*. 2007). The major effect of thrombomodulin on thrombin involves the conversion of thrombin as a procoagulant to an anticoagulant enzyme (Walker & Fay 1992). In conjunction with the alteration of the function of thrombin, a dramatic alteration in the activation properties also occur, resulting in an increase of the activation of protein C by approximately 20, 000 fold. Ion specificity is also altered and calcium changes from an inhibitor to the major cation activator of the protein C pathway (Walker & Fay 1992).

Thrombomodulin has three effects on the activity of thrombin:

- Thrombomodulin removes thrombin from the blood by binding it with high-affinity to the cell surface
- Thrombomodulin initiates a conformational change in protein C structure which results in an alteration in the calcium dependence of the reaction
- The glycosylated regions of thrombomodulin modify the substrate specificity of thrombin (Walker & Fay 1992)

The binding of thrombin to thrombomodulin on the cell surface results in a localized effect and prevents a widespread activation of the coagulation cascade and also functions to prevent excessive procoagulant activity (Walker & Fay 1992).

2.8.3 The Anticoagulant Activity of Activated Protein C

Activated protein C is a powerful anticoagulant. The specific effect of activated protein C is the inhibition of two procoagulant factors, activated factor V and activated factor VIII (Walker & Fay 1992; Lu *et al*. 1996; Rau *et al*. 2007). Factor V is essential in the prothrombinase enzyme complex and factor VIII is an intrinsic factor enzyme complex or tenase complex (Esmon *et al*. 1982; Walker & Fay 1992; Dahlbäck & Villoutreix 2005). When protein S is present as a cofactor, protein C does not only inactivate factors V and VIII, but also prevents excessive or further thrombin generation in the area surrounding injury (Esmon 2003; Rau *et al*. 2007).

The proteolytic activity of protein C is controlled mainly by protein C inhibitors such as PAI-1 and α_1-protease inhibitor (Pike *et al*. 2005; Rau *et al*. 2007). Protein C activation can be inhibited if thrombomodulin is absent. Since calcium is an essential cation in certain concentrations, the excess of calcium in the blood and the ion specificity thereof prevents the activation of protein C in solution by thrombin (Walker & Fay 1992).

Thrombin, which is responsible for the activation of the protein C pathway, can also be inhibited by antithrombin. Antithrombin is a protease inhibitor and its function is assisted by heparin as well as thrombin-specific heparin cofactor (Gettins 2002, Olson & Chuang 2002; Tollefsen 2006; Di Cera 2008).

2.8.4 The Role of Protein S as a Cofactor in the Protein C Pathway

Activated protein C activity is increased by two factors: firstly, protein S that acts as a cofactor and secondly, the inactive form of factor V (Dahlbäck & Villoutreix 2005). Although protein S does not appear to possess enzymatic activity, it plays a very important role as a cofactor in the protein C pathway.

Protein S is similar to protein C in the sense that protein S is also vitamin K-dependent (Walker & Fay 1992). Protein S is the cofactor necessary for the binding of protein C to the phospholipids cell surfaces, by the formation of a stoichiometric complex (Walker & Fay 1992). The stoichiometric complex is formed on cell surfaces as well as the surface of the thrombocytes. The complex also accelerates the function of protein C by increasing the rate at which factors V and VIII are inactivated. Protein S is sufficient for the inhibition of factor V, but for the inhibition of factor VIII a synergy between protein S and factor V is necessary (Walker & Fay 1992; Dahlbäck & Villoutreix 2005). Protein S also enhances the cleavages necessary for the inactivation of various factors (Walker & Fay 1992).

Phospholipids appear to be essential in the role of protein S, since protein S has no effect on activated protein C when phospholipids are absent (Walker & Fay 1992). The inhibition of factors V and VIII by the protein C pathway in conjunction with protein S is a highly effective regulatory mechanism of the blood coagulation system (Dahlbäck & Villoutreix 2005).

A defect in either or both protein S and protein C have severe consequences and has been identified as a major risk factor for the development of thromboembolic diseases (Walker & Fay 1992). Protein C has also been linked to anti-inflammatory and anti-apoptotic functions, which could involve a wide variety of effects if this protein is defected (Dahlbäck & Villoutreix 2005).

A very important factor that will be observed in this study involves the alterations that occur in healthy individuals with increasing age. It is, however, important to understand what factors play a role in alterations that occur in older individuals.

2.9 Aging and Coagulation

It is important to first understand what is meant by aging individuals. Aging can be explained by a short description of clinical factors, inflammatory factors and also vasculopathy that is characteristic of an elderly person.

Clinical factors indicative of, especially frailty in aging individuals include the presence of a number of different disorders that are present at one time interval. The factors could involve a chronic disease with frequent hospitalizations. Sarcopenia occurs and overall physical activity decreases. Malnutrition can occur with a change in psychosocial status (Gharacholou & Becker 2009).

The inflammatory or pro-coagulant factors, associating with aging, involve the typical hypercoagulability in elderly individuals, chronic inflammation may occur, difficulties with anti-coagulation could be present and the possible appearance of ischaemic heart disease has been noted (Gharacholou & Becker 2009).

Lastly the vasculopathy aspect involves impaired vascular regeneration mechanisms, impaired wound healing and endothelial dysfunction that could initiate thrombocyte activation (Gharacholou & Becker 2009).

2.9.1 General Changes that occur in Aging Individuals

The changes that occur during old age have been studied in recent years and various reasons for these changes have been hypothesized and include, but are not restricted to, a widespread increase in fibrinogen and other coagulation factors (Meade *et al*. 1977; Tracy & Bovill 1992; Tracy *et al*. 1992; Ershler 1993; Wilkerson & Sane 2002).

It is known that with increasing age healthy individuals undergo an assortment of changes. The changes include alterations in vasculature, haemostasis and endothelium and affects thrombocytes, coagulation and fibrinolytic factors (Franchini 2006; Pretorius *et al*. 2010). Some of the key changes can be attributed to a heightened coagulation enzyme activity which is enhanced by an increase in the formation of fibrin and also secondary hyperfibrinolysis (Mari *et al*. 2008; Pretorius *et al*. 2010). Thrombin action is also increased with aging (Wilkerson & Sane 2002). The frequency of atherothrombotic events with increasing age is a well documented fact that can be due to an accumulation of the effects of various factors. These factors include an upregulation of coagulation proteins, decreased fibrinolytic activity, and impaired regeneration and gene-environment interactions (Gharacholou & Becker 2009).

In 1961 Hume already suggested that the fibrin content present in blood increases with age (Hume 1961). Various factors have been shown to increase with age. The factors that have increased concentration in the plasma includes fibrinogen, factors V, VII, VIII and IX (involved in the coagulation cascade that results in the formation of fibrin), Von Willebrand factor, kininogen, pre-kallikrein, D-dimer levels, plasma-antiplasmin complex and plasminogen activator inhibitor-1 (PAI-1). PAI-1 is the major inhibitor in the fibrinolytic process (Wilkerson & Sane 2002; Mari *et al*. 2008; Pretorius *et al*. 2010). Since PAI-1 levels increase with increasing age, fibrinolysis is inhibited which results in an increase in fibrin levels within the blood. However, some studies have reported that PAI-1 increase up to the age of 60, after which it decreases slightly (Mehta *et al*. 1987; Dolan *et al*. 1994; Wilkerson & Sane 2002). The changes in concentrations could be due to increased endothelial disturbances and also an increased risk of atherosclerotic disease (Lee *et al*. 1995; Pretorius *et al*. 2010).

It is important to note that Von Willebrand factor is an independent predictor of atherosclerotic disease in older individuals (Mari *et al*. 2008; Pretorius *et al*. 2010). Von Willebrand factor levels increase with age, as already mentioned, and is responsible for thrombocytes being activated by damaged endothelium or subendothelium (Conlan *et al*. 1993; Wilkerson & Sane 2002).

Thrombocyte activity also seems to increase with age and this could be due to an increase in thrombocyte transmembrane signaling or due to an accumulation of second messengers (Bastyr *et al*. 1990; Wilkerson & Sane 2002; Franchini 2006; Pretorius *et al*. 2010). Since thrombocyte activation generates a major assembly for prothrombinase complexes it plays a major role in the propensity of thrombus formation (Wilkerson & Sane 2002).

Tracy and co-worker in 1992 as well as Meade and associates (1977) found an increase in fibrinogen between the ages of 18 and 85 (Meade *et al*. 1977; Tracy & Bovill 1992). The exact mechanism by which an increase in fibrinogen levels can result in cardiovascular disease is not clear, but it could include the following possibilities: a greater production of fibrin due to a greater substrate mass, increased thrombocyte aggregation and also an increase in blood viscosity (Tracy & Bovill 1992). However, an increase in fibrinogen may also be in response to increased levels of Interleukin-6 (IL-6), which also increases with advancing age (Ershler 1993; Wilkerson & Sane 2002). IL-6, IL-1, Tumor Necrosis Factor α (TNF-α), Tissue Growth Factor β (TGF-β) and other cytokines, hormones and growth factors influence the activity of PAI-1 (an acute phase reactant) (Healy & Gelherter 1994; Koike *et al*. 1996; Seki & Gelehrter 1996; Loskutoff & Samad 1998; Wilkerson & Sane 2002).

It can be concluded that high levels of IL-6 can result in increased levels of fibrinogen since IL-6 has other prothrombotic effects as well, such as increased thrombocyte count (Mestries *et al*. 1994) and a promotion of thrombocyte aggregation (Burstein 1994; Soslau *et al*. 1997; Wilkerson & Sane 2002).

The decreased function of endothelial vasodilators is another characteristic that has been found with advancing age (Taddei *et al*. 1997; DeSouza *et al*. 1998; Smith *et al*. 2003). The loss of vasodilatation is accompanied by a decreased ability of the endothelial cells to release t-PA. Aging is therefore further characterized by the decrease in plasma fibrinolytic activity as well as an increase in system concentrations of t-PA and PAI-1 in some cases (Smith *et al*. 2003). However, the systemic concentration of certain factors does not determine the anti-coagulation potential, but it is rather the local endothelial release of factors that influence the thrombolysis potential (Kooistra *et al*. 1994; Jern *et al*. 1997; Smith *et al*. 2003). Since the thrombolytic activity decreases with age it can be noted that the capacity of endothelial t-PA release decreases significantly (Smith *et al*. 2003).

It is very likely that oxidative stress in older age could impair the release of t-PA and together with the diminished capacity of endothelial cells; it can lead to serious physiological and clinical implications (Smith *et al*. 2003). Interestingly, it was found by Smith and colleagues (2003) that cardiovascular exercise could improve the capacity of endothelial cells even in advancing age (Smith *et al*. 2003).

2.9.2 Aging and Coagulation: Comparison between Males and Females

The presence of differences between males and females has resulted in ambiguous findings. Hume (1961) studied three age groups that consisted of young individuals, middle-aged individuals and old individuals. Hume's results indicated that there is an increase in the fibrin content within the blood of older individuals, but he found a difference in the fibrin content of young and middle-age individuals between males and females (Hume 1961).

Hume (1961) concluded that there is no difference between the sexes in old age individuals, but his findings indicated that a difference exists between males and females in the younger individuals. An increase in fibrin content was found to be higher in females in the youngest age group but in middle aged individuals, the males have higher fibrin content (Hume 1961). From these results it can be concluded that females have a higher fibrinolytic activity and that they are less susceptible to the development of thrombi (Hume 1961). This could be a result of hormone levels within the females, especially with regard to estrogen.

Tracy and co-worker, however, concluded that even though fibrinogen levels steadily increase with age, there is no significant difference between the sexes (Tracy & Bovill 1992; Wilkerson & Sane 2002).

2.9.3 Specific Alterations occurring in Older Individuals and "Thrombotic Preparedness"

Gharacholou and Becker (2009) described a state of "thrombotic preparedness" in which the entire system is characterized by thrombophilia which includes increased inflammatory response as well as impaired fibrinolytic activity. The systemic activity is out of proportion with the physiological needs (Gharacholou & Becker 2009).

Pretorius and associates (2010) further proved these findings by adding ultrastructural evidence, especially the ultrastructure of the fibrin networks and to a smaller extent in the thrombocytes (Pretorius *et al*. 2010).

It has been observed that thrombocytes do not aggregate normally in older individuals. In younger individuals thrombocytes tend to aggregate into large, bulbous structures (Pretorius *et al*. 2010). The ultrastructure of the thrombocytes seems to remain relatively constant in all age groups. The alterations in morphology are most likely due to impaired aggregation and not as a result of impaired fibrinolysis or increased thrombotic activity (Pretorius *et al*. 2010). Thrombocytes in elderly individuals may also be less susceptible to inhibitory factors such as prostacyclin, due to a decrease in high- and low-affinity receptors (Modesti *et al*. 1985; Wilkerson & Sane 2002).

Bastyr *et al*. (1990) found that the increased platelet activity is due to an increase in thrombocyte polyphosphoinositide turnover, but is independent of metabolic and haemodynamic perturbations (Bastyr *et al*. 1990). This research group also incorporated the biochemical alterations that may cause platelet hyperaggregation and atherosclerotic disease. A major factor influencing hyperaggregation is β-thromboglobulin (β-TBG). β-TBG is a thrombocyte specific protein that is secreted once the thrombocyte is activated (Bastyr *et al*. 1990).

The activation of thrombocytes is associated with phosphoinositide turnover and also with a few second messengers. The second messengers, 1,2-diacylglycerol (DAG) and 1,4,5-triphosphate (IP3), are formed by the hydrolysis of phosphoinositol-4,5-bisphosphate (PIP2) (Lloyd *et al*. 1973; Billah & Lapetina 1982; Vickers *et al*. 1982; Rink *et al*. 1983; Berridge 1984; Bastyr *et al*. 1990). DAG is phosphorylated by an enzyme (DAG kinase) to form phosphatidic acid. Protein kinase C, calcium and thrombocyte granular release act as second messengers (Rink *et al*. 1983; Nishizuka 1984; Bastyr *et al*. 1990). Since thrombocyte aggregation increased with an elevation of ADP in advanced age as well as β-TBG, evidence indicates that thrombocyte activity increases with age both *in vivo* and *in vitro* (Bastyr *et al*. 1990).

The major differences found between old and young individuals are found in the fibrin network structure, more so than in the thrombocyte morphology. It was observed by Pretorius and co-workers (2010) that the major, thick fibres become thinner and are more sparsely arranged than in healthy younger individuals (Pretorius *et al*. 2010). Minor, thin fibres are found more commonly in older individuals, where in younger individuals they are sparsely arranged. It appears as if the thinner fibres dominate the fibrin network in contrast to those in younger individuals. The thin fibres form a “net-like” structure that is common throughout the fibrin network in older individuals. The changes are most likely the result of increased coagulation enzymatic activity (Pretorius *et al*. 2010).

It would appear that anti-coagulation factors in general also increase with age, but not to the same extent as coagulation factors. This results in increased blood coagulation even though fibrinolytic factors are also increased (Sakkinen *et al*. 2000; Wilkerson & Sane 2002). Plasminogen levels however decrease with advancing age in women, but not in men (Dolan *et al*. 1994; Wilkerson & Sane 2002).

Alterations in the cardiovascular system of the elderly could also influence the propensity for thrombosis. Structural alterations occur in older individuals especially in the walls of arteries when elastic fibres are lost and collagen content increases (Lakotta *et al*. 1987; Wilkerson & Sane 2002).

The relationship between coagulation and inflammation in the elderly has also been studied extensively in the past few years. Gharacholou and Becker (2009) concluded that inflammatory factors also increase during aging and not only haemostatic factors. According to Gharacholou and Becker (2009) the following haemostatic factors increase during advancing age: Factors V, VII, VIII, IX and XIII, Von Willebrand factor, PAI-1, D-dimer, fibrinogen, plasmin-antiplasmin complexes, fibrinopeptide A/B and Factor IX and X activation peptides. At the same time during inflammation the following factors are upregulated: C-reactive proteins, IL-6, TNF-α, TGF-β and Angiotensin II (Gharacholou and Becker 2009).

The simultaneous occurrence of advancing age, environmental stressors and chronic inflammation on especially fibrinolytic activity mediated by the increased expression of PAI-1 can be a contributing factor to both venous and arterial thrombosis in the elderly (Wilkerson & Sane 2002; Yamamoto *et al*. 2005; Garacholou & Becker 2009).

With regards to the completed literature review, the following research objectives direct this thesis:

- The comparative study of the fibrin networks of different age groups using Scanning Electron Microscopy (SEM)
- The comparative study of the thrombocytes of different age groups using Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM)

- The comparative study of the coagulation profiles of the different age groups using Thromboelastography® (TEG®)

Once these research objectives have been reached, the following research questions can be answered:

- What characteristics does a fibrin network need to consist of to be classified as a healthy normal fibrin network?
- When studying platelets what characteristics need to be present to classify a platelet as normal and healthy?
- It is known that age related changes occur in normal healthy individuals, but what are these changes and in what age group can the changes be observed, with regard to the fibrin networks, thrombocytes and coagulation profiles?
- The role of sex in the alterations in the fibrin networks and thrombocytes have been briefly described, but can it be concluded that sex plays a role in the changes that are observed with advancing age?

Chapter 3

A Comparative Study of the Coagulation Profiles of Different Age Groups Using Thromboelastography®

3.1 Introduction

The coagulability (both hypo- and hypercoagulability) of an individual's blood has, in recent years, been monitored by routine, plasma-based tests (Johannson *et al*. 2009; Powner 2010). These tests form part of a general collection of tests that are used in general surveillance of coagulation potential (Powner 2010). The usual tests included are: Prothrombin Time (PT) and Partial Thrombin Time (PTT), both measuring the time that is needed for clot formation. PT and PTT can also be used as an indicator of the concentration and function of a variety of coagulation proteins (Powner 2010). Serum Fibrinogen Levels which measures the specific concentration of fibrinogen is also included in this collection (Powner 2010). Lastly, a Platelet Count is performed which quantifies the number of thrombocytes present in one ml of blood (Powner 2010).

The measurement of the quantity of thrombin generation during the entire coagulation process is used to assess the risk of bleeding and to detect hypercoagulability (Rivard *et al*. 2005) Plasma-based assays, however, are not adequately sensitive, since they only reflect a very small amount of thrombin present at the initiation of coagulation and not the entire amount of thrombin present in whole blood (Johansson *et al*. 2009). Therefore, a point-of-care test is necessary that is appropriate for the monitoring of coagulopathies and the evaluation of haemostasis (Artang *et al*. 2009; Johansson *et al*. 2009).

A reliable and effective point-of-care test is Thromboelastography® (TEG®) (Segal & Dzik 2005; Levi *et al*. 2006; Johansson *et al*. 2009). TEG® can be performed at the bed-side and provides immediate results (Scarpelini *et al*. 2009). This test is useful in emergency situations (associated with coagulopathy) such as in trauma to guide therapy and to assess the coagulation potential of the individual as quickly as possible (Brohi *et al*. 2007; Hess *et al*. 2008; Johansson *et al*. 2009).

TEG® provides a complete review of the entire coagulation process and not just an evaluation of certain discreet portions; it can also be used to analyze coagulation factors and cellular elements in a single test (Scarpelini *et al*. 2009; Roeloffzen *et al*. 2010).

Since the role of platelets and other blood cells are better understood with regard to the coagulation process, tests such as visco-elastic haemostatic assays (which measures both the viscosity and elasticity of blood) such as TEG® that focus more on the generation of thrombin throughout the entire coagulation process, from initiation through to fibrinolysis (Rivard *et al*. 2005). It is consequently necessary to evaluate whole blood with all the coagulation relevant factors present, rather than determining the concentration of these factors in the plasma alone. The technique is valuable because it is possible to assess the global integrity of the coagulation process in terms of TEG® and not only sections thereof (Hartert 1948; Rivard *et al*. 2005).

TEG® assesses whole blood to determine the global, real-time coagulation potential by providing an evaluation of the entire coagulation cascade (Mallett & Cox 1992; Rivard *et al*. 2005; Scarpelini *et al*. 2009). TEG® evaluation starts with an assessment of the initial interaction between platelets and activation factors, followed by platelet aggregation, then the strengthening of the clot by fibrin formation and fibrin cross-linking and ends with fibrinolysis (Mallett & Cox 1992; Scarpelini *et al*. 2009). TEG® can be used to measure normal coagulation as well as hyper- or hypocoagulable states (Powner 2010).

TEG® can detect haemostatic imbalances by measuring the visco-elastic properties of the blood (O'Shaugnessy *et al*. 2005). This test also offers information on parts of the coagulation process that conventional tests (such as INR (International Normalized Ratio), PT and aPPT (activated PPT)) cannot measure and produces an overview of the clotting process. The overview includes the interaction of various elements and their activity. TEG® assessment is especially accurate since it can be performed without adding additional buffers (Scarpelini *et al*. 2009).

The basic principle of TEG® is based on the idea that at the end of the coagulation process a clot will be formed and dissolved. The measurement of certain physical properties of the clot, such as the mechanical strength of the clot, the stability of the fibrin network and the kinetics of the clot formation and lysis is essential in TEG®

(O'Shaugnessy *et al*. 2005). By taking the variables into account it is possible to determine if a person has normal haemostasis or if a coagulopathy has occurred (O'Shaugnessy *et al*. 2005). TEG® measures the formation of the clot (from liquid to fibrin formation) as well as the subsequent fibrinolysis (Rivard *et al*. 2005).

The result of TEG® is a graphic presentation (Figure 3.1) depicting the thrombus formation as well as fibrinolysis on the horizontal axis (Johansson *et al*. 2009; Powner 2010). The resulting graph is divided into regions, with individual points representing different stages in the haemostatic process (Rivard *et al*. 2005; Johansson *et al*. 2009). The stages include the clotting time necessary for the clot to form, the kinetics of clot formation, the mechanical strength of the clot as well as fibrinolysis (Johansson *et al*. 2009).

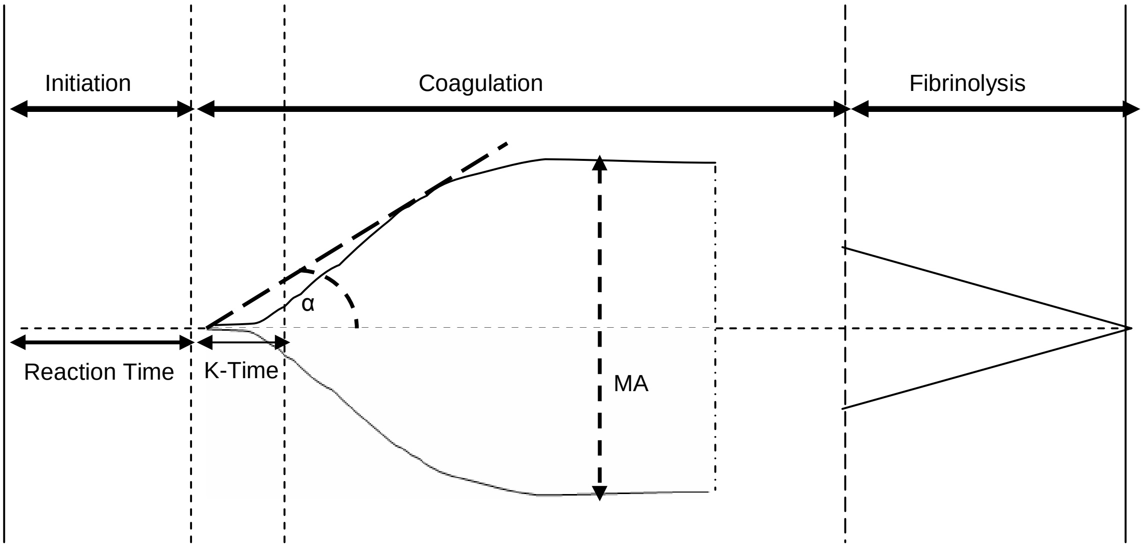


Figure 3.1: Thromboelastography® Resultant Graph (redrawn from O'Shaugnessy *et al*. 2005)

A number of variables that influence haemostasis are fundamental to the TEG® analysis (O'Shaugnessy *et al*. 2005). The Reaction Time (R-Value) is measured from the sample placement to the point where the graph reaches an amplitude of two mm. The R-Time represents the rate of initial fibrin formation and is associated functionally to the plasma clotting factors related to intrinsic and circulating inhibitory activity. The prolonged or decrease of the R-Value can indicate coagulation factor deficiencies or hypercoagulation respectively (O'Shaugnessy *et al*. 2005).

The K-Time or coagulation time represents the time necessary for a clot to form (fixed degree of visco-elasticity is reached due to fibrin accumulation). The K-Value is measured from the R-Time to the point where the amplitude on the graph reaches 20 mm. The K-Time is influenced by intrinsic clotting factors, thrombocytes and fibrinogen (O'Shaugnessy *et al*. 2005).

The Alpha Angle is a line tangent from the start of clot formation to the Maximum Amplitude and represents the speed at which the solid thrombus is formed. A decreased Alpha Angle could indicate hypofibrinogenemia or thrombocytopenia (O'Shaugnessy *et al*. 2005).

The Maximum Amplitude (MA) is depicted on the graph where the greatest amplitude is reached and is indicative of the strength of the formed thrombus. It is directly related to the maximum dynamic properties of the interaction of fibrin and thrombocytes. Platelet abnormalities greatly disturb the MA (O'Shaugnessy *et al*. 2005).

Fibrinolysis can be indicated by a number of characteristics represented on the TEG® graph. A narrowing of the maximum amplitude indicates the start of fibrinolysis (O'Shaugnessy *et al*. 2005).

The influence of sex, age, oral contraceptives, ethnic background, body mass and blood type on TEG® has been studied and produced interesting results. With regards to sex, recent studies conducted by Gorton and co-workers (2000), Scarpelini and associates (2009) and Roeloffzen and colleagues (2010) indicated that healthy, non-pregnant women are more hypercoagulable than men when comparing TEG® profiles. The study performed by Gorton and co-workers (2000) did not necessarily compare individuals within the same age range, and this could possibly have an influence on the results. Ng (2004) found that men and women have different normal ranges when studying TEG® which also indicates sex-related differences between males and females. Sex-related differences may occur due to pregnancy, the use of oral contraceptives as well as other endogenous hormones (Gorton *et al*. 2000; Ng 2004; Scarpelini *et al*. 2009; Roeloffzen *et al*. 2010).

With regards to the changes that occur in the TEG® profiles of aging individuals, the results have been contradictory. In 2004 Ng found that advancing age is associated with hypercoagulability when all the TEG® variables (R-value, K-Time, MA and Alpha Angle) are compared. He further found that in older individuals the R-value and the K-Time are shorter and MA and Alpha Angle are wider than in younger individuals (Ng 2004). Ng's results were supported by the findings of Roeloffzen and associates (2010) as they also found hypercoagulability to be associated with aging (Ng 2004; Roeloffzen *et al*. 2010). However, Scarpelini and co-workers (2009) found that elderly participants do not necessarily have a hypercoagulable state when using TEG® to study coagulation profiles (Scarpelini *et al*. 2009). It is well known that changes in coagulation occur with aging – indicating hypercoagulability - but due to contradictory results it is not known whether this is visible in TEG® results.

It has been found that low haematocrit levels as well as the use of oral contraceptives leads to hypercoagulability (Roeloffzen *et al*. 2010). According to the literature other factors such as ethnic background, body mass and blood type have no effect on the TEG® profile (Scarpelini *et al*. 2009).

In the current chapter the coagulation profiles of different age groups will be compared to determine whether any age- or sex-related differences exist between the respective groups. Although this study includes fewer individuals than Ng (2004), this study excluded many factors that were included by him (Ng 2004). The participants in Ng (2004) can not be considered as healthy since all the participants presented with fractures. Smokers were also included in his study. Both these factors are known contributors to hypercoagulability. Since alterations in coagulation have been found with advancing age, this chapter determines whether this is true for healthy individuals as well.

3.2 Materials and Methods

3.2.1 Samples

Whole blood samples for this study were collected by venipuncture from 36 voluntary participants. The samples were collected from individuals arranged in six different age groups. Informed written consent was obtained from all the volunteers after this study was approved by the Student Ethics Committee (SEC) of the University of Pretoria in 2010. Each group includes six individuals and the groups were arranged as follows:

Table 3.1: Experimental Groups and Age Ranges of the Participants

Experimental Group	Age range
1	< 30 yr
2	30-39yr
3	40-49yr
4	50-59yr
5	60-69yr
6	>70yr

A medical history was taken from each participant to exclude any factors that could influence the coagulation potential of the volunteers. The participants in this study were regarded as healthy since individuals with known fibrin network altering changes were excluded from the study, to ensure that the fibrin networks, platelets and coagulation profiles are as close as possible to normal.

Known exclusion factors that alter the ultrastructure of fibrin networks include smoking, obesity, asthma, diabetes, other inflammatory conditions, metabolic diseases, hormone replacement therapy (HRT) or contraceptive medication and anticoagulant medication such as Warfarin (Pretorius & Oberholzer 2009; Pretorius *et al*. 2009b; Pretorius *et al*. 2010).

3.2.2 Thromboelastography®

Thromboelastography® is a test that is used to measure the different phases that occur in coagulation and the subsequent fibrinolysis of whole blood (Saraf *et al*. 2009). Prior to the initiation of TEG®, the whole blood is recalcified with calcium dichloride ($CaCl_2$). No activator is needed since whole blood is used which contains the necessary substances.

The platelets contained in the blood provide the phospholipids surface for the coagulation reactions to take place. The platelets therefore enhance fibrin formation (Chandler 1995; Gonano *et al*. 2006).

During the process of TEG®, the whole blood is held in a cylindrical sample cup (cuvette). The cup oscillates to an angle of about five degrees (5°). A pin (which is attached to a torsion wire) is then suspended into the oscillating cup (Saraf *et al*. 2009). Initially before the formation of the clot the liquid blood does not transmit any torque, which produces no amplitude on the TEG® (Chandler 1995; Gonano *et al*. 2006). The pin monitors the motion and is thereby able to determine the strength of the bond formed between the fibrin reaction and the platelets (Saraf *et al*. 2009). As the fibrinogen is converted to fibrin and the clot forms the amplitude increases due to the increased torque that is produced (Chandler 1995; Gonano *et al*. 2006). Each rotation lasts 10 seconds (Rivard *et al*. 2005).

TEG® provides a reliable and overall assessment of the haemostatic functions which correlates with routine coagulation tests. However, the TEG® is more sensitive than most routine tests (McCrath *et al*. 2005; Shore-Lesserson *et al*. 1999; Gonano *et al*. 2006).

3.2.3 Statistical Analysis

Blood derived from 36 volunteers in six different age groups was assessed in terms of its fibrinogen content and TEG® variables: TEG® Index, Reaction Time, K-Time, Alpha Angle and the Maximum Amplitude. The acquired data was analyzed in two manners. Firstly, the data in each age group was compared by means of a one-sample t-test or Wilcoxon Signed-Rank Test, depending on whether the necessary assumptions for the parametric tests were met or not, to the medically set norms for these coagulation properties. The tests were performed to determine whether the coagulation properties of the various age groups fell within the described medical ranges. Secondly, the different age groups were compared to each other, by means of one-way ANOVAs or Kruskal-Wallis one-way ANOVAs depending on whether the necessary assumptions for the parametric test were met or not, for each of the assessed coagulation properties. The statistical program NCSS was used in order to perform statistical analysis on the data and the level of significance was set at 0.05.

3.3 Results

3.3.1 Averages (Means) of the Fibrinogen Level and TEG® Variables in Each Age Group

The averages (means) of the Fibrinogen Level as well as the averages (means) for all the TEG® variables (Thromboelastography® Index, Reaction Time, K-Time, Alpha Angle and Maximum Amplitude), within each group, were determined using the data obtained from each individual participant. The averages for each variable are depicted in Table 3.2. These values were used in the statistical analysis of this chapter. The TEG® Index is determined through the use of a mathematical formula, provided by the manufacturer, which determines the net effects of the four variables and describes the overall assessment of coagulability (Donahue & Otto 2005).

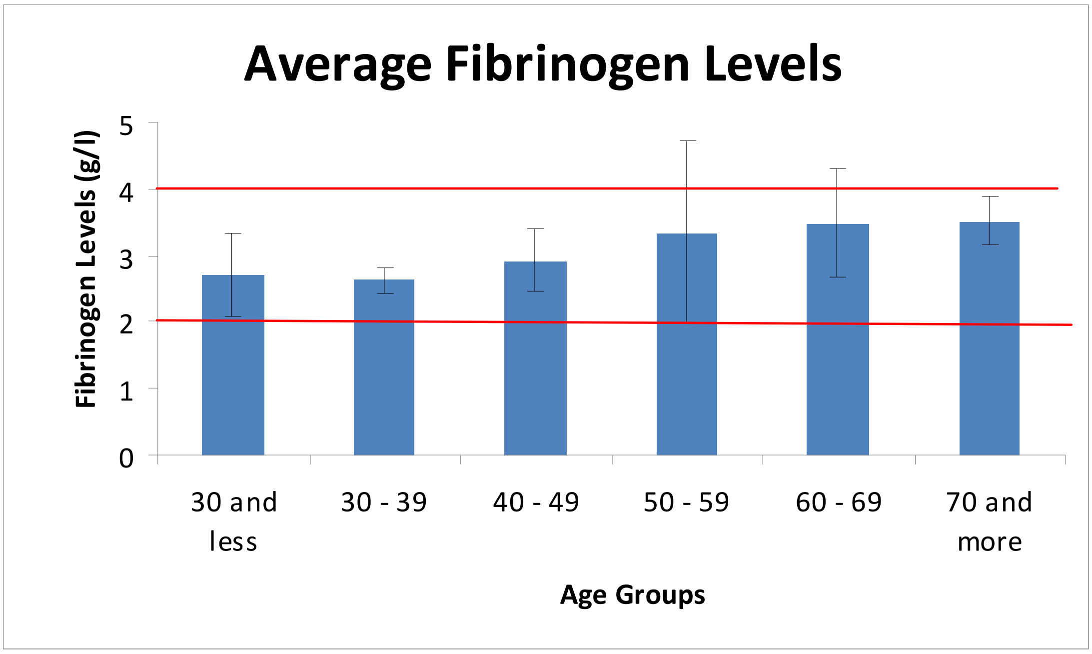
Table 3.2: Averages for Fibrinogen and Thromboelastography® Variables of the Different Age Groups

	<30	**30-39**	**40-49**	**50-59**	**60-69**	**70+**
Fibrinogen Level	2.71	2.63	2.93	3.35	3.47	3.52
Thromboelastography® Index	-2.48	-0.8	0.2	0.63	0.73	0.58
Reaction Time	6.47	5.58	5.35	4.58	5.1	5.05
K-Time	2.45	1.95	1.78	1.67	1.62	1.57
Alpha Angle	51.25	60.3	65.12	63.12	61	66.2
Maximum Amplitude	53.4	55.37	58.8	59.33	63.63	59.6

On all graphs the y-error bar represents the standard deviation. The resultant graphs are depicted in Figures 3.2 to 3.7. Each graph represents a different variable and includes the average for each assessed age group.

3.3.1.1 Fibrinogen Level

As illustrated in Figure 3.2 the average Fibrinogen Levels increase with advancing age. It can be observed that the Fibrinogen Levels are the lowest in the group of individuals under the age of 30 and the highest in the group of individuals in excess of 70 years of age.

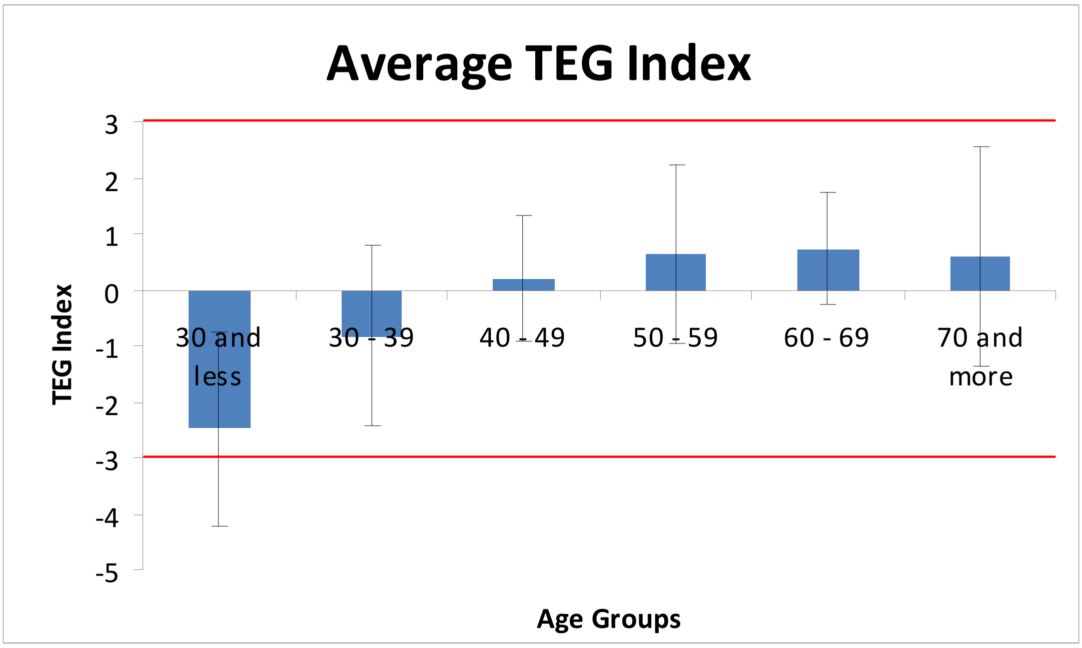


*Red lines indicate maximum and minimum of the set medical range for Fibrinogen Levels

Figure 3.2: The Average Fibrinogen Levels within each Age Group

3.3.1.2 TEG® Index

In Figure 3.3 it can be seen that the average TEG® Index is directly proportional to the age of the individual. An increase in the TEG® Index is observed with advancing age.

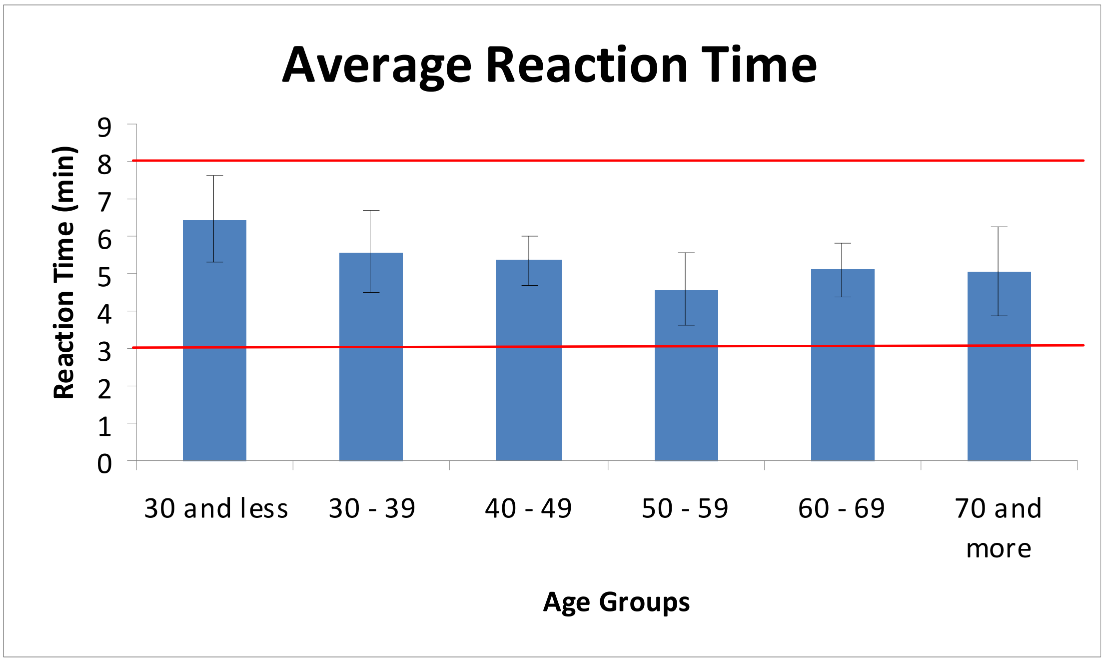


*Red lines indicate maximum and minimum values of the set medical ranges for the TEG® Index

Figure 3.3: The Average Thromboelastography® Index of each Age Group

3.3.1.3 Reaction Time

The average Reaction Time (R-Value) is depicted in Figure 3.4. In this graph it is shown that an increase in age is correlated with a decrease in the R-Value.

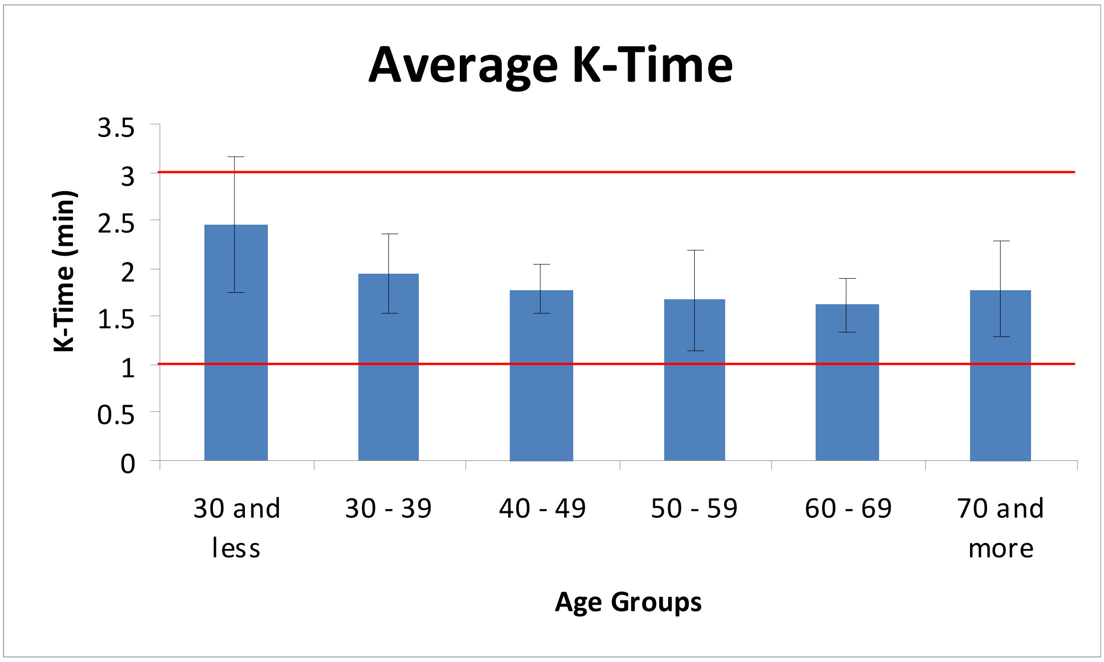


*Red lines indicate maximum and minimum values of the set medical range for R-Time

Figure 3.4: The Average Reaction Time of the Six Different Age Groups

3.3.1.4 K-Time

The Average K-Time is illustrated in Figure 3.5 and this representation shows a decrease in K-Time with an increase in age.

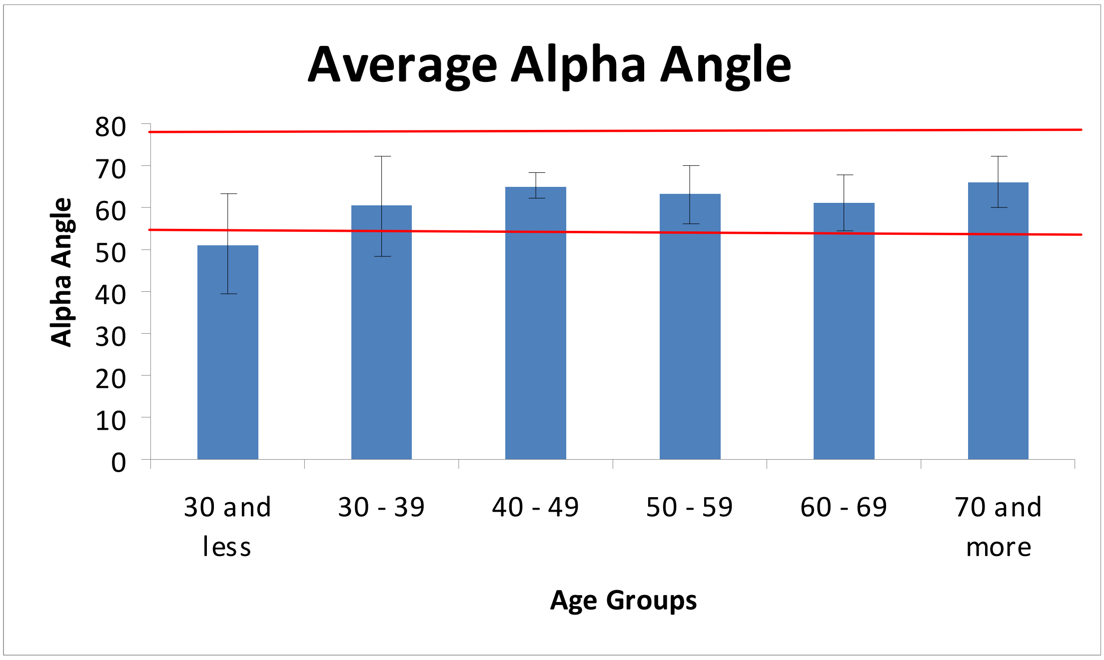


*Red lines indicate the maximum and minimum values of K-Time of the set medical range

Figure 3.5: The Average K-Time of the Six Assessed Age Groups

3.3.1.5 Alpha Angle

In contrast to the K-Time and the R-value, the Alpha Angle increases with advancing age. This is revealed in Figure 3.6.

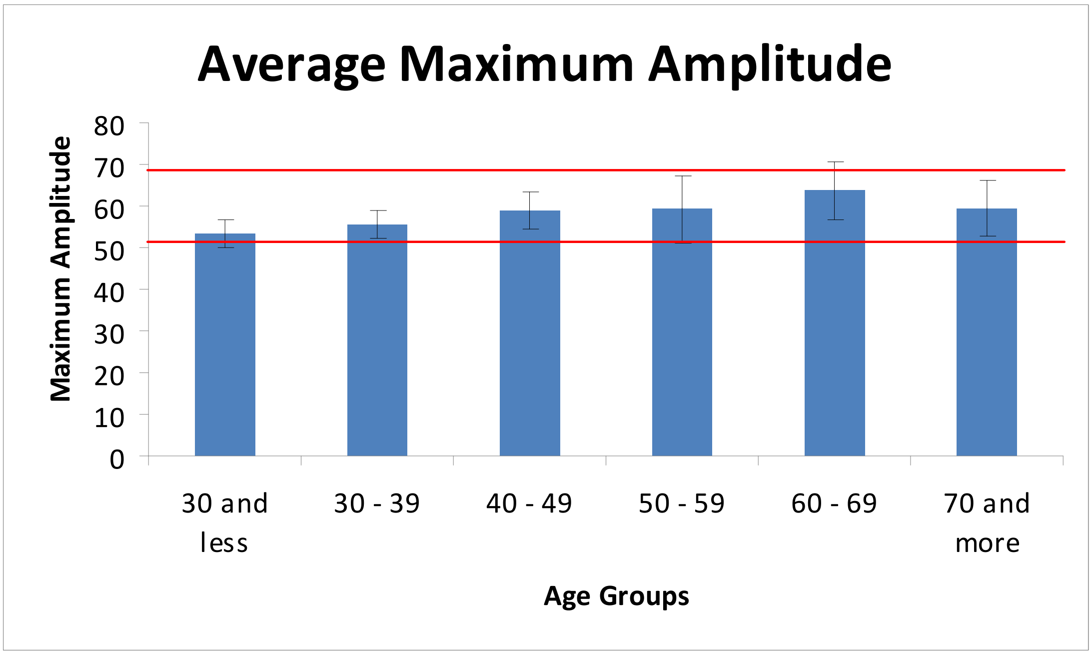


* Red lines indicate the maximum and minimum values of the set medical range for the Alpha Angle

Figure 3.6: The Average Alpha Angle of Each Age Group

3.3.1.6 Maximum Amplitude

The Maximum Amplitude follows a similar phenomenon to the Thromboelastography® Index and the Alpha Angle and is shown, in Figure 3.7, to increase with age.



*Red lines indicate maximum and minimum values of the set medical range relating to the Maximum Amplitude

Figure 3.7: The Average Maximum Amplitude of the Six Age Groups

3.3.2 Comparison of Variables of Age Groups to Set Medical Ranges

The aim of the statistical analysis pertaining to the set medical ranges in this chapter is to determine whether the obtained values fall within set medical ranges since healthy individuals would be expected to have normal values. Alterations in coagulation profiles have been determined (Ng 2004), but it is not known whether the alterations fall within the healthy medical range.

In order to determine if the assessed blood coagulation properties, for each age group, fell within the medically set ranges (for the Fibrinogen Levels: 2 – 4 g/l; for the

Thromboelastography® Index: -3.0 – 3.0; Reaction Time: 3.0 – 8.0 min; K-Time: 1.0 – 3.0 min; Alpha Angle: 55.0 – 78.0; Maximum Amplitude: 51.0 – 69.0) one-sample t-tests were performed on the data. The parametric one-sample t-test could be performed in all cases as all the necessary assumptions were met by the data. The results of the tests are presented in Tables 3.3 to 3.8.

3.3.2.1 Fibrinogen Levels

The six different age groups were compared to each other by means of a Kruskal-Wallis one-way ANOVA, due to a lack of normal distribution, in order to determine if the age groups themselves differed from each other significantly in terms of their fibrinogen content. The results of the parametric test are shown in Table 3.3. The test showed that no statistically significant difference (P=0.066) existed between the six assessed age groups.

Table 3.3: Comparison of Blood Fibrinogen Levels, of Various Age Groups, to the Set Medical Range

Standard medical Fibrinogen limits used for comparison (g/l)^	**Age Group**	**P value***	**Overall result from the two t-tests**
2.0 (Lower limit)	Less than 30	0.981344	The mean blood Fibrinogen Levels for each age group were within the set medical range for normal individuals
	30 – 39	0.999732	
	40 – 49	0.997644	
	50 – 59	0.969151	
	60 – 69	0.996562	
	70 and above	0.999928	
4.0 (Upper limit)	Less than 30	0.99808	
	30 – 39	0.999994	
	40 – 49	0.998715	
	50 – 59	0.850689	

	60 – 69	0.912629	
	70 and above	0.988576	

^ The null hypothesis using lower medical set limit was that the age groups mean Fibrinogen Level would be greater than or equal to the lower limit value. The null hypothesis using upper medical set limit was that the age groups mean fibrinogen level would be smaller than or equal to the upper limit value.

* Significance was set at a level of 0.05

3.3.2.2 Thromboelastography® Indexes

As the necessary assumptions were met for the use of a one-way ANOVA, the six different age groups were compared (as seen in Table 3.4), in terms of the Thromboelastography® Index, to each other by means of this parametric test in order to determine if the age groups differed from each other significantly. The test showed that there existed a statistically significant difference (P=0.0065) between the six assessed age groups. Pos hoc comparisons, by means of the Tukey-Kramer Multiple-Comparison Test, showed that the Thromboelastography® Index for the participants below 30 years of age was significantly smaller than that of the 50 to 59, 60 to 69 and individuals above 70 years.

Table 3.4: Comparison of Blood Thromboelastography® Indexes, of Various Age Groups, to the Set Medical Range

Standard medical Thromboelastography® Index used for comparison^	**Age Group**	**P value***	**Overall result from the two t-tests**
-3.0 (Lower limit)	Less than 30	0.751472	The mean blood Thromboelastography® Indexes for each age group were within the set medical range for
	30 – 39	0.989886	
	40 – 49	0.999543	
	50 – 59	0.998674	
	60 – 69	0.999866	

	70 and above	0.996777	normal individuals
3.0 (Upper limit)	Less than 30	0.999717	
	30 – 39	0.998978	
	40 – 49	0.999161	
	50 – 59	0.992242	
	60 – 69	0.998667	
	70 and above	0.985454	

^ The null hypothesis using lower medical set limit was that the age groups mean Fibrinogen Level would be greater than or equal to the lower limit value. The null hypothesis using upper medical set limit was that the age groups mean fibrinogen level would be smaller than or equal to the upper limit value.

* Significance was set at a level of 0.05

3.3.2.3 Reaction Times

One-way ANOVA was used to compare the six different age groups (since all the necessary assumptions were met for the use of the parametric test) with regards to their Reaction Times to determine if a statistically significant difference existed between the six groups. The test showed that there was no statistically significant difference (P=0.053) between the six different age groups. See Table 3.5 for the results of the one-way ANOVA.

Table 3.5: Comparison of Blood Reaction times, of Various Age Groups, to the Set Medical Range

Standard medical Reaction Times used for comparison (minutes)^	**Age Group**	**P value***	**Overall result from the two t-tests**
3.0 (Lower limit)	Less than 30	0.999629	The mean blood Reaction Times for
	30 – 39	0.998944	

	40 – 49	0.999822	each age group were within the set medical range for normal individuals
	50 – 59	0.994778	
	60 – 69	0.999565	
	70 and above	0.995649	
8.0 (Upper limit)	Less than 30	0.988543	
	30 – 39	0.998582	
	40 – 49	0.999899	
	50 – 59	0.999825	
	60 – 69	0.999905	
	70 and above	0.999082	

^ The null hypothesis using lower medical set limit was that the age groups mean Fibrinogen Level would be greater than or equal to the lower limit value. The null hypothesis using upper medical set limit was that the age groups mean fibrinogen level would be smaller than or equal to the upper limit value.

* Significance was set at a level of 0.05

3.3.2.4 K-Times

The necessary assumptions were met for the use of one-way ANOVA and therefore this parametric test was used to compare the different age groups in terms of the K-Times. The comparison was done to determine if a statistically significant difference existed between the six groups. The comparison is shown in Table 3.6. The test showed that there was no statistically significant difference (P=0.051) between the six assessed groups.

Table 3.6: Comparison of Blood K-Times, of Various Age Groups, to the Set Medical Range

Standard medical K-Times used for comparison (minutes)^	**Age Group**	**P value***	**Overall result from the two t-tests**
1.0	Less than 30	0.998064	The mean blood K-

(Lower limit)	30 – 39	0.998708	Times for each age group were within the set medical range for normal individuals
	40 – 49	0.999615	
	50 – 59	0.987484	
	60 – 69	0.998711	
	70 and above	0.994313	
3.0 (Upper limit)	Less than 30	0.943726	
	30 – 39	0.999173	
	40 – 49	0.999952	
	50 – 59	0.999272	
	60 – 69	0.999971	
	70 and above	0.999119	

^ The null hypothesis using lower medical set limit was that the age groups mean Fibrinogen Level would be greater than or equal to the lower limit value. The null hypothesis using upper medical set limit was that the age groups mean fibrinogen level would be smaller than or equal to the upper limit value.

* Significance was set at a level of 0.05

3.3.2.5 Alpha Angles

Due to a lack in normal distribution the six different age groups were compared to each other by means of a Kruskal-Wallis one-way ANOVA, shown in Table 3.7, in order to determine if a statistically significant difference existed between the assessed groups with regards to their Alpha Angles. The test showed no statistically significant difference (P=0.192) existed between the six age groups.

Table 3.7: Comparison of Blood Alpha Angles, of Various Age Groups, to the Set Medical Range

Standard medical Alpha Angles used for comparison^	**Age Group**	**P value***	**Overall result from the two t-tests**

55.0 (Lower limit)	Less than 30	0.236764	The mean blood Alpha Angles for each age group were within the set medical range for normal individuals
	30 – 39	0.840048	
	40 – 49	0.999802	
	50 – 59	0.981696	
	60 – 69	0.960743	
	70 and above	0.99687	
78.0 (Upper limit)	Less than 30	0.998671	
	30 – 39	0.992902	
	40 – 49	0.999938	
	50 – 59	0.998255	
	60 – 69	0.999232	
	70 and above	0.997485	

^ The null hypothesis using lower medical set limit was that the age groups mean Fibrinogen Level would be greater than or equal to the lower limit value. The null hypothesis using upper medical set limit was that the age groups mean fibrinogen level would be smaller than or equal to the upper limit value.

* Significance was set at a level of 0.05

3.3.2.6 Maximum Amplitudes

All the necessary assumption were met for use of the one-way ANOVA as parametric test. The six different age groups were compared to each other, with regards to their Maximum Amplitudes to determine if a statistical significance existed between the groups. The test showed no statistically significant difference (P=0.064) between the six assessed age groups. The results are illustrated in Table 3.8.

Table 3.8: Comparison of Blood Maximum Amplitudes, of Various Age Groups, to the Set Medical Range

Standard medical Maximum Amplitude used	**Age Group**	**P value***	**Overall result from the two t-tests**

for comparison^			
51.0 (Lower limit)	Less than 30	0.932827	The mean blood Maximum Amplitudes for each age group were within the set medical range for normal individuals
	30 – 39	0.988939	
	40 – 49	0.99645	
	50 – 59	0.973852	
	60 – 69	0.996827	
	70 and above	0.988048	
69.0 (Upper limit)	Less than 30	0.999958	
	30 – 39	0.999923	
	40 – 49	0.998874	
	50 – 59	0.983854	
	60 – 69	0.943213	
	70 and above	0.991371	

^ The null hypothesis using lower medical set limit was that the age groups mean Fibrinogen Level would be greater than or equal to the lower limit value. The null hypothesis using upper medical set limit was that the age groups mean fibrinogen level would be smaller than or equal to the upper limit value.

* Significance was set at a level of 0.05

3.4 Discussion

Thromboelastography® is a laboratory test mainly used as a point-of-care test rather than a diagnostic tool. TEG® differs from other coagulation tests, because it includes sections of the clotting process that cannot usually be studied using standard laboratory test such as platelet count and a PTT. TEG® gives a global overview of the individual's coagulation profile (Spiess *et al*. 1995; Pfanner *et al*. 2007; Scarpelini *et al*. 2009).

Although it has been found, in some studies, that women are more hypercoagulable than men; this study does not support that theory, since women using oral contraceptives as well as pregnant individuals were included in previous studies. This study excluded any individuals with characteristics that could influence the coagulation potential in order to obtain a normal healthy coagulation profile. Reference values supplied by the manufacturer were identical for men and women; this further supports the theory that there is no sex-related difference between males and females. In light of these findings this study, with regards to TEG®, mainly focused on age-related changes that occur with advancing age.

It is widely accepted that Fibrinogen Levels increase with advancing age and this was true for this study (Figure 3.2). Although the values fell within the normal medical ranges, there was a marked increase in Fibrinogen Levels when comparing the six assessed age groups. However, this increase was not found to be statistically significant, as seen in Table 3.3. An increase in fibrinogen leads to a larger amount of fibrinogen being converted into fibrin by thrombin. An increase in the fibrin levels in the blood will lead to hypercoagulability in the event of the activation of fibrinogen, leading to possible coagulopathies such as strokes.

The comparison between the six different age groups in terms of the TEG® Index was found to be statistically significant with an increase in the overall coagulation profile with increased age (Table 3.4). Although the values still fell within the normal range, it was found that the Thromboelastography® Index differences were most pronounced in the age group below 30 years when compared to the 50 to 59, 60 to 69, and 70 and older age groups, depicted in Figure 3.3. This supports the theory that the overall coagulability increases with advancing age with a resultant hypercoagulation in old age.

With regards to the Reaction Time (indicative of the initial rate of fibrin formation) other studies have concluded that the R-Value decreases with advancing age, since a decrease in the R-Value indicates hypercoagulability. A decreased reaction time leads to an abundance of inactive fibrinogen which is suddenly converted to active fibrin. The large amount of fibrin can then lead to excessive activation and subsequent thrombus formation. According to the TEG® results this was found to be true with a decrease in the averages of the R-Values, shown in Figure 3.4. The decrease, however, was found to not be statistically significant as can be seen in Table 3.5.

The average K-Time of each group was compared and although there was a decrease in the values when comparing the averages, these differences were not statistically significant. The K-Time averages are illustrated in Figure 3.5 and the calculations are shown in Table 3.6. A decreased K-Time is indicative of a shorter time period necessary for the full vasoelastic potential of the thrombus to be reached. If this is too rapid, fibrinolysis will be prolonged.

The Alpha Angle represents the speed at which the thrombus is formed and it has been found that this angle widens with increasing age – indicating hypercoagulability. When comparing the age group below 30 years of age to the 70 years and older there seemed to be a marked increase (Figure 3.6), but this difference is not statistically significant (Table 3.7).

The Maximum Amplitude was found to increase with age (Figure 3.7), but not with statistical significance (Table 3.8). The Maximum Amplitude represents the strength of the formed thrombus and it would therefore be expected to increase with advancing age, since a more rigid thrombus would lead to an increase in coagulation potential and also result in a longer fibrinolytic process.

3.5 Conclusion

In the current analysis, sex-related differences in terms of Thromboelastography® were not detected. However, after a careful interpretation of the data accumulated in this chapter, it can be concluded that the overall coagulability potential (represented by the TEG® Index) increases with advancing age when comparing the Fibrinogen Levels as well as the TEG® Index of the six age groups. A statistically significant difference (P=0.0065) was found with regard to the TEG® Index results, when the six groups were compared with each other. It is unclear however; exactly which variable is responsible for the increase in coagulation potential, since the differences in the comparisons of the individual variables associated with TEG® were not statistically significant.

The conclusion that coagulability increase with age, and that it is detectable using the technique of Thromboelastography®, is important. This support previous research by Gharacholou and Becker (2009) that a state of thrombotic preparedness” exists in older individuals where the entire system is characterized by hypercoagulation.

Their findings were further enforced by the ultrastructural evidence, indicating hypercoagulability in fibrin networks, provided by Pretorius and associates (2010) (Gharacholou & Becker 2009; Pretorius *et al*. 2010).

Chapter 4

A Comparative Study of the Fibrin Networks of Different Age Groups Using Scanning Electron Microscopy

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4.1 Introduction

Fibrinogen is the precursor of fibrin and can be observed as an elongated dimeric molecule (Hall & Slater 1959; Fowler & Erickson 1979; Williams 1981; Brown *et al*. 2000; Yang *et al*. 2001; O'Brien *et al*. 2008). In the presence of vascular damage, fibrinogen is activated to form fibrin which, together with thrombocytes, is essential for maintaining haemostasis (Blomback 2001; Mosesson 2005; Lord 2007; Weisel 2007; O'Brien *et al*. 2008; Wolberg & Campbell 2008).

The polymerization of fibrin is achieved by the cleavage of fibrinopeptides A and B from fibrinogen (Chernysh & Weisel 2008). The cleavage of the fibrinopeptides exposes the "A" and "B" knobs, which are located in the centre of the molecule. The knobs are catalyzed by the presence of thrombin and results in the formation of fibrin monomers (protofibrils) (Chernysh & Weisel 2008; O'Brien *et al*. 2008; Smith & Morrissey 2008). Protofibrils are relatively mobile and consequently come into contact with one another. The contact results in the formation of initiation points (Chernysh & Weisel 2008). The knobs then interact in a complementary manner, with the "a" and "b" ends of the molecules (Chernysh & Weisel 2008). The interaction between the "A" knob and the "a" end causes the longitudinal polymerization of fibrin monomers to form double-stranded protofibrils (Doolittle 1984; Cierniewski *et al*. 1986; Litvinov *et al*. 2005). The protofibrils aggregate laterally, in a highly specific manner (Fowler *et al*. 1981; Weisel *et al*. 1983; Weisel 1986), to form fibres (Chernysh & Weisel 2008). Fibrin fibres are described as elastic, adhesive and highly extensible (Guthold *et al*. 2004; Liu *et al*. 2006; O'Brien *et al*. 2008). The network is then further extended by branching as well as the formation of new initiation points. The thickness of the fibrils is also increased by the addition of new protofibrils (Chernysh & Weisel 2008). Activated Factor XIII, which is also activated by thrombin covalently links fibers to increase both the elasticity as well as the strength of the formed thrombus (Wolberg 2007; Smith & Morrissey 2008).

The limiting factor on the lateral growth is the natural twisting of the protofibrils. An increase in diameter requires an increase in the stretching ability (elasticity) that is necessary to accommodate an increase in path length. Consequently lateral aggregation ceases when the energy necessary to add more protofibrils is equal to the energy necessary for binding of new protofibrils (Weisel *et al*. 1987; Chernysh & Weisel 2008). Fibres branch to form a three-dimensional network (Chernysh & Weisel 2008).

The fibrin network along with thrombocytes play an essential role in the blood coagulation cascade and accordingly in the maintenance of haemostasis (Herd & Page 1994). The final product of the procoagulant part of coagulation, is the formation of a blood clot (thrombus) which is achieved when inactive fibrinogen is converted to fibrin, and the fibrin is subsequently polymerized. Fibrin forms the structural component of the thrombi (O'Brien *et al*. 2008; Smith & Morrissey 2008).

The role of fibrin, however, is not restricted to normal haemostasis, since it has a physiological role in allergic processes, other immunological mechanisms and the formation of pathological thrombi (Blomback 2001; O'Brien *et al*. 2008; Pretorius *et al*. 2009a).

Fibrin clot structure is dependent on a large number of possibly fluctuating variables. Factors that influence clot structure include, but are not limited to, the concentration of fibrinogen and thrombin (Wolberg 2007), the pH of the blood (Nair *et al*. 1986; Di Stasio *et al*. 1998), chloride ion concentration (Di Stasio *et al*. 1998) and the presence of calcium ions (Smith & Morrissey 2008). The thickness of the fibrin fibrils is also dependent on a multitude of parameters such as the rate of cleavage of fibrinopeptide A, the initiation of protofibrils formation, the growth of fibre as well as the aggregation of the fibres (Carr *et al*. 1986).

Typically, in healthy individuals, the fibrin network consists of major (thick) fibres and minor (thin) fibres (Nair *et al*. 1986) and possesses a bimodal distribution pattern when considering the diameters of the fibrin fibrils (Pretorius *et al*. 2009a; Pretorius *et al*. 2009b).

Once fibrin has formed, elongated, twisted and highly branched fibres combine with thrombocytes, and other blood cells, to form thrombi. The interaction is essential in the presence of vascular damage (Blomback 2001; Mosesson 2005; Lord 2007; Weisel 2007; O'Brien *et al*. 2008; Wolberg & Campbell 2008).

During normal circumstances the plasmin should be completely solubilized once the site of vascular injury has healed (Morris *et al*. 2006). However, any dysfunction of fibrin is associated with the presence or the development of certain pathologies such as thrombotic disease (Pretorius *et al*. 2009a). If the fibrin is not completely solubilized, it can act as a stimulus which results in the proliferation of neighbouring cells. Fibrinolysis ends in the remodeling of the fibrin network and will end with newly formed organized tissue (Morris *et al*. 2006).The newly formed tissue can therefore result in formation of rigid dense fibrin networks and chronic organized scar tissue which would increase the possibility of pathologies developing (Morris *et al*. 2006; Pretorius *et al*. 2009a).

In the current chapter the fibrin networks of participants in the different age groups were compared to establish whether any differences exist in fibrin size and distribution by using Scanning Electron Microscopy, in the current sample of control individuals.

4.2 Materials and Methods

4.2.1 Samples

Blood samples for this study were collected from 36 voluntary participants by venipuncture, performed by a qualified professional. The participants were selected after a careful medical history was taken to ensure that the individuals are healthy. The samples were collected from individuals from different age groups in citrate tubes. The six different age groups are depicted in Table 4.1.

Table 4.1: Experimental Groups and Age Ranges of the Participants

Experimental Group	Age range
1	< 30 yr
2	30-39yr
3	40-49yr
4	50-59yr
5	60-69yr
6	>70yr

The participants in this study is regarded as healthy since individuals with fibrin network altering changes were excluded, to ensure that the fibrin networks, platelets and coagulation profiles of the participants are as close as possible to normal.

Known factors that alter the ultrastructure of fibrin networks include smoking, overweight individuals, asthma, diabetes, other inflammatory conditions, hormone replacement therapy or contraceptive medication and anticoagulant medication. Therefore, in the current study the above-mentioned factors were regarded as exclusion criteria.

4.2.2 Preparation of Fibrin Clots

Blood from 36 individuals were collected after ethical clearance was obtained from the Student Ethics Committee of the University of Pretoria, South Africa. Freshly prepared human platelet-rich plasma (FPRP) was prepared by drawing four ml of blood in a citrate tube. The blood samples were allowed to separate without centrifugation into plasma and a pellet. Fibrin clots were prepared in order to provide platelet aggregates as well as fibrin fibres for all the participants. The fibrin clots were prepared by using Human Thrombin (Supplied by the South African National Blood Service).

Human Thrombin is prepared from a single regular donor by the calcium chloride activation of a euglobulin fraction of plasma. The plasma was obtained by apheresis. Each individual unit is tested and has to be non-reactive to a number of infectious agents including hepatitis B surface antigen, HIV-1 antibody, HIV-2 antibody and HIV p-24 antigen, hepatitis C virus (HCV) antibody and antibodies to *Treponema palladium*. These tests are performed by using licensed assay methods. The thrombin solution is at a concentration of 20 U/ml and is made up in a biological buffer containing 0.2% human serum albumin.

When thrombin is added to the freshly prepared human platelet-rich plasma (FPRP), the fibrinogen is converted to fibrin (as discussed in the coagulation cascade) and other intracellular platelet components are released into the coagulum. These components include, but are not limited to transforming growth factor, platelet-derived growth factor and fibroblastic growth factor (Pretorius *et al*. 2010). 10 µl of FPRP was mixed with 10 µl of the human thrombin on a 0.2 µm Millipore membrane (MICROSEP (PTY) LTD) to form the fibrin clot. The millipore membrane was then placed on a filter paper dampened with Phosphate Buffer Solution (PBS) and placed in an incubator at 37ºC for 10 minutes. The Millipore membrane was consequently placed in phosphate buffer solution on a microplate shaker where it was washed for 20 minutes. The wash step was included to remove any blood proteins that may be entangled in the formed blood clot.

4.2.3 Preparation of Washed Fibrin Clots for Scanning Electron Microscopy

The washed fibrin clots formed on the millipore membrane were fixated for 30 minutes. The fixative consisted of one ml 25% gluteraldehyde, one ml 25% formaldehyde, three ml double distilled water and five ml phosphate buffer solution (PBS). After the fixation step the clots were washed three times in PBS for three minutes to remove any residual fixative. The clots were then fixated further for 15 minutes in 1% osmium tetraoxide (OsO_4). The osmium tetraoxide fixated clots were washed three times for three minutes in PBS. The samples were dehydrated, for three minutes, serially in 30%, 50%, 70%, 90% and finally three times in 100% ethanol. The samples were dried by the critical point drying technique and were then mounted and coated with a layer of carbon. After coating the samples were examined using a Zeiss ULTRA plus FEG Scanning Electron Microscope.

4.3.4 Statistical Analysis

Fibrin clots derived from 36 volunteers, distributed over six age groups, were assessed by using statistical analysis. Initially the fibrin clots were viewed and photographed using a ZEISS ULTRA plus FEG SEM. Subsequently, the diameters of 100 randomly selected fibrins, constituting the clots of each participant, were measured with the aid of Image Tool© Version 3. The measured diameters were then used for statistical analysis using the statistical program NCSS and Statistix 8, using a significance level of 0.05. The fibrin diameters of each participant were assessed by means of the Shapiro Wilk W test for normality. A histogram of the fibrin fibre diameters was constructed for each participant to see the distribution of the diameters graphically. The thickest fibres quantified for the various participants according to age, by means of a one-way ANOVA and Tukey-Kramers Multiple comparison pos hoc tests.

Figure 4.1 represents an example of the measurements taken of all the fibrin networks. Each white dot indicates a measurement.

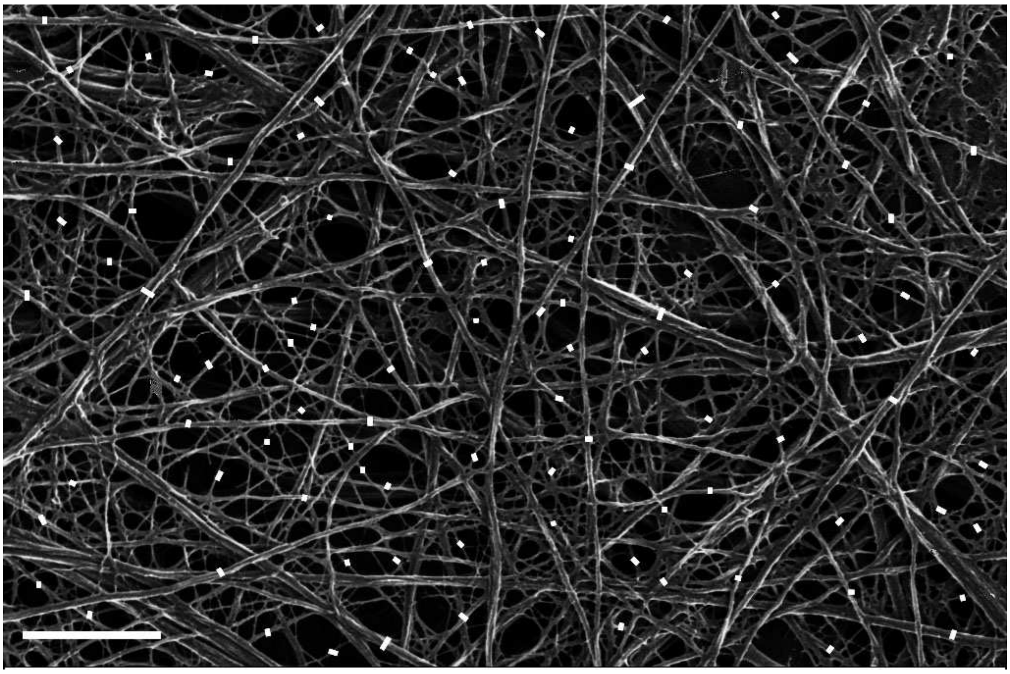


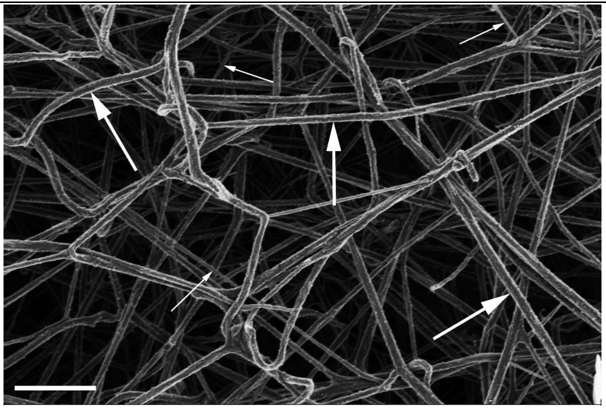
Figure 4.1: Representation of the Measurements Taken to Determine the Fibre Diameters (Scale = 1µm)

4.3 Results & Discussion

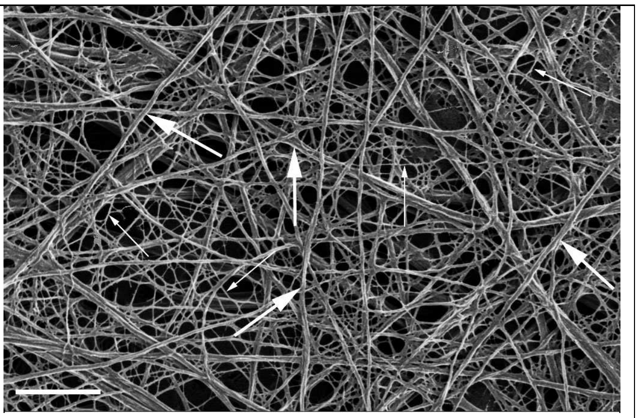
After the addition of human thrombin to the platelet rich plasma of 36 individuals (ranging in ages from 21 years to 86 years of age) a thrombus was formed. The 36 individuals were divided into six age groups and assessed together with the rest of their age group. When viewing the formed thrombus a fibrin network could be observed, by using SEM. The use of SEM allowed the entire thrombus area to be studied methodically to ensure that the resultant micrographs depict an accurate representation of the entire clot area. The SEM micrographs representing the 36 individuals are depicted in Figures 4.2 through 4.7. Major and minor fibres were observed in all the individuals, but with differing predominance.

4.3.1 Scanning Electron Microscopy Micrographs

4.3.1.1 Age Group 30 Years and Under



a



b

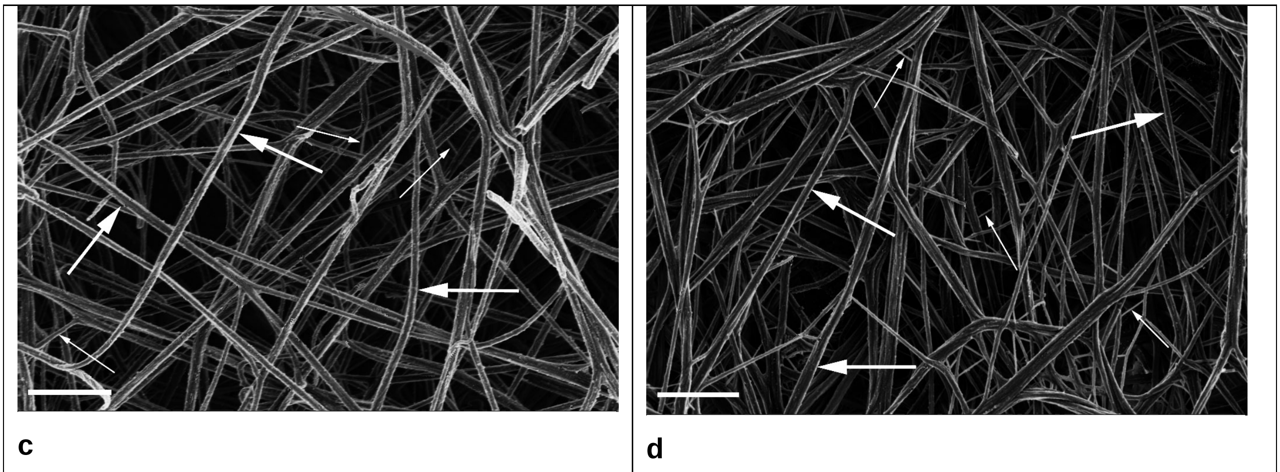


Figure 4.2: Fibrin Networks of Individuals below 30 Years of Age

a: Fibrin network of a 28 year old male. b: Fibrin network of a 23 year old female. c: The fibrin network of a 21 year old female. d: The fibrin network of a 25 year old male. The fibrin networks were created by the addition of human thrombin to platelet rich plasma. The thick white arrows indicate major, thick fibers and the small white arrows indicate thin, minor fibres. Scale = 1 µm.

In Figure 4.2a uniformly packaged fibres can be observed consisting predominantly of thick, major fibres (thick white arrows). Thin, minor fibres (thin white arrows) are sparsely arranged in the spaces between the major fibres. Major fibres appear as single individual fibres and tend to branch off each other. Major fibres are distributed evenly.

Major fibres (thick white arrows) are observed as being predominant in Figure 4.2b. The major fibres are uniformly packed and are distributed evenly. The major fibres appear as individual fibres instead of fibre bundles. The minor fibres (thin white arrows) are sparsely arranged in between the major fibres.

In Figure 4.2c the major fibres (thick white arrows) are predominant and the minor fibres (thin white arrows) are more sparsely arranged. However, macroscopically, there is not an immense difference in the diameter of the different types of fibres. Major fibres appear to branch off each other and are uniformly packaged and evenly spaced. Major fibres are predominantly observed as individual fibres instead of fibre bundles.

Both major and minor fibres are present in Figure 4.2d. indicated by the thick white and thin white arrows respectively. The major fibres seem to make up the majority of the present fibres and in between these thick fibres, the thinner (minor fibres) are present. A few fibre bundles are present, but the major fibres are mainly present as individual fibres.

4.3.1.2 Age Group 30 to 39 Years

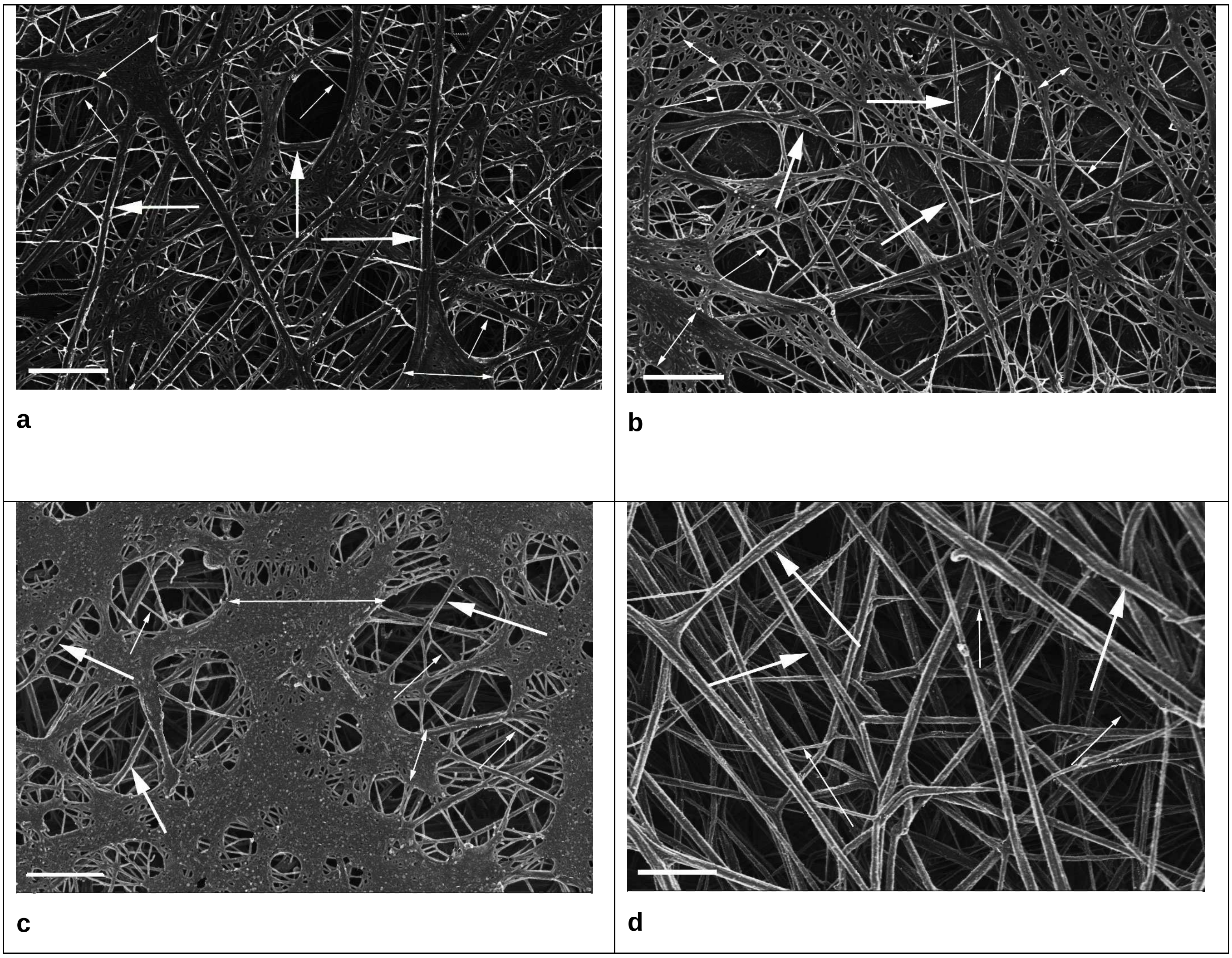


Figure 4.3: Fibrin Networks of Individuals between 30 and 39 Years of Age
a: Fibrin network of a 39 year old female. b: Fibrin network of a 38 year old female. c: The fibrin network of a 31 year old male. d: The fibrin network of a 30 year old male. The fibrin networks were formed by adding human thrombin to

platelet rich plasma. The thick white arrows indicate major, thick fibers and the small white arrows indicate thin, minor fibres. The double arrows indicate "net-like" structures. Scale = 1 µm.

Minor fibres (thin white arrows) seem to be more predominant than major fibres (thick white arrows) in Figure 4.3a. The thin, minor fibres are not sparsely arranged but form dense net-like structures in areas. Minor fibres form between the thicker major fibres. The entire fibrin network appears denser than the previous age group.

In Figure 4.3b the minor fibres (thin white arrows) appear to be more predominant than the major fibres (thick white arrows). Some minor fibres are deposited as "net-like" structures (double arrows) in some areas, covering the major fibres. The major fibres are densely surrounded by the minor fibres.

The 31 year old male in Figure 4.3c illustrates a fibrin network consisting of mainly minor fibres (thin white arrows) with a limited amount of major fibres (thick white arrows) in between. It appears as if the dense fibres were polymerized first after which the minor fibres were formed and deposited as a dense "net-like" structure (double arrows).

The fibrin network of a 30 year old male in Figure 4.3d shows predominantly major fibres (thick white arrows) as in the younger groups and a limited amount of minor fibres (thin white arrows) that formed between the thick fibres. In this micrograph no "net-like" structures were observed which is in conflict with the other individual's fibrin networks.

4.3.1.3 Age Group 40 to 49 Years

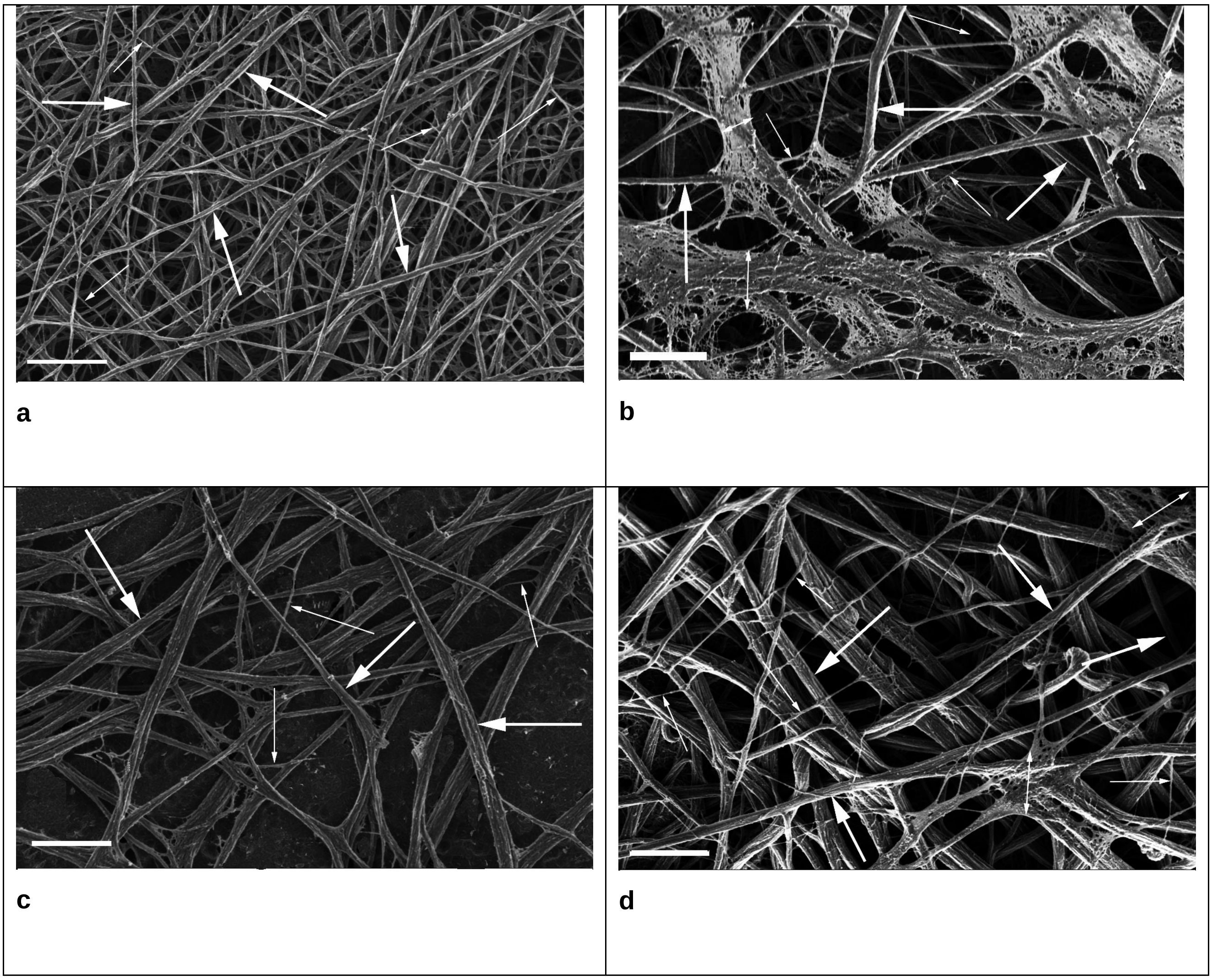


Figure 4.4: The Fibrin Networks of Individuals between 40 and 49 Years
a: Fibrin network of a 47 year old male. b: Fibrin network of a 49 year old female. c: The fibrin network of a 43 year old male. d: The fibrin network of a 42 year old female. The fibrin network was formed by adding human thrombin to platelet rich plasma. The thick white arrows indicate major, thick fibers and the small white arrows indicate thin, minor fibres. Scale = 1 µm.

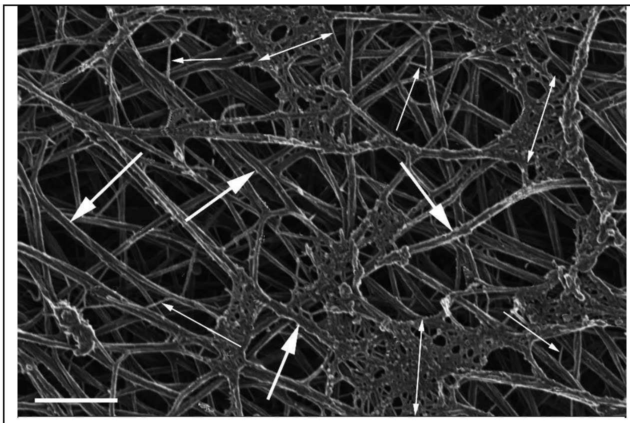
In Figure 4.4a a 47 year old male's fibrin network is depicted. The minor fibres (thin white arrows) are sparsely arranged between the abundance of major, thick fibres (thick white arrows). Dense “net-like” structures are observed which appear to have been formed by minor fibres deposited over the major fibres.

The fibrin network of a 49 year old female is depicted in Figure 4.4b. Major fibres (thick white arrows) appear to be the dominant fibres with a few areas covered by dense “net-like” structures (double arrows) of minor, thin fibres (thin white arrows). Minor fibres are also present in between the spaces created by the formation of the major fibres.

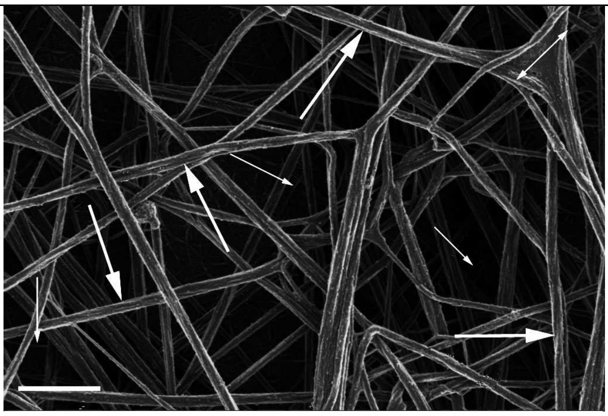
The micrograph in Figure 4.4c illustrates a less dense fibrin network of a 43 year old male. The major, thick fibres (thick white arrows) are abundant with sparsely arranged minor fibres (thin white arrows) in between. The minor fibres are not dominant in this fibrin network.

Figure 4.4d represents the fibrin network of a healthy 42 year old female. Major fibres (thick white arrows) are the prevailing fibres. Some minor fibres (thin white arrows) are visible either in between the major fibres or covering them. It appears as if the minor fibres were deposited after the formation of the major fibres in this thrombus. A small area covered by a “net-like” structure (double arrows) is also visible.

4.3.1.4 Age Group 50 to 59 Years



a



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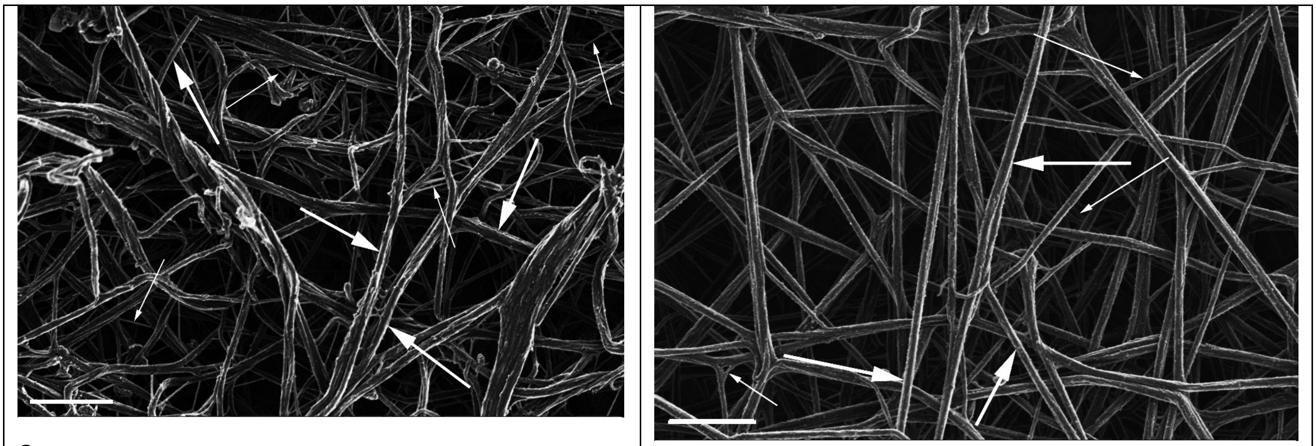

c

d

Figure 4.5: The Fibrin Networks of Individuals between 50 and 59 Year
a: Fibrin network of a 56 year old female. b: Fibrin network of a 54 year old female. c: The fibrin network of a 56 year old male. d: The fibrin network of a 53 year old male. The fibrin networks were produced by the addition of human thrombin to platelet rich plasma. The thick white arrows indicate major, thick fibers and the small white arrows indicate thin, minor fibres. The double arrows indicate “net-like” structures. Scale = 1 µm.

In Figure 4.5a the major fibres (thick white arrows) are more abundant than the minor fibres (thin white arrows). “Net-like” structures (double arrows) consisting of densely deposited minor fibres are visible in some areas. The overall structure appears relatively uniformly packaged and regular.

The fibrin network of a 54 year old female in Figure 4.5b can be observed as a regular, uniformly packed network. The major fibres (thick white arrows) are predominant and abundant and the minor fibres (thin white arrows) are distributed sparsely and are present in a much smaller amount than the major fibres. The minor fibres are much thinner than the major fibres.

Figure 4.5c represents the fibrin network of a healthy 56 year old female. The major fibres (thick white arrows) are in abundance and present in a much larger amount than the minor, thin fibres (thin white arrows). The major fibres, however, form large fibre bundles in some areas. The fibres appear “sticky” and appear to be glued together. Both the diameter of the individual as well as the fibre bundles is greater than the diameter of the minor fibres.

The fibrin network of a 53 year old male in Figure 4.5d can be observed as a healthy fibrin network which is predominantly formed by major fibres (thick white arrows). The network is regular and uniformly packed with a normal branching tendency and is arranged in an ordered manner. The thin fibres (thin white arrows) form the minority of the total fibrin count.

4.3.1.5 Age Group 60 to 69 Years

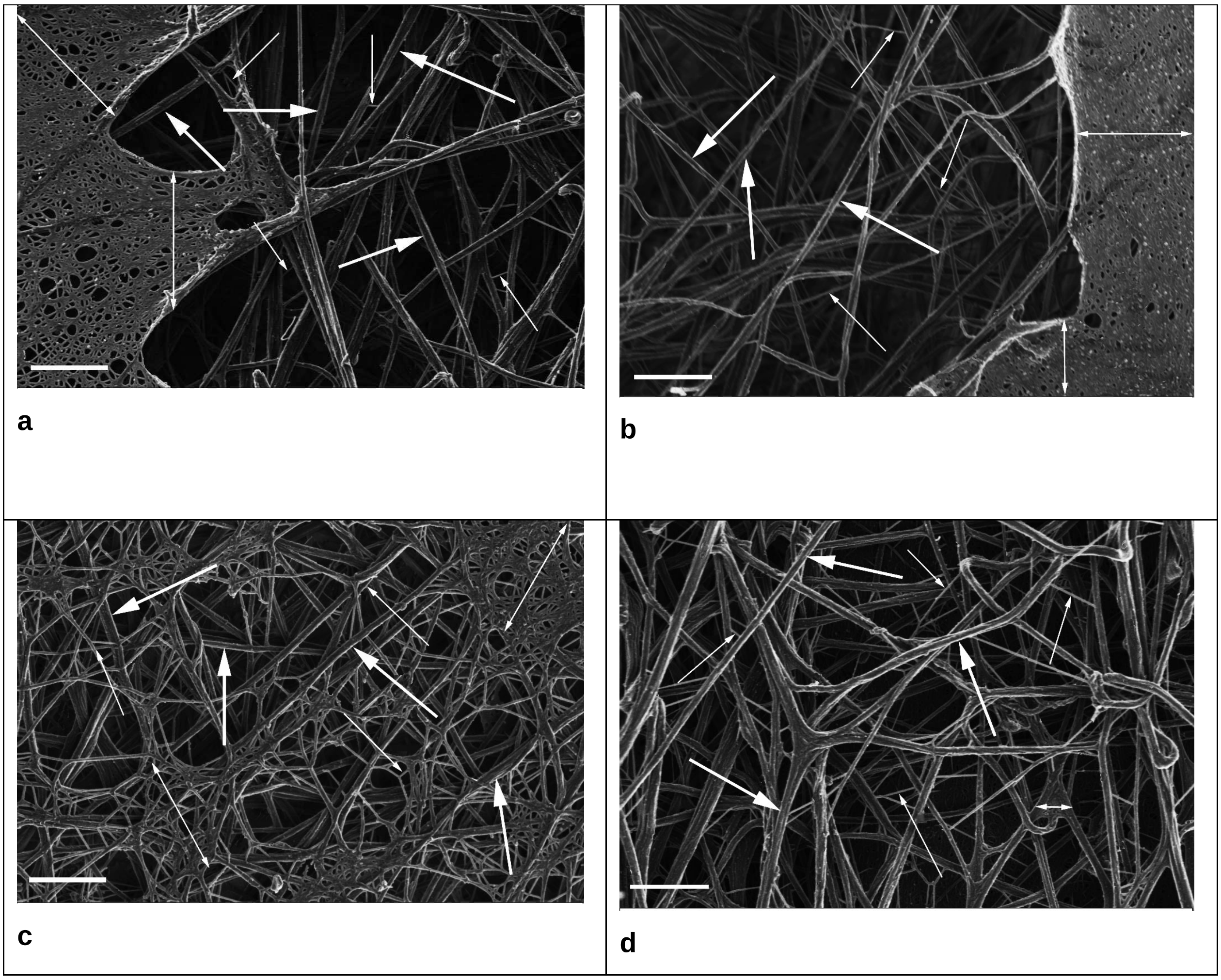

Figure 4.6: Fibrin Networks of Individuals between 60 and 69 Years of Age

a: Fibrin network of a 62 year old female. b: Fibrin network of a 61 year old male. c: The fibrin network of a 65 year old female. d: The fibrin network of a 65 year old female. The fibrin network was created by adding human thrombin to platelet rich plasma. The thick white arrows indicate major, thick fibers and the small white arrows indicate thin, minor fibres. The double arrows indicate "net-like" structures formed by minor fibres. Scale = 1 µm.

In Figure 4.6a the fibrin network of a 62 year old female is represented. The network is made up of both major and minor fibres. Major fibres (thick white arrows) are present in some areas with minor fibres (thin white arrows) in between the regular packed fibres. A large area of the major fibres, however, is covered by a dense structure of minor fibres that forms a "net" (double arrows) across the thrombus. The "net-like" structure appears to have been deposited subsequent to the formation of the major, thick fibres.

Figure 4.6b depicts the healthy fibrin network of a 61 year old male. Major thick fibres (thick white arrows) are predominant in some areas with thin fibres (thin white arrows) in between the major fibres. Large areas of the thrombus however are covered by a dense deposit of minor, thin fibres to form a "net-like" structure across the formed thrombus and the resulting fibrin network.

In Figure 4.6c the minor fibres (thin white arrows) appear to be present in a larger number than the major fibres (thick white arrows). The major fibres are still regularly arranged and uniformly packed, but it appears as if there was a dramatic increase in minor fibres. The thin fibres make up large parts of the fibrin network. "Net-like" structures (double arrows) also cover some areas of the formed fibrin network of this healthy 65 year old female.

Figure 4.6d appears to consist mainly of minor thin fibres (thin white arrows). The major fibres (thick white arrows) are present, but a striking increase in thin, minor fibres can be observed. "Net-like" structures (double arrows) often formed by minor fibres are also visible.

4.3.1.6 Age Group 70 Years and Above

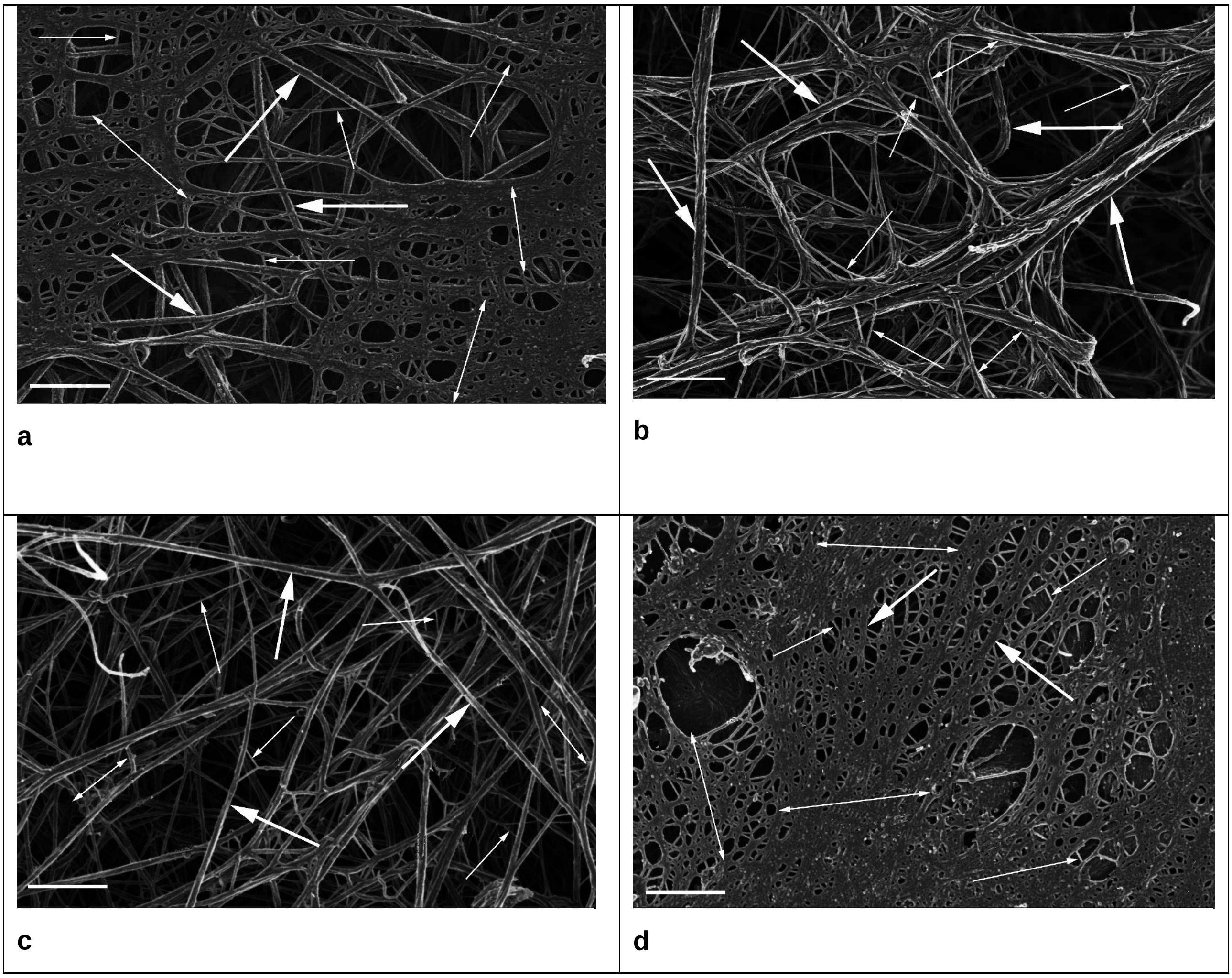

Figure 4.7: Fibrin Networks of Individuals Exceeding 70 Years of Age
a: Fibrin network of a 79 year old female. b: Fibrin network of a 86 year old female. c: The fibrin network of a 80 year old female. d: The fibrin network of a 76 year old female. The fibrin networks were obtained by adding human thrombin to platelet rich plasma. The thick white arrows indicate major, thick fibers and the small white arrows indicate thin, minor fibres. The double arrows indicate “net-like” structures. Scale = 1 µm.

In Figure 4.7a the network of a healthy 79 year old woman is depicted. Minor fibres (thin white arrows) make up the majority of the network with major, thick fibres (thick white arrows) in the minority. “Net-like” structures (double arrows) cover a large part of the obtained thrombus and consequently the represented fibrin network. The major fibres appear to be mostly covered by a dense network of minor fibres.

Figure 4.7b represents the thrombus and consequent fibrin network of a healthy 86 year old female. Thin fibres (thin white arrows) appear to be predominant although dense “net-like” structures of thin fibres are not readily visible. The minor fibres are clearly visible and much thinner than the relatively straight major fibres (thick white arrows). The major fibres appear “sticky” and in some areas seem glued together to form thicker fibre bundles.

The fibrin network of a healthy 80 year old female is illustrated in Figure 4.7c. Major fibres (thick white arrows) display regular branching patterns. The minor fibres (thin white arrows) are arranged in the spaces between the major fibres. Minor fibres are much thinner than the major fibres. The minor fibres are abundant but less so than the major fibres.

The fibrin network of a healthy 76 year old female is depicted in Figure 4.7d. The minor fibres (thin white arrows) are in abundance, so much so that the major fibres (thick white arrows) appear to be absent. The minor fibres are arranged in thick, dense “net-like” structures (double arrows) and make up the majority of the fibrin network.

With an advance in age minor fibres appear to become the predominant fibre type, especially after the age of 60. “Net-like” structures appear and seem to be deposited after the formation of the primary fibrin network. The major fibres appear more flimsy in old age and tend to form fibre bundles in individuals over the age of 50 years. These changes could indicate hypercoagulability in older individuals.

4.3.2 Statistical Analysis

4.3.2.1 Normality

The results of the fibrin diameters studied using the Sharpiro Wilk W test for normality is presented in Table 4.2. Normality refers to the arrangement of samples that have a normal distribution pattern and are clustered around a single mean. In all the cases the data failed to assume a normal distribution pattern.

Table 4.2: Statistical Analysis of Normality for Fibrin Diameter Distribution

Age Group (Years)	**Participant**	**Sharpiro-Wilk W Test for Normality***
Less than 30	1	Reject normality (P = 2.42 x 10^{-7})
	2	Reject normality (P = 4.28 x 10^{-8})
	3	Reject normality (P = 6.73 x 10^{-6})
	4	Reject normality (P = 1.26 x 10^{-3})
	5	Reject normality (P = 1.26 x 10^{-4})
	6	Reject normality (P = 2.28 x 10^{-8})
30-39	7	Reject normality (P = 3.46 x 10^{-7})
	8	Reject normality (P = 3.91 x 10^{-12})
	9	Reject normality (P = 6.68 x 10^{-10})
	10	Reject normality (P = 1.22 x 10^{-6})
	11	Reject normality (P = 3.38 x 10^{-12})
	12	Reject normality (P = 1.96 x 10^{-5})
40-49	13	Reject normality (P = 3.09 x 10^{-12})
	14	Reject normality (P = 4.56 x 10^{-9})
	15	Reject normality (P = 6.68 x 10^{-8})
	16	Reject normality (P = 5.85 x 10^{-11})
	17	Reject normality (P = 8.74 x 10^{-11})
	18	Reject normality (P = 3.93 x 10^{-11})
50-59	19	Reject normality (P = 1.95 x 10^{-8})
	20	Reject normality (P = 2.83 x 10^{-07})
	21	Reject normality (P = 4.45 x 10^{-9})

	22	Reject normality (P = 1.14 x 10^{-4})
	23	Reject normality (P = 1.17 x 10^{-12})
	24	Reject normality (P = 2.11 x 10^{-10})
60-69	25	Reject normality (P = 3.10 x 10^{-4})
	26	Reject normality (P = 7.78 x 10^{-9})
	27	Reject normality (P = 2.72 x 10^{-5})
	28	Reject normality (P = 5.998 x 10^{-9})
	29	Reject normality (P = 1.86 x 10^{-7})
	30	Reject normality (P = 6.99 x 10^{-11})
70 and greater	31	Reject normality (P = 4.36 x 10^{-10})
	32	Reject normality (P = 1.51 x 10^{-11})
	33	Reject normality (P = 2.34 x 10^{-6})
	34	Reject normality (P = 3.84 x 10^{-8})
	35	Reject normality (P = 5.81 x 10^{-8})
	36	Reject normality (P = 2.64 x 10^{-10})

* Significance was set at a level of 0.05

4.3.2.2 Fibrin Fibre Size Classes

The histograms depicting the fibrin fibre diameters for each age group is depicted in Figures 4.8 to 4.13.

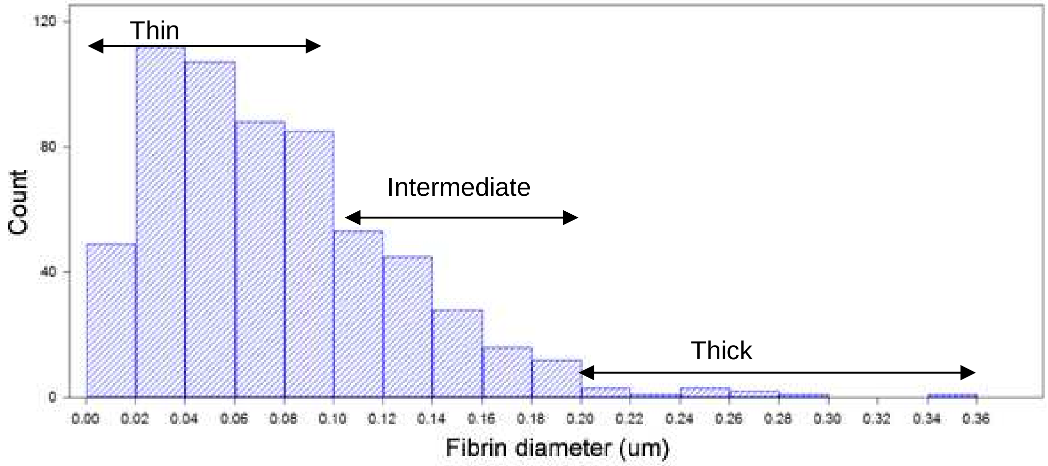

Figure 4.8: A Histogram presenting the Fibrin Fiber Diameter Distribution Pattern for all Six Less than 30 Year Old Participants of this Study

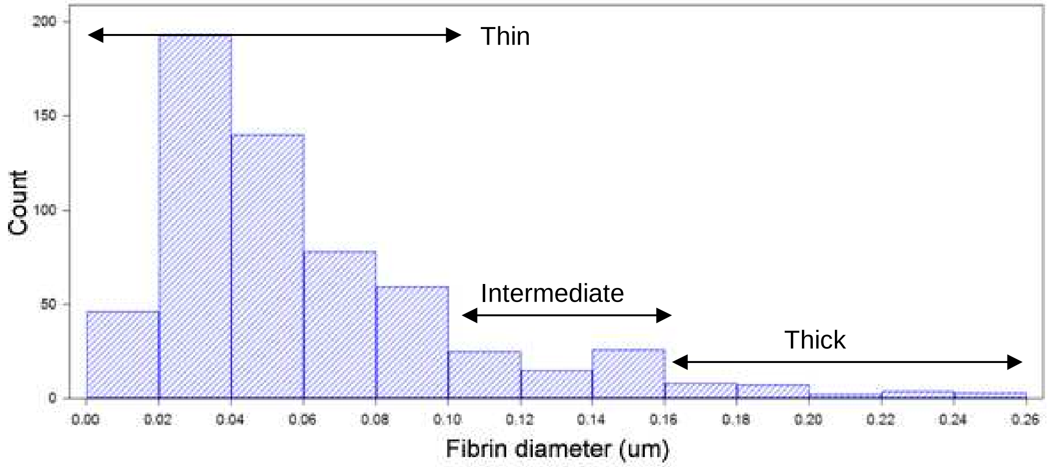

Figure 4.9: A Histogram presenting the Fibrin Fiber Diameter Distribution Pattern for all Six 30 to 39 Year Old Volunteers of this Study

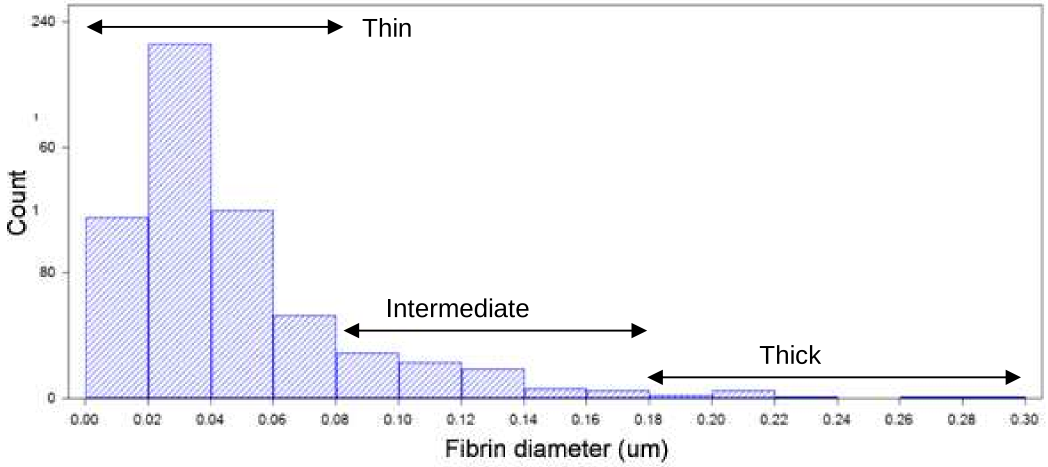

Figure 4.10: A Histogram presenting the Fibrin Fiber Diameter Distribution Pattern for all Six 40 to 49 Year Old Participants

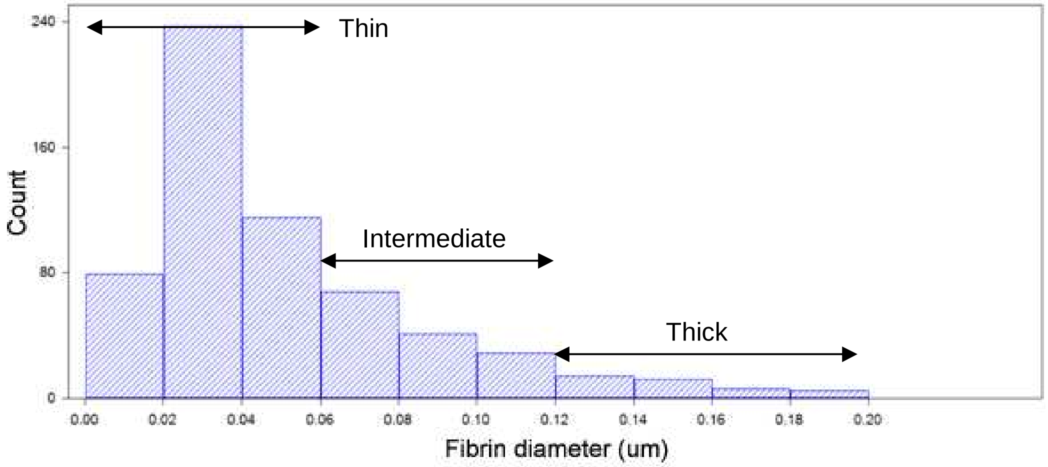

Figure 4.11: A Histogram presenting the Fibrin Fiber Diameter Distribution Pattern for all Six the 50 to 59 Year Old Volunteers

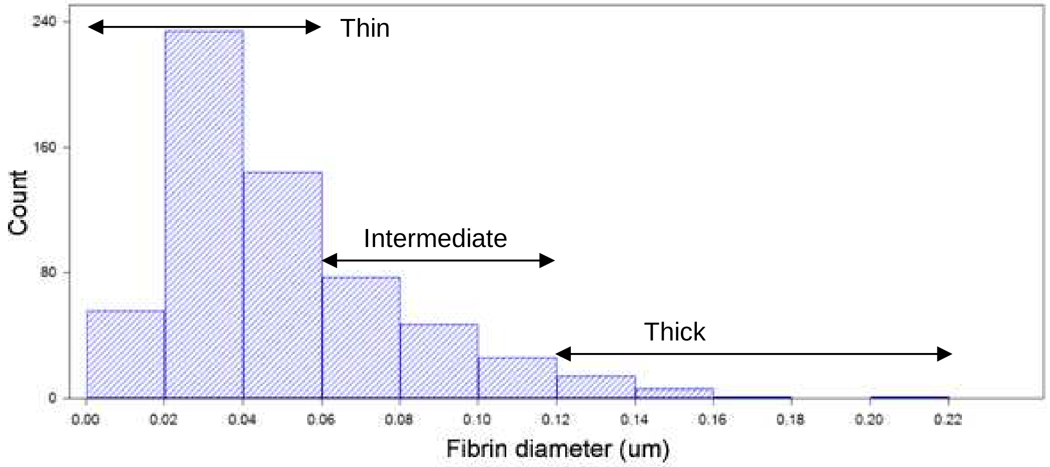

Figure 4.12: A Histogram presenting the Fibrin Fibre Diameter Distribution Pattern for all Six 60 to 69 Year Old Participants of this Study

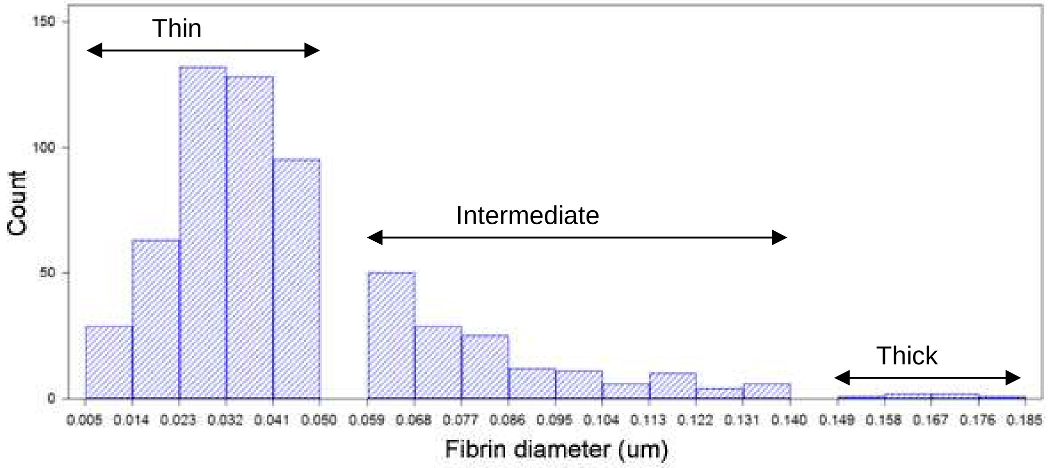

Figure 4.13: A Histogram presenting the Fibrin Fiber Diameter Distribution Pattern for all Six Participants Aged 70 Years or Older

All the histograms (Figure 5.8 to Figure 5.13) show similar patters of fibrin distribution. The overall pattern seen amongst the volunteers studied, in terms of their fibrin diameter distributions, was not one of a distinct bimodal distribution pattern (Table 4.2). A bimodal pattern would have been expected since classical literature (Pretorius & Oberholzer 2009) indicates the occurrence of two distinct sections in the histograms, each region corresponding to either the classical thick (major) or thin (minor) fibres. No clear distinction could be made between thick (major and thin (minor) fibres, but the majority of the individuals rather presented a “merged” distribution pattern (See Figure 4.8 to 4.13). The “merged” distribution pattern did not hold a clear separation between thick and thin fibres of the formed thrombus and may be due to the presence of an intermediate sized set of fibres within the fibrin clot. Thus, these fibres would aid to constitute a continuum or tri-modal distribution pattern.

The size range of the most prominent fibres for each age group of this study, determined with the aid of Figures 4.8 to 4.13, is presented in Table 4.3.

Table 4.3: Most Prominent Fibre Size Range per Age Group

Age Group (n=6)	**Most Prominent Fiber Size Range (µm)**
Less than 30	0.02 – 0.04
30-39	0.02 – 0.04
40-49	0.02 – 0.04
50-59	0.02 – 0.04
60-69	0.02 – 0.04
70 and greater	0.02 – 0.03

Table 4.4 presents the average diameters of the thickest fibre measured in each of the studies age groups. One-way ANOVA and Tukey-Kramers Multiple comparison pos hoc tests were used for the comparison. As the diameters of the fibres were measured randomly and the thin fibres were amongst the highest proportions of fibres counted, it can be concluded that the thin fibres constitute a large proportion of the clot.

Table 4.4: Average Diameter of the Thickest Fibrin Fibre per Age Group

Age Group (Years)	*less than 30*	*30-39*	*40-49*	*50-59*	*60-69*	*70 and more*
average diameter of thickest quantified fiber (µm) (mean ± standard deviation) (n=6)	0.250 ± 0.065	0.198 ± 0.043	0.215 ± 0.060	0.187 ± 0.010	0.162* ± 0.027	0.152* ± 0.025

*significantly smaller than the average thickest diameter of the less than 30 year old group.

The most prominent fibre diameter for the group older than 70 years is smaller than the other age groups as seen in Table 4.3. If one examines the histogram in Figure 4.13, it can be seen that the size range is 0.032 – 0.041 is very close in count to the 0.021-0.032 group and the two are almost indistinguishable from the one another. Amongst all the age groups the thinnest fibre noted was 0.01 µm. Thus, it appears that age does not appear to affect the fibrin clot in terms of its prominent thin fibre presence.

In Table 4.4 it could be seen that the thickest fibre measured amongst the 60 to 69 year olds and 70 year olds and more was significantly smaller than the thickest fibre measured amongst the less the 30 year olds (P=0005350).

Thus once over the age of 60 individuals' fibrin fibres, produced during the coagulation process are significantly thinner in comparison is to the individuals younger than 30 years of age.

The decrease in diameter thickness is roughly 0.02 µm over the 50 year period. As the 60 to 69 and 70 and older age groups were not significantly smaller that the other age groups, one could assume that the thick fibres gradually decrease in diameter with age but only becomes significantly smaller when comparing the extremities of the age scale.

Due to the great variation in the data and the unclear presence of distinct diameter groups no size classes could be determined for the participants of this study when using statistical analysis.

4.5 Conclusion

As described in literature the thrombi consisted of major, thick fibres and minor, thin fibres and this was found to be true for all the participants. The major fibres in all age groups displayed a regular branching pattern. It can be concluded that the amount of thin fibres remain stable in all the age groups, but that the thickness of the major fibres decrease with age, especially after the age of 60, where the fibres become more flimsy. The thinner fibres contribute to the appearance of a network consisting mostly of minor, thin fibres. The alterations noted in aged individuals can contributed to an increased coagulation potential and thus hypercoagulability in old age.

It could also be concluded that fibrin fibres can not only be divided into thick and thin fibre diameters, but that a third, intermediate diameter fibre may also exist in the formed thrombus. The results of this study therefore support the findings of Pretorius and associates in 2007 (Pretorius *et al*. 2007).

“Net-like” structures formed by thin fibres appear after the age of 30, but becomes the predominant part of the thrombus after the age of 60. After the age of 50, fibre bundles start appearing. Networks appear denser after the age of 30, especially in females.

Sex-related differences are present with regards to SEM study of the fibrin networks. The differences are absent before the age of 30 and become more prominent from 30 onwards. The difference becomes more evident with advancing age. After the age of 60, however, the differences disappear (similar to the under 30 year old age group) and the thrombin characteristics are similar for males and females.

Chapter 5

A Comparative Study of the Thrombocytes of Different Age Groups Using Transmission Electron Microscopy and Scanning Electron Microscopy

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5.1 Introduction

Thrombocytes normally circulate in blood, close to the endothelial lining of the blood vessel (Freson *et al*. 2007). Thrombocytes are not whole cells since they lack nuclei, but can rather be described as cell fragments that are enclosed by a membrane (Solomon *et al*. 2005). The fragments are pinched off of large cells called megakaryocytes (Solomon *et al*. 2005). Thrombocyte development takes approximately four to five days and the life span of the platelets ranges between nine and 10 days (Coetzee *et al*. 2003). Normal thrombocyte concentration is between 250 000 to 350 000 thrombocytes per one µl of blood (Coetzee *et al*. 2003). When vascular damage occurs, the circulating thrombocytes adhere to the subendothelial structures and initiate the coagulation cascade (Freson *et al*. 2007).

Studies done with light microscopy reveals that thrombocytes are anucleated cells with a diameter of two to five microns (Coetzee *et al*. 2003). Inert platelets can usually be observed as discoidal, however the shape may change once the thrombocytes have been activated (Freson *et al*. 2007). The platelet membrane is usually smooth in normal controls (Pretorius & Oberholzer 2009). Two structural areas can be defined under the light microscope, the outer hyalomere and the central or inner region (the granulomere) (Coetzee *et al*. 2003). The cytoplasm, when studied under an electron microscope, indicates the presence of two tubular systems. The first system consists of tubules that connect the cytoplasm to the surface, known as the open canalicular system (OCS); this is probably for the transportation of substances (Coetzee *et al*. 2003). The OCS enlarge the platelet surface, since the invaginations are continuations of the plasma membrane (Gartner & Hiatt 2007). The second system (with an unknown function) is found in the central portion of the cytoplasm and is derived from the Golgi complex of the megakaryocyte (Coetzee *et al*. 2003).

The microtubules and filaments observed in the hyalomere, function as support structures for the platelet to maintain its shape and also function to secrete granules, as the microtubules and filaments play a role in the retraction of the clot during fibrinolysis (Coetzee *et al*. 2003; Freson *et al*. 2007). The cytoplasm contains cellular elements such as mitochondria, ribosomes, glycogen (energy source of the platelet), and storage granules (Coetzee *et al*. 2003).

The storage granules can be divided into two distinct groups, alpha granules and dense bodies. The alpha granules contain a variety of proteins such as platelet factor 4, β-thromboglobulin (β-TBG), platelet derived growth factor (PDGF), fibrinogen, PAI-1 and Von Willebrand Factor. On activation of the thrombocyte, the contents of the alpha granules are released. The dense bodies on the other hand contain serotonin, ATP and calcium. The substances contained in the dense bodies facilitate platelet aggregation and adhesion (Freson *et al*. 2007).

Platelets play a major role in both the formation and growth of the thrombus and the prevention of early fibrinolysis. The clot structure is greatly influenced by the number of adherent thrombocytes that are present. By initiating the coagulation cascade, thrombocytes promote the cross-linking of fibrin (Wohner 2008).

The function of thrombocytes is not restricted to temporary blockage of the damaged vessel; they are also responsible for procoagulation enzymatic events by exposing the phospholipids of the vessel wall as well as the sustaining of the procoagulant reactions by preventing premature fibrinolysis (Sachs & Nieswandt 2007; Wohner 2008). Only about 10, 000 thrombocytes are necessary for adequate haemostasis, therefore, the large number of thrombocytes present could be indicative of other functions. These alternative functions could include as a possible role in innate immunity and could be regarded as anti-pathogenic due to platelets' role in the generation of thrombin (Clemetson 2010).

The presence of thrombocytes is essential in the initial reaction after vascular injury (Wohner 2008). Platelets adhere to the rough edges of the vessel wall around the damaged area, where the platelets are activated to attract other thrombocytes to the site of injury. The thrombocytes aggregate to form a primary platelet plug and also attract leukocytes to the damaged area (Solomon *et al*. 2005). The aggregated and adhered thrombocytes form the surface or substrate for the subsequent coagulation pathway reactions (Wohner 2008).

In this chapter platelets of the participating individuals were compared to determine whether sex- or age-related differences exist between individuals of different age groups. Scanning Electron Microscopy was used to compare the surface morphology and Transmission Electron Microscopy to compare the internal composition of the platelets.

5.2 Materials and Methods

Two types of electron microscopy were used to study the platelets for this chapter. Firstly Scanning Electron Microscopy (SEM) was used to study the platelet surface morphology of platelets obtained from a plasma smear. Secondly, Transmission Electron Microscopy (TEM) was used to study the internal morphology of the platelet. The platelets for TEM were obtained from the mixture of plasma and thrombin to form a fibrin clot. The fibrin clot was sectioned to visualize the platelets.

5.2.1 Samples

Blood for this study was drawn from 36 voluntary participants. The samples were collected from individuals from six different age groups. The six are groups are depicted in Table 5.1.

Table 5.1: Experimental Groups and Age Ranges of the Participants

Experimental Group	Age range
1	< 30 yr
2	30-39yr
3	40-49yr
4	50-59yr
5	60-69yr
6	>70yr

The participants in this study is regarded as healthy since a careful medical history was obtained in order to exclude any individuals with fibrin network altering changes, to ensure that the fibrin networks, platelets and coagulation profiles are as close as possible to normal. Known factors that alter the ultrastructure of fibrin networks served as exclusion factors in this study. The factors are smoking, overweight individuals, asthma, diabetes, other inflammatory conditions, hormone replacement therapy or contraceptive medication and anticoagulant medication.

5.2.2 Preparation of Fibrin Clots for Transmission Electron Microscopy

Blood were collected from 36 individuals (ethical clearance was obtained from the Research Ethical Committee of the University of Pretoria, South Africa). Freshly prepared human platelet-rich plasma (FPRP) was prepared from the four ml blood obtained by venipuncture. The blood samples were allowed to separate naturally in order to separate the whole blood into plasma and the pellet. Fibrin clots were prepared in order to provide platelet aggregates as well as fibrin fibres for all the participants. The fibrin clots were prepared by using Human Thrombin (Supplied by the South African National Blood Service) in the case of the Transmission Electron Microscopy (TEM) samples.

Human Thrombin is prepared from a single regular donor by the calcium chloride activation of a euglobulin fraction of plasma. The plasma was obtained by apheresis.

Each individual unit is tested and has to be non-reactive to a number of infectious agents including hepatitis B surface antigen, HIV-1 antibody, HIV-2 antibody and HIV p-24 antigen, hepatitis C virus (HCV) antibody and antibodies to *Treponema palladium*. These tests are performed by using licensed assay methods. The thrombin solution is at a concentration of 20 U/ml and is made up in a biological buffer containing 0.2% human serum albumin.

When thrombin is added to freshly prepared human platelet-rich plasma (FPRP), the fibrinogen is converted to fibrin and other intracellular platelet components are released into the coagulum. These components include, but are not limited to transforming growth factor, platelet-derived growth factor and fibroblastic growth factor (Pretorius *et al*. 2010). 10 µl of FPRP was mixed with 10 µl of the human thrombin on a 0.2 µm Millipore membrane (MICROSEP (PTY) LTD) to form the fibrin clot. The Millipore membrane was then placed on a filter paper dampened with Phosphate Buffer Solution (PBS) and placed in an incubator at 37ºC for 10 minutes. After incubation the Millipore membrane was placed in phosphate buffer solution on a microplate shaker for 20 minutes to remove any blood proteins that may have been caught in the fibrin mesh.

5.2.3 Preparation of Washed Fibrin Clots for Transmission Electron Microscopy and Plasma Smears for Scanning Electron Microscopy

The initial preparations are identical for both the SEM and the TEM samples: The washed fibrin clots formed on the Millipore membrane and the plasma smears also made on Millipore membranes were fixated for 30 minutes. The fixative consisted of one ml 25% gluteraldehyde, one ml 25% formaldehyde, three ml water and five ml phosphate buffer solution (PBS). After the fixation step the clots and smears were washed three times in PBS for three minutes to remove any residual fixative. The clots and smears were fixated for 15 minutes in 1% osmium tetraoxide (OsO_4). The osmium tetraoxide fixated clots and smears were washed three times for three minutes in PBS. Both types of samples were then dehydrated serially in 30%, 50%, 70%, 90% and finally three times in 100% ethanol. Each dehydration step lasted three minutes.

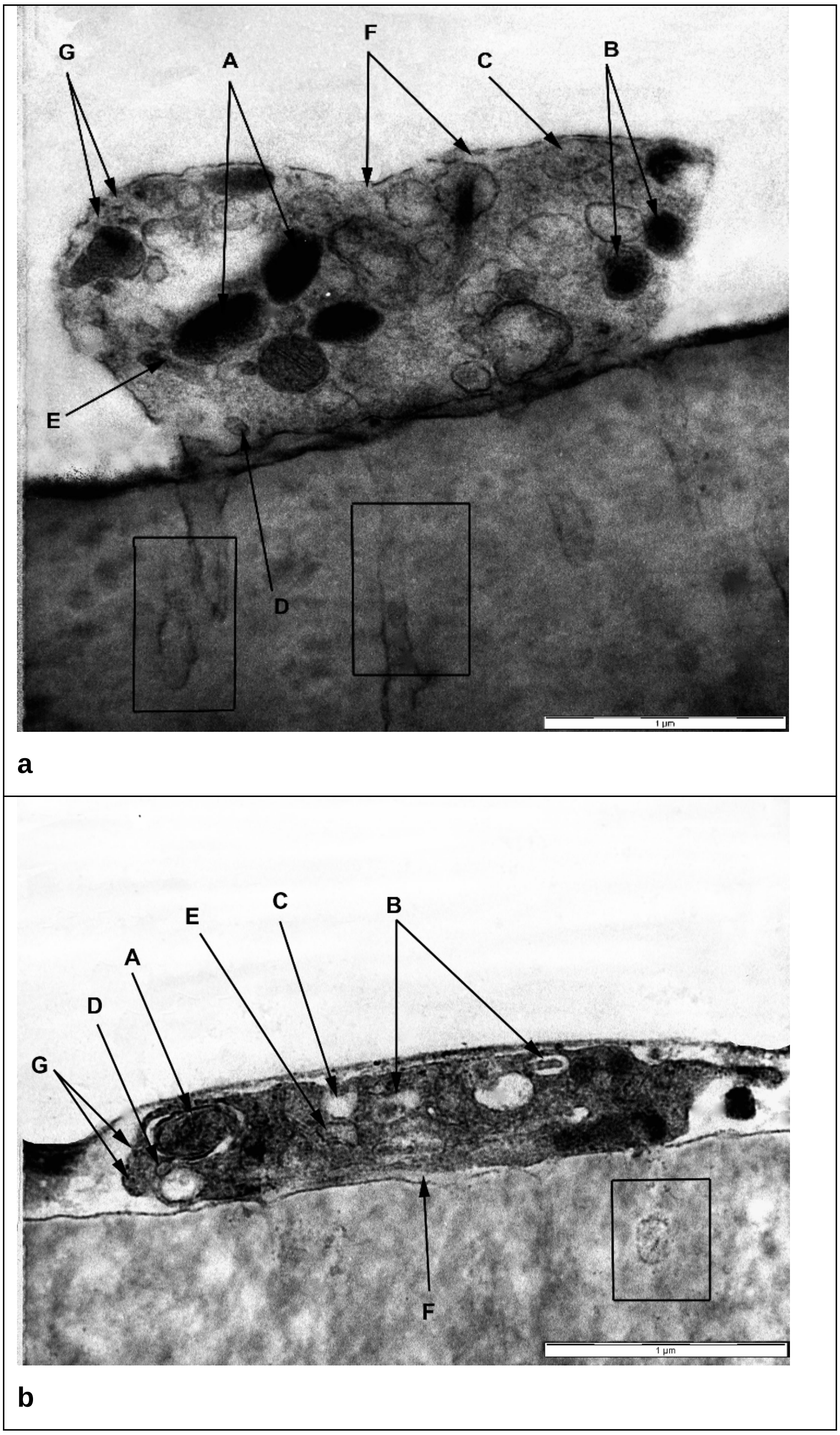
For TEM samples the following method was used: A small section of the Millipore membrane (containing the fibrin clot) was sectioned out and this section was infiltrated with Quetol resin. Infiltration consisted of two steps: the samples were infiltrated with 50% ethanol and 50% Quetol resin for an hour and then with 100% Quetol resin for one day overnight. The Quetol resin infiltrated samples were then embedded with a sample number in rubber moulds. The embedding was done over three days in an oven. The samples were sectioned using an ultramicrotome. Contrastaining is done with uranyl acetate and lead citrate. The stained samples were subsequently viewed using a JEOL Transmission Electron Microscope (JEM 2100F).

For the SEM samples: The plasma smears on the Millipore membranes were dried by the critical point drying technique and were mounted and coated with carbon. After coating the samples were examined using a Zeiss ULTRA plus FEG Scanning Electron Microscope.

5.3 Results

Thrombocytes representative of the 36 participants of this study is presented in Figures 5.1 to 5.12. TEM micrographs represent the interior composition of the platelets and the SEM micrographs depict the surface morphology of the platelets.

5.3.1 Age Group Under 30 Years of Age



A: Alpha granules
B: Dense Bodies
C: OCS
D: Mitochondria
E: Dilated Channels
F: Microtubules
G: Glycogen
Black rectangles indicate pores in the Millipore membrane
Scale = 1 µm

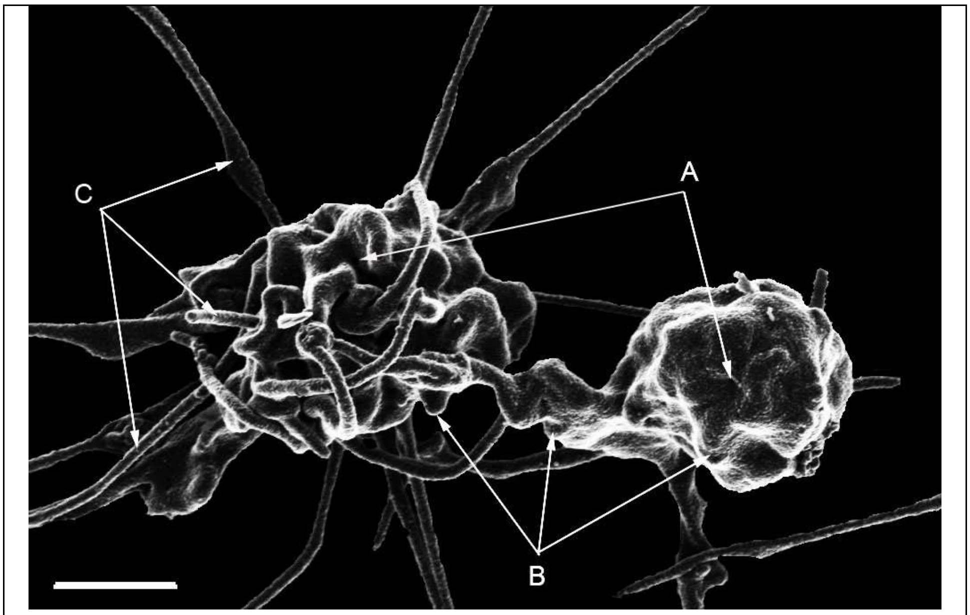
A: Alpha granules
B: Dense Bodies
C: OCS
D: Mitochondria
E: Dilated Channels
F: Microtubules
G: Glycogen
Black rectangles indicate pores in the Millipore membrane
Scale = 1 µm

Figure 5.1: TEM Micrographs of Individuals Younger than 30 Years

a: The platelet of a 24 Year Old Female. b: Platelet of a 27 Year Old Male.

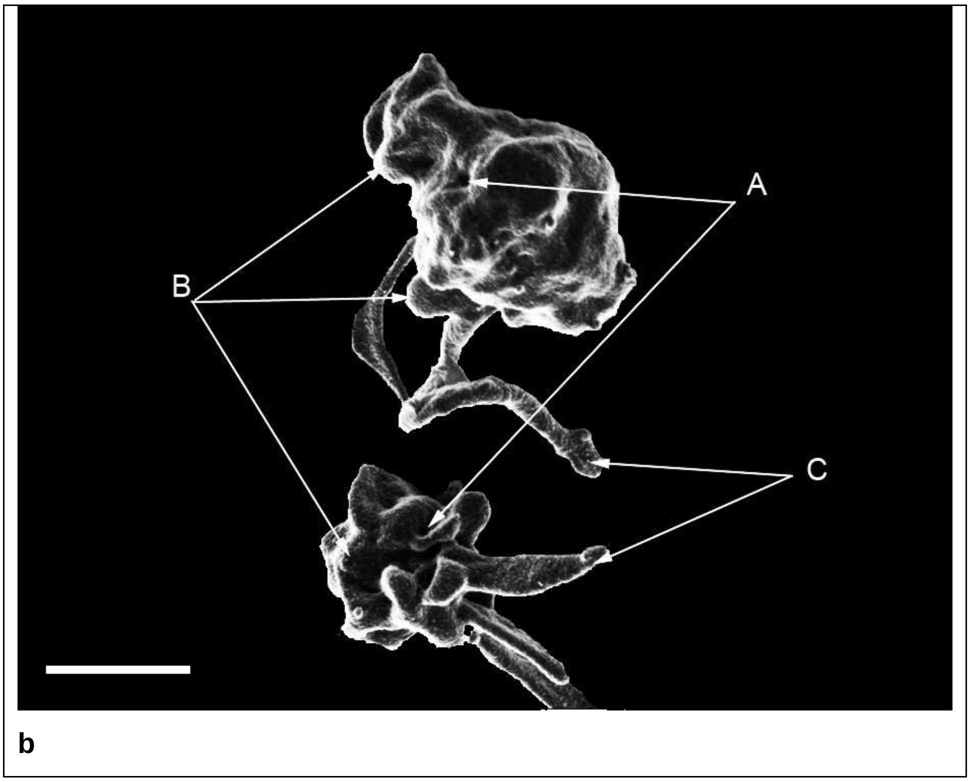
Figure 5.1a and Figure 5.1b depicts the platelets of individuals of the age group below 30 years of age. Alpha granules as well as dense bodies are visible in both micrographs. OCS channels can be observed together with mitochondria, dilated channels, microtubules and glycogen granules. The alpha granules are approximately 300 nm in size and the dense bodies approximately 200nm in size.

In Figure 5.2a and Figure 5.2b the SEM micrographs of platelets of two individuals under the age of 30 years can be observed. The platelet membranes appear smooth and a few OCS openings can be seen. Pseudopodia are formed on the platelet surface and fibrin fibres can be observed as long protrusions extending from the platelet surface.



a

A: OCS
B: Pseudopodia
C: Fibrin fibres

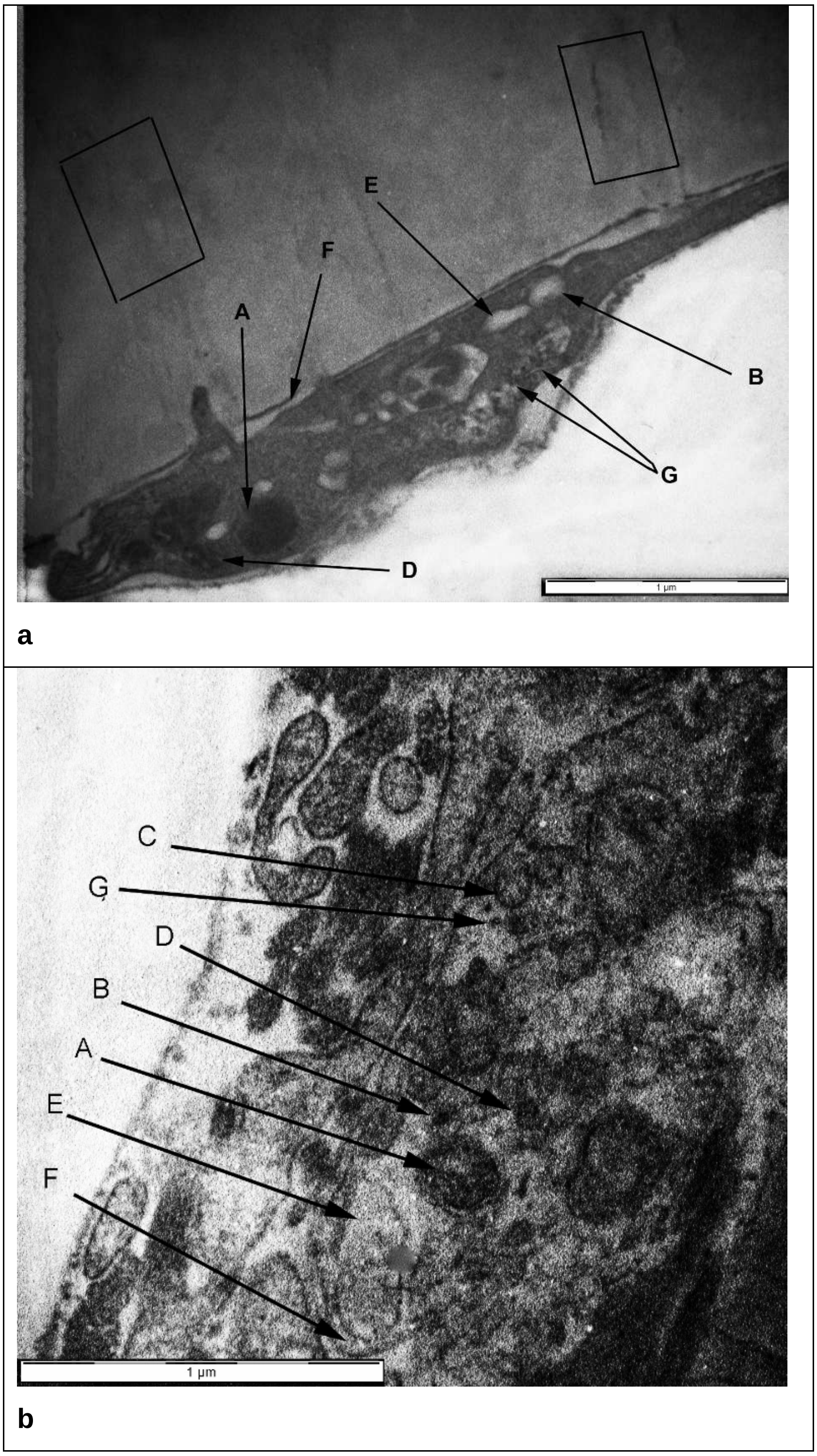


A: OCS
B: Pseudopodia
C: Fibrin fibres

Figure 5.2: SEM Micrographs of Individuals below 30 Years of Age
a: Thrombocyte of a 25 Year Old Male. b: The thrombocyte of a 27 Year Old Male. Scale = 1µm.

5.3.2 Age Group between 30 and 39 Years of Age

In Figure 5.3a and b the thrombocytes of individuals between 30 and 39 years of age are depicted. The thrombocytes contain alpha granules (approximately 300nm in size) and dense bodies (approximately 200nm in size). Mitochondria, dilated channels, microtubules and glycogen granules are present in Figure 5.3a and b. In Figure 5.3b, the OCS can also be observed. The black rectangle indicates pores found in the Millipore membrane.

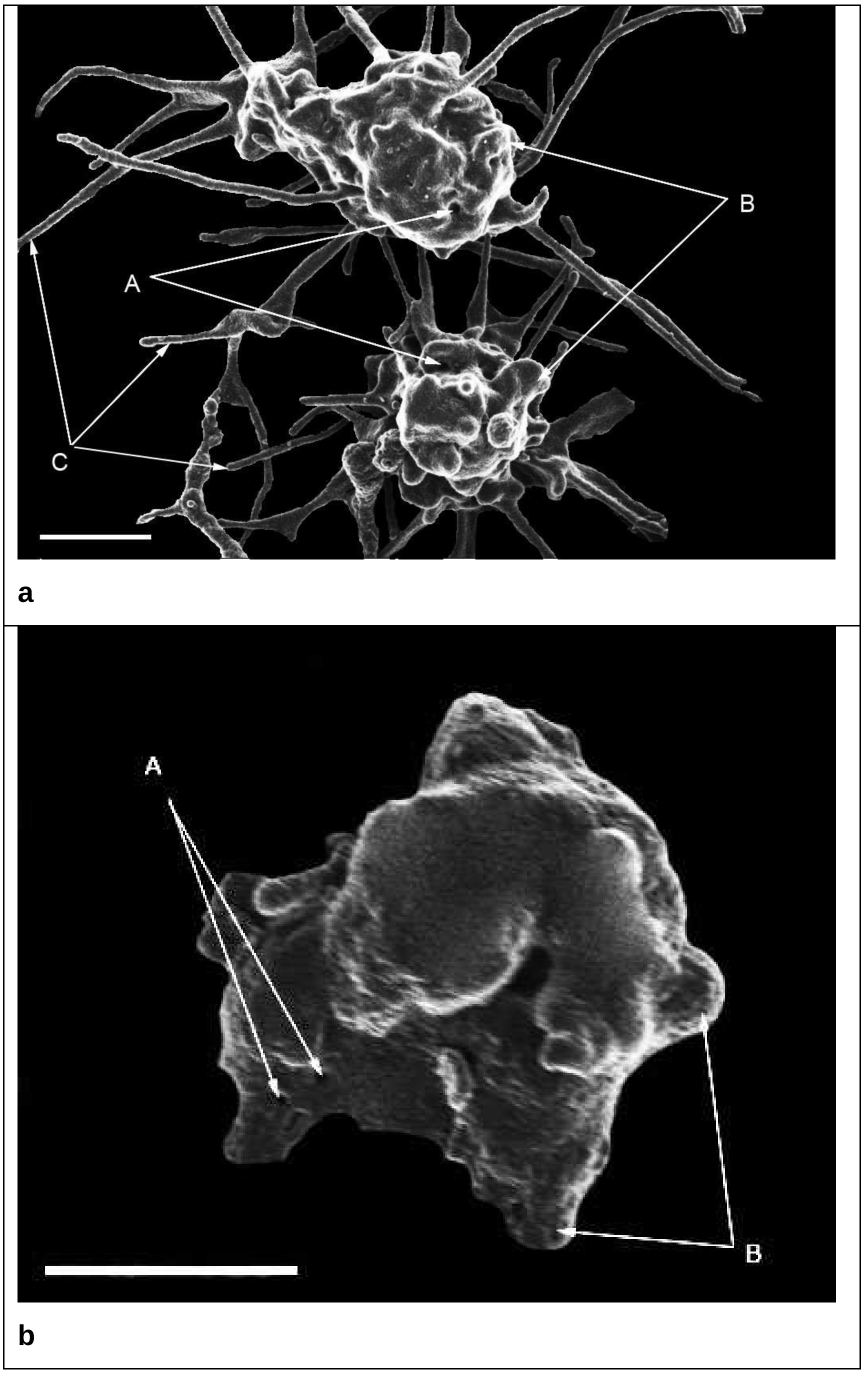


A: Alpha granules
B: Dense Bodies
D: Mitochondria
E: Dilated Channels
F: Microtubules
G: Glycogen
Black rectangles indicate pores in the Millipore membrane
Scale = 1 µm

A: Alpha Granules
B: Dense Bodies
C: OCS
D: Mitochondria
E: Dilated Channels
F: Microtubules
G: Glycogen
Scale = 1 µm

Figure 5.3: Micrographs of Platelets of Individuals between 30 and 39 Years of Age using TEM

a: Platelet of a 39 Year Old Female. b: The platelet of a 31 Year Old Male.



A: OCS
B: Pseudopodia
C: Fibrin fibres

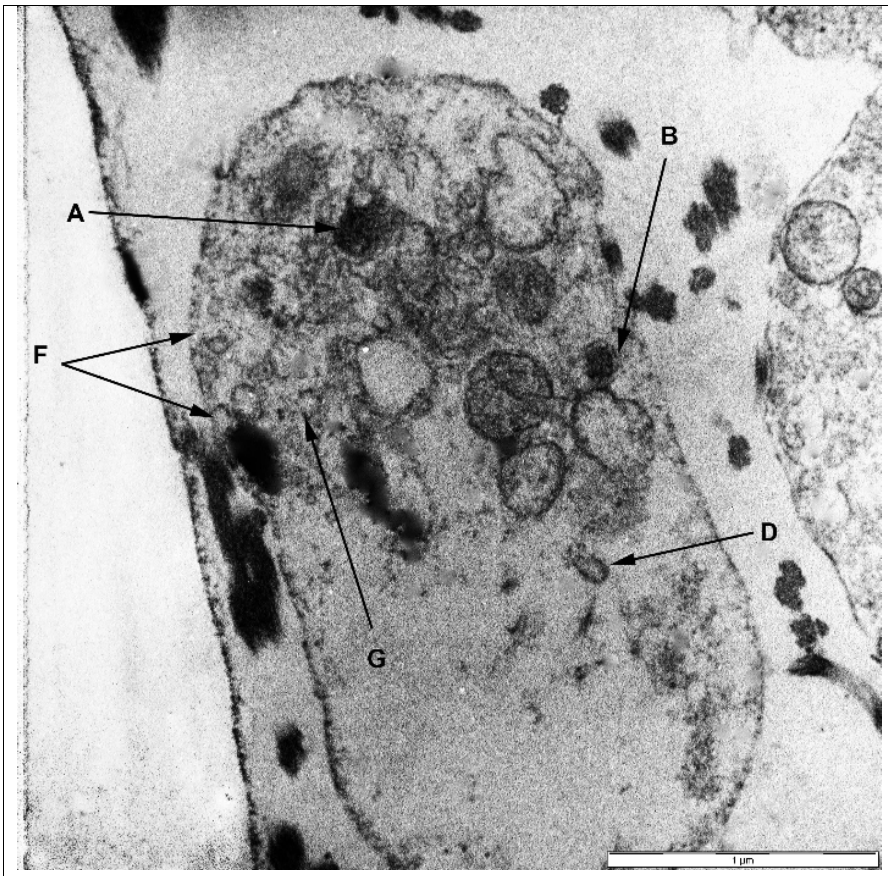
A: OCS
B: Pseudopodia

Figure 5.4: The SEM Micrographs of Individuals between 30 and 39 Years of Age

a: Platelets of a 33 Year Old Male. b: Platelets of a 30 Year Old Male. Scale = 1µm.

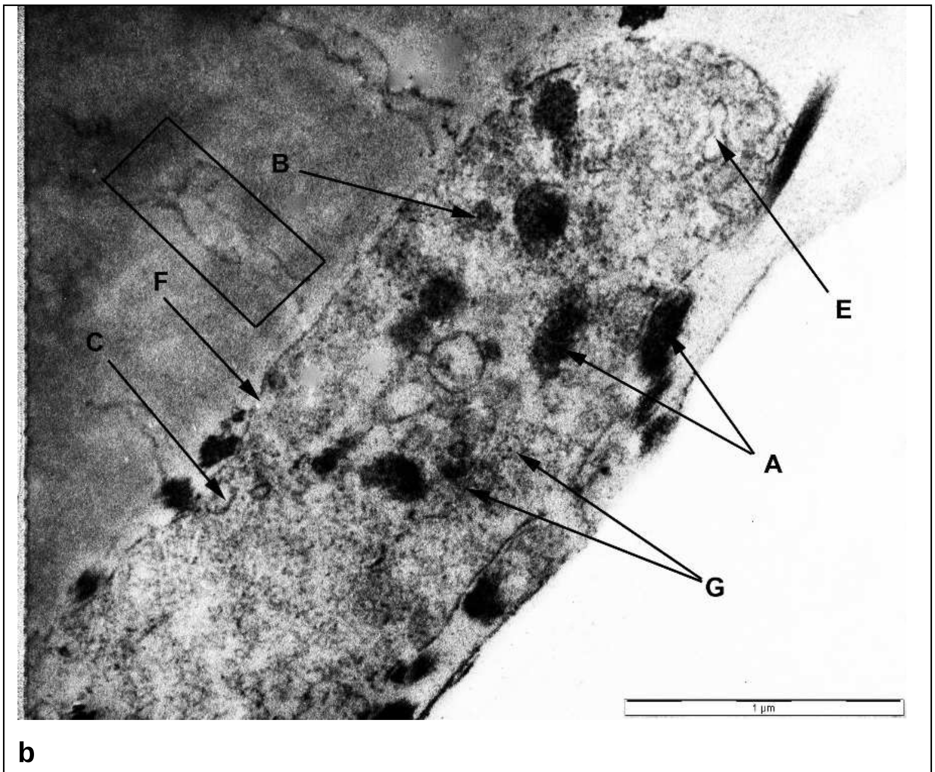
Figure 5.4a and b represents the platelets of individuals in the age range 30 to 39 years of age. Pseudopodia and OCS openings can be observed in both figures and the fibrin fibres appear in abundance in Figure 5.4a.

5.3.3 Individuals between 40 and 49 Years of Age



a

A: Alpha granules
B: Dense Bodies
D: Mitochondria
F: Microtubules
G: Glycogen
Scale = 1 µm



A: Alpha granules
B: Dense Bodies
C: OCS
E: Dilated Channels
F: Microtubules
G: Glycogen
Black rectangles indicate pores in the Millipore membrane
Scale = 1 µm

Figure 5.5: TEM Images of Platelets of Individuals between 40 and 49 Years of Age

a: Platelets of a 47 Year Old Female. b: Platelets of a 49 Year Old Female.

Figure 5.5a and b illustrates the platelets of two individuals in the age group 40 to 49 years. Alpha granules and dense bodies can be observed in both pictures and have approximate sizes of less than 300 nm and under 200 nm respectively. Figure 5.5a also depicts mitochondria, microtubules and glycogen granules. No OCS or dilated channels can be observed in Figure 5.5a. In Figure 5.5b no mitochondria can be observed, but dilated channels, microtubules and glycogen granules are present. The black rectangle indicates pores found in the Millipore membrane.

Figure 5.6a and Figure 5.6b illustrates the activated platelets of individuals between the ages of 40 and 49 years. Both micrographs show pseudopodia and OCS openings, but fibrin fibres are only visible in Figure 5.6b.

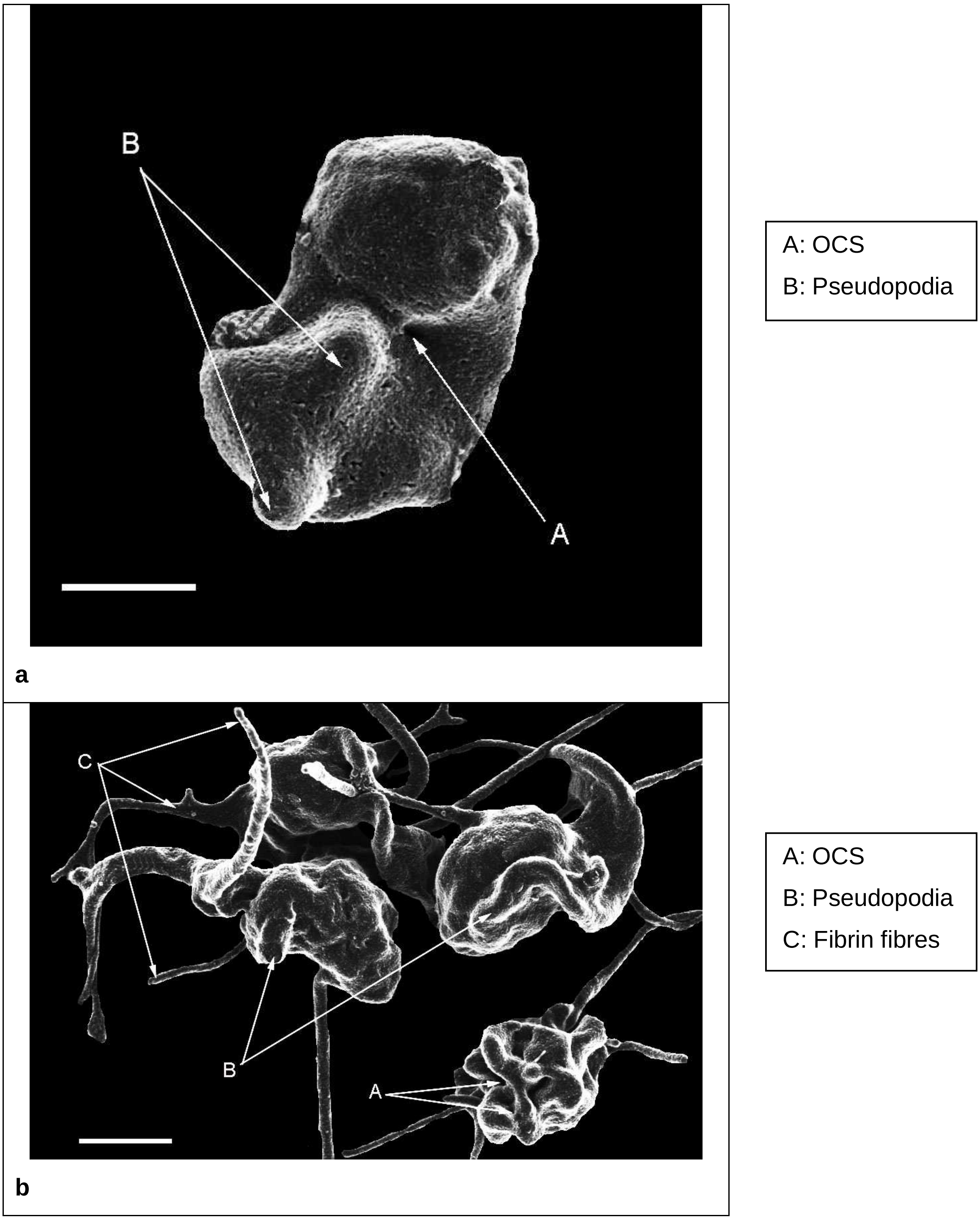
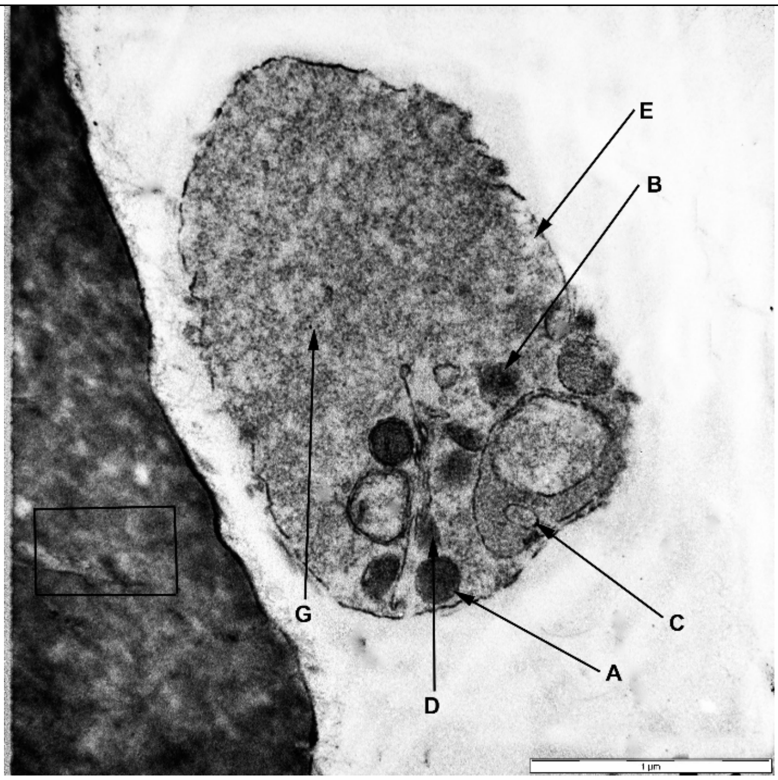


Figure 5.6: Micrographs of Platelets of Individuals between 40 and 49 Years of Age

a: Platelets of a 47 Year Old Female. b: Platelets of a 47 Year Old Male. Scale = 1µm.

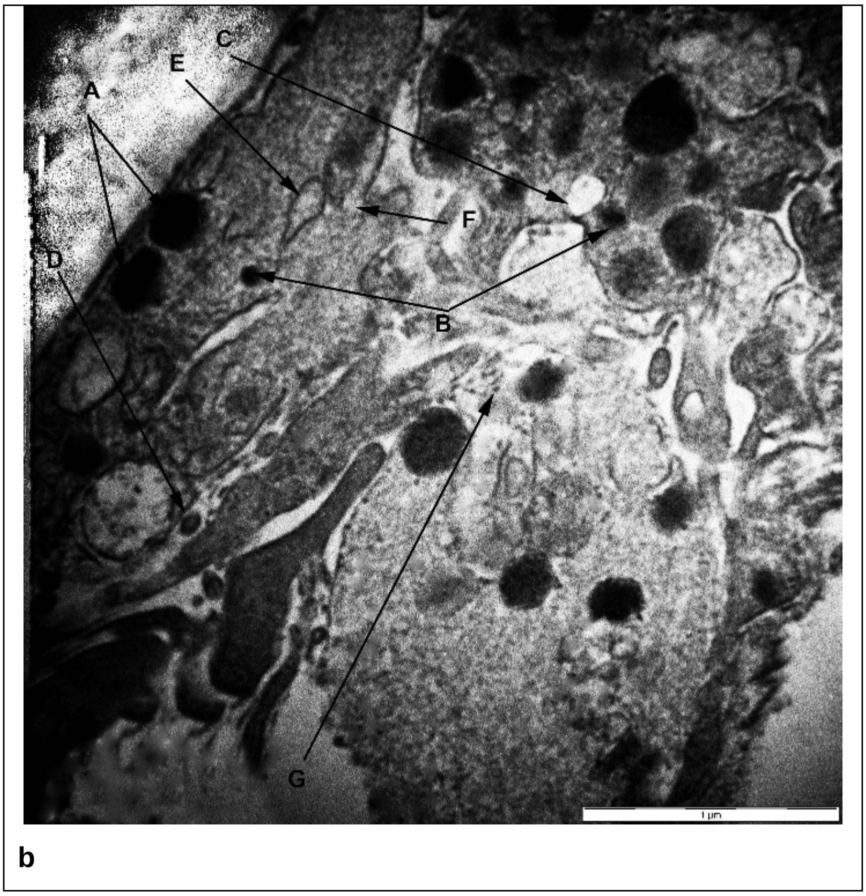
5.3.4 Participants Aged Between 50 and 59 Years of Age

The platelets of individuals in the age range 50 to 59 years are presented in Figure 5.7a and b. Alpha granules (less than 300nm); dense bodies (under 200 nm in size), the OCS, mitochondria as well as dilated channels can be observed in both micrographs. Glycogen granules are illustrated in both micrographs. Microtubules are only visible in Figure 5.7b. The black rectangle indicates pores found in the Millipore membrane in which the fibrin clot is prepared.



a

A: Alpha granules
B: Dense Bodies
C: OCS
D: Mitochondria
E: Dilated Channels
G: Glycogen
Black rectangles indicate pores in the Millipore membrane
Scale = 1 µm



A: Alpha granules
B: dense bodies
C: OCS
D: Mitochondria
E: Dilated Channels
F: Microtubules
G: Glycogen
Scale = 1 µm

Figure 5.7: TEM Micrographs of Platelets of Individuals between 50 and 59 Years of Age

a: Platelets of a 56 Year Old Female. b: Platelets of a 59 Year Old Male.

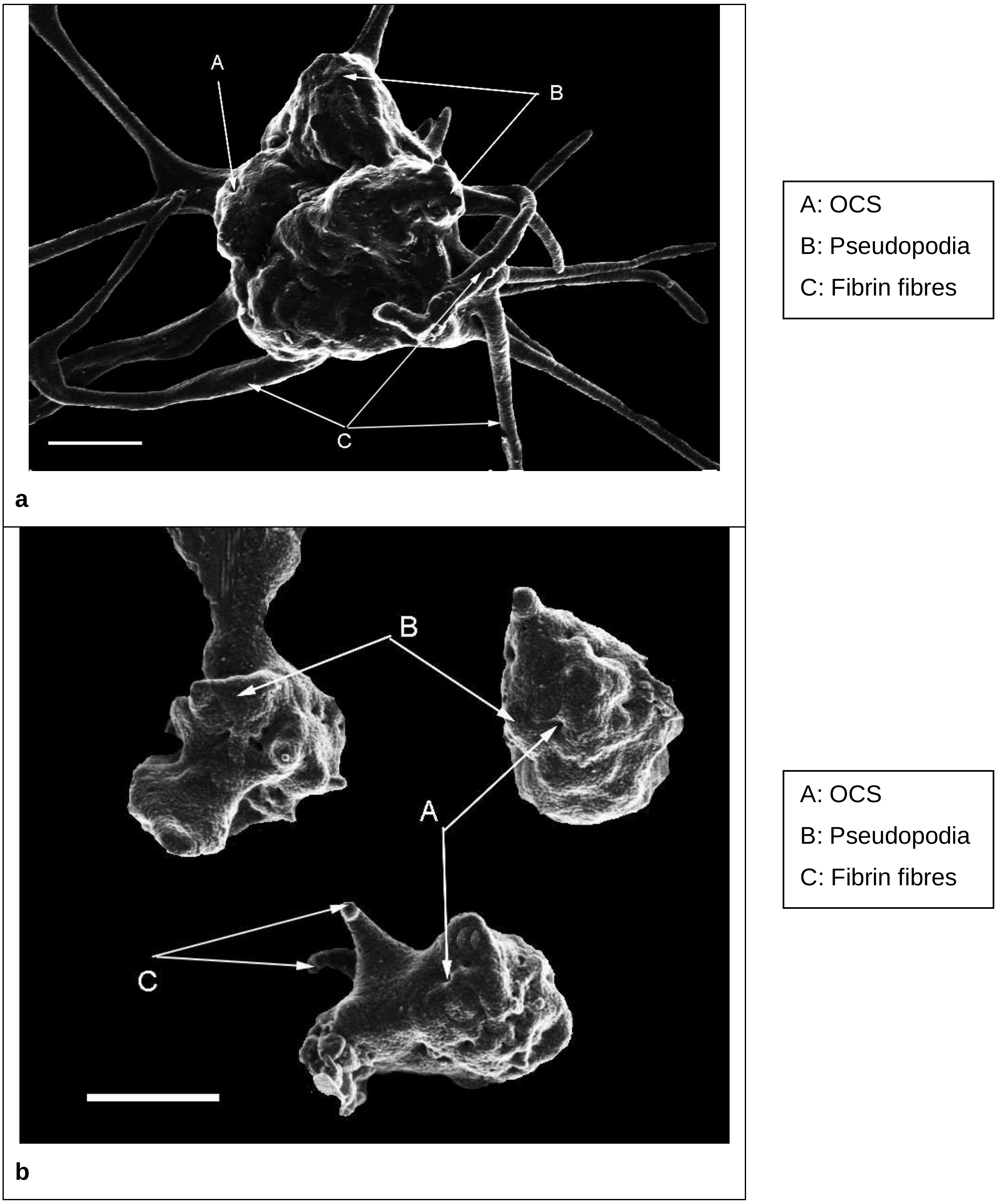


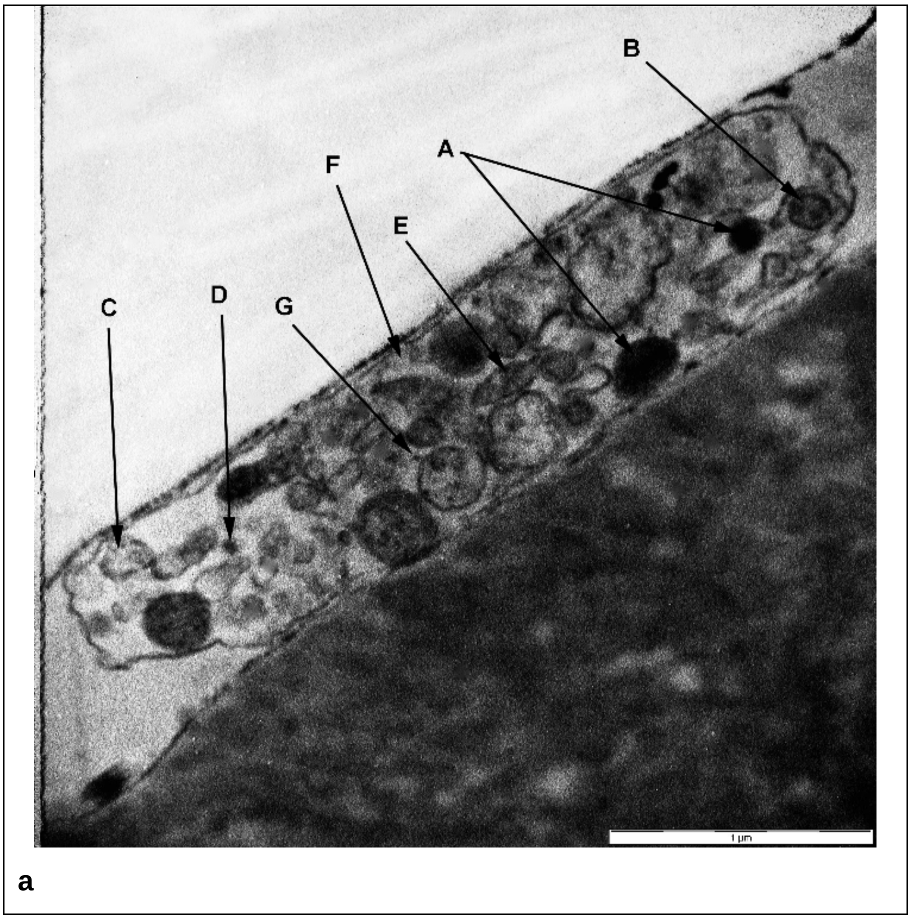
Figure 5.8: SEM Micrographs of Platelets of Individuals between 50 and 59 Years of Age

a: Platelets of a 54 Year Old Female. b: Platelets of a 53 Year Old Male. Scale = 1µm.

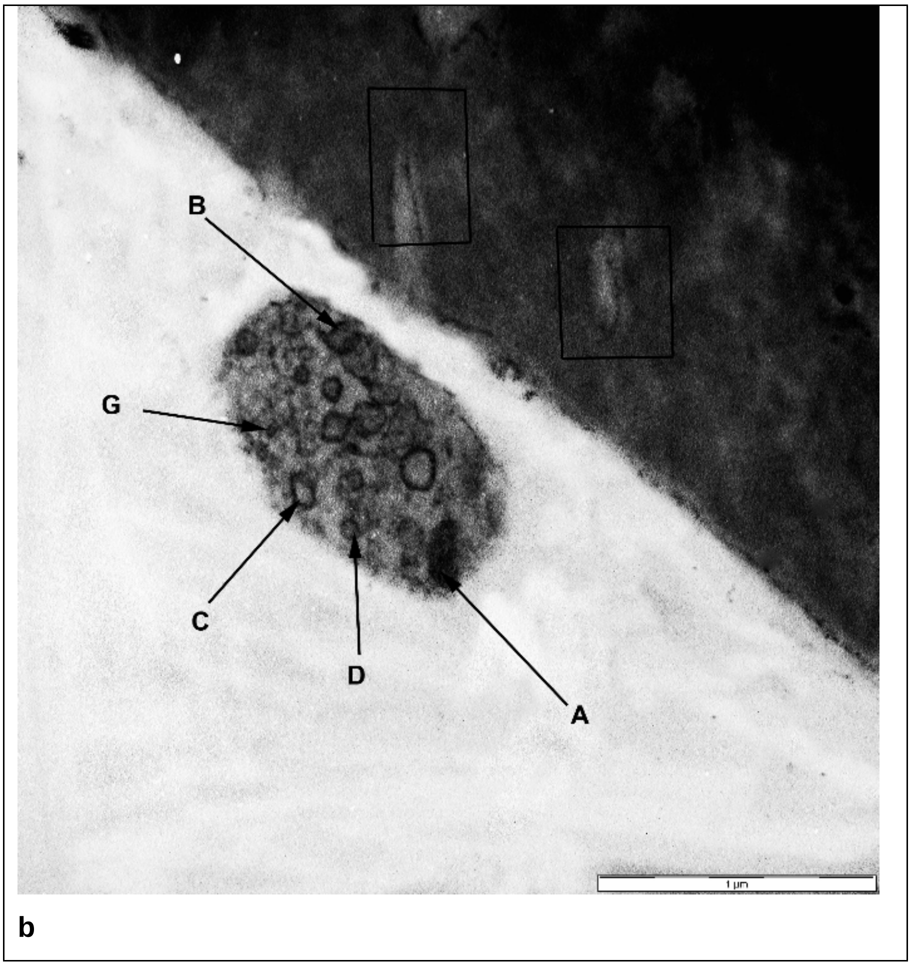
In Figure 5.8a and Figure 5.8b represents the thrombocytes of individuals between the ages of 50 and 59 years. OCS openings, formed pseudopodia and fibrin fibres extending from the platelet membrane are visible in both micrographs.

5.3.5 Age Group between 60 and 69 Years Old

Figure 5.9a and Figure 5.9b depicts the thrombocytes of individuals between the ages of 60 and 69 years of age. Alpha granules and dense bodies are present in both micrographs. The alpha granules are less than 300 nm in size and the dense bodies are less than 200 nm in size. Both micrographs depict OCS openings, mitochondria, and glycogen granules. Microtubules and dilated channels are only visible in Figure 5.9a. Black rectangles indicate pores in the Millipore membrane.



A: Alpha granules
B: Dense Bodies
C: OCS
D: Mitochondria
E: Dilated Channels
F: Microtubules
G: Glycogen
Scale = 1 µm



A: Alpha granules
B: dense bodies
C: OCS
D: Mitochondria
G: Glycogen
Black rectangles indicate pores in the Millipore membrane
Scale = 1 µm

Figure 5.9: Platelets of Individuals between 60 and 69 Years of Age Viewed with TEM

a: Platelets of a 65 Year Old Female. b: Platelets of a 62 Year Old Male.

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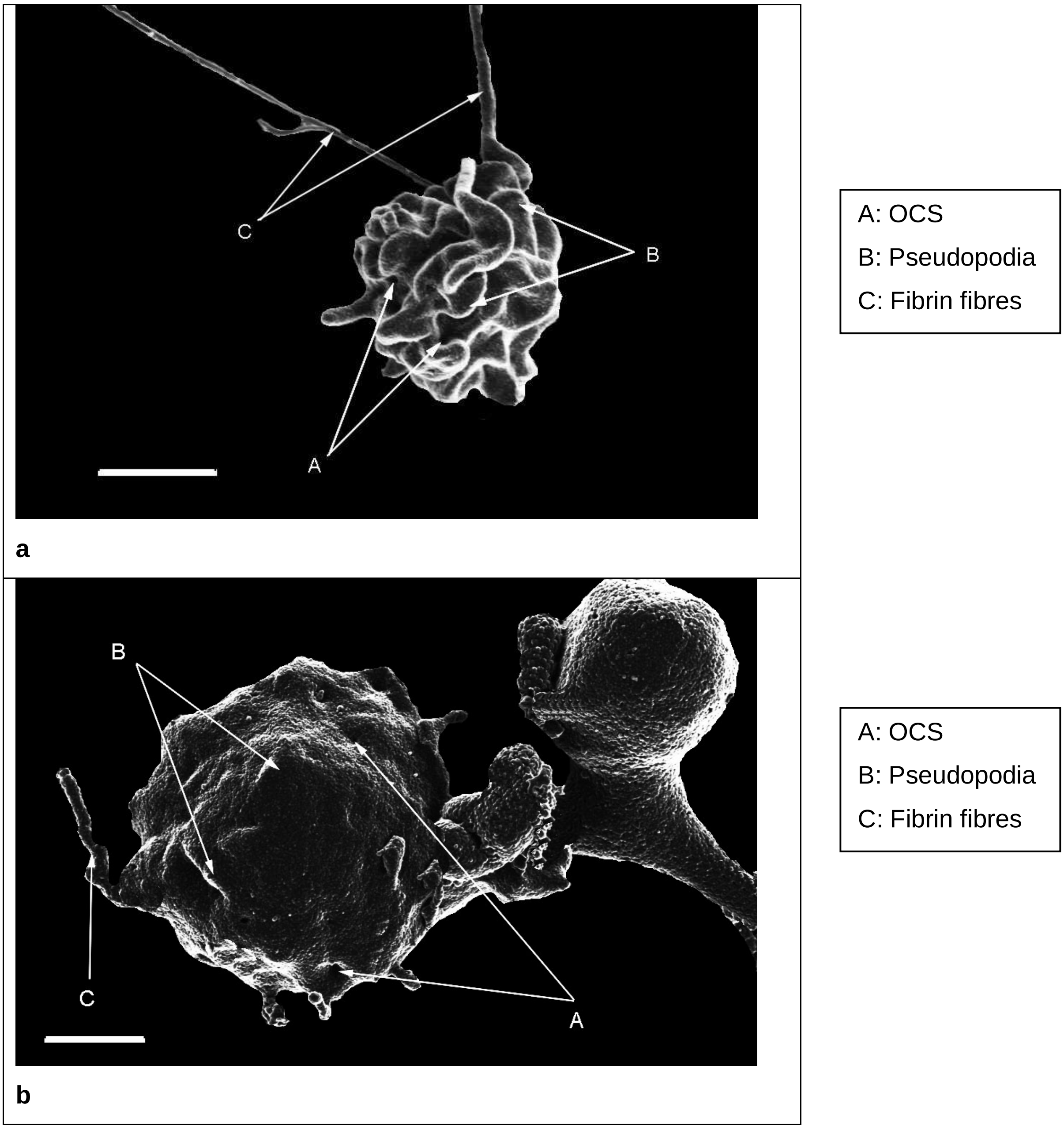
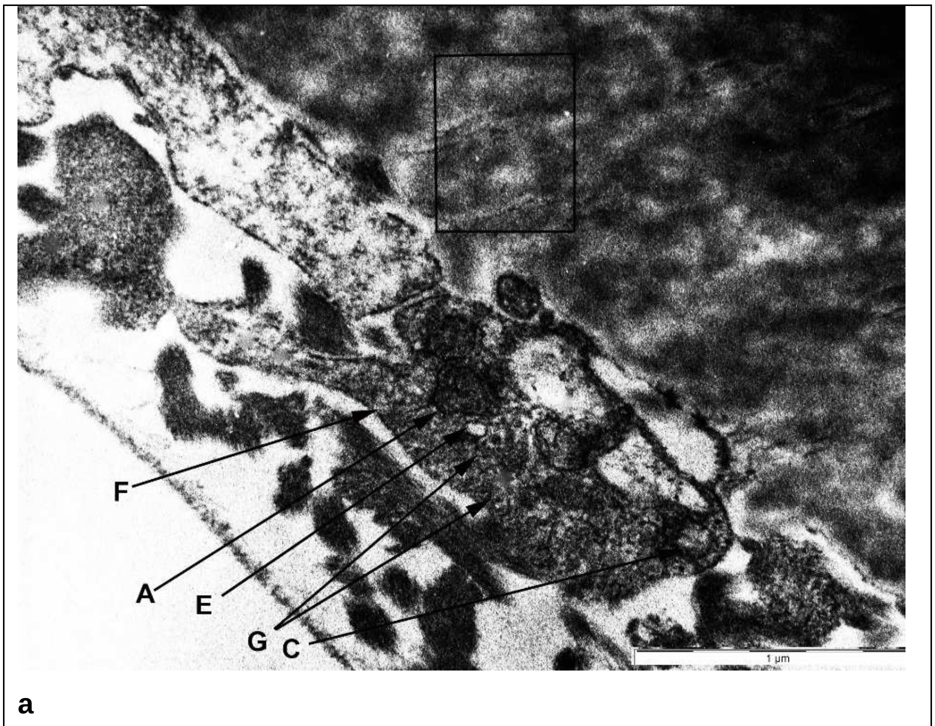


Figure 5.10: The Platelets of Individuals between 60 and 69 Years of Age
a: Platelet of a 62 Year Old Female. b: Thrombocyte of a 62 Year Old Female. Scale = 1µm.

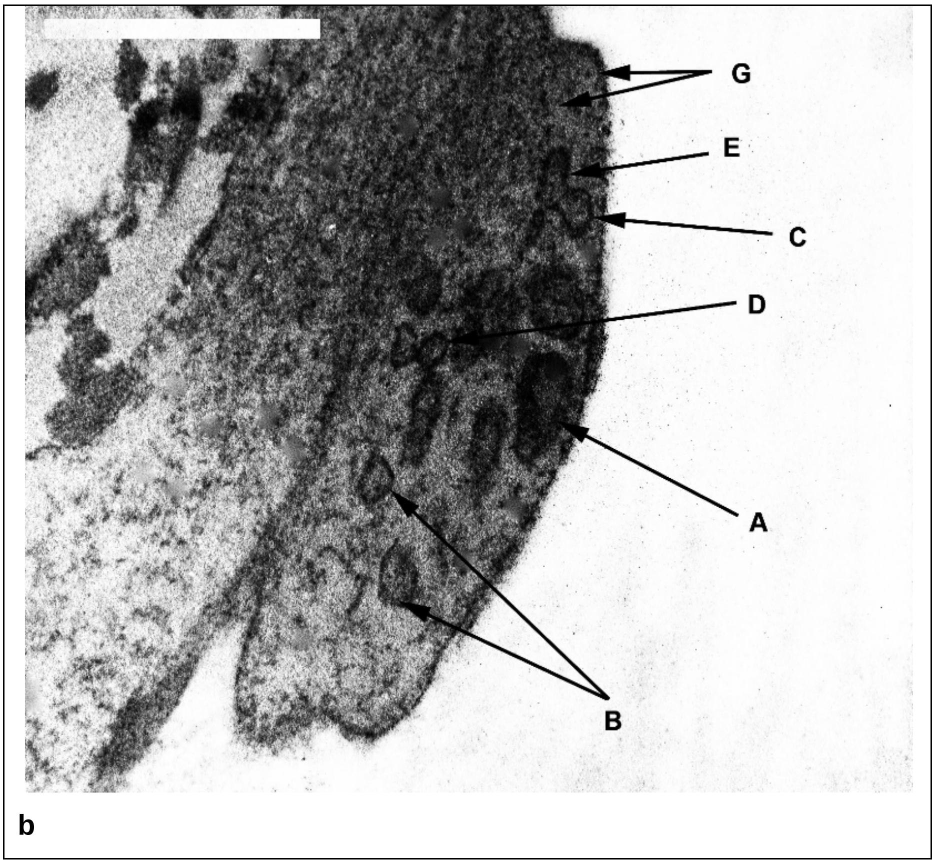
Figure 5.10 (both a and b) represents the thrombocytes of individuals between the ages of 60 and 69 years. OCS, pseudopodia and fibrin fibres are visible in both micrographs. One platelet in Figure 5.10b shows slight spreading.

5.3.6 Individuals Over 70 Years of Age

Figure 5.11a and Figure 5.11b indicates thrombocytes of individuals over the age of 70 years. Alpha granules with approximate sizes of 300 to 500 nm can be observed in Figure 5.11a together with OCS channels, dilated channels, microtubules and glycogen granules. Dense bodies and mitochondria appear absent. Figure 5.11b contains both alpha granules (300-500 nm in size), and dense bodies (approximately 250 nm in size) as well as OCS openings, mitochondria, dilated channels and glycogen granules. Microtubules appear absent in Figure 5.11b.



A: Alpha granules
C: OCS
E: Dilated Channels
F: Microtubules
G: Glycogen
Black rectangles indicate pores in the Millipore membrane
Scale = 1 µm



A: Alpha granules
B: Dense Bodies
C: OCS
D: Mitochondria
E: Dilated Channels
G: Glycogen
Scale = 1 µm

Figure 5.11: TEM Micrographs of Individuals Over 70 Years of Age
a: Platelet of a 79 year old female. b: Thrombocyte of a 77 year old female.

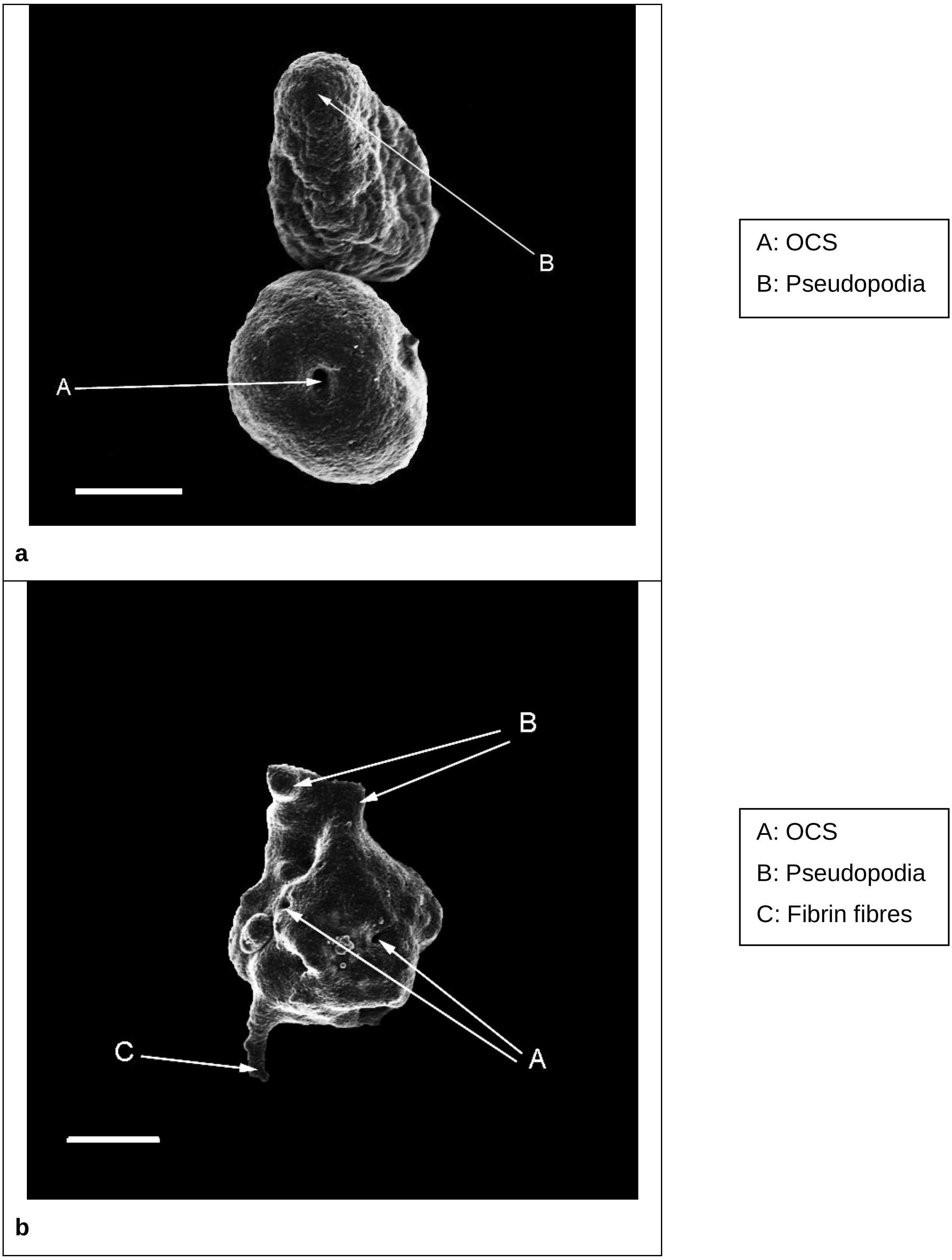


Figure 5.12: The Platelets of Individuals Over 70 Years of Age

a: Platelets of an 86 Year Old Female. b: Thrombocyte of a 77 Year Old Female. Scale = 1µm.

In Figures 5.12a and b platelets of individuals over the age of 70 is presented. OCS openings and pseudopodia are visible in both pictures. Fibrin fibres are absent in Figure 5.12a and only present as short little fibres in Figure 5.12b. The platelet membrane appears relatively smooth in Figure 5.12b, but slightly rough in Figure 5.12a.

5.4 Discussion

5.4.1 Transmission Electron Microscopy

TEM micrographs representative of the 36 individuals' thrombocytes are presented in Figure 5.1, 5.3, 5.5, 5.7, 5.9 and 5.11. It appears that the overall amount of granules within the granulomere of the thrombocytes remain constant until the age of 40. After the age of 40, there seems to be a decrease in the overall amount of granules. The platelets are all assumed to be activated due to the thrombin (necessary for activation) that is usually present in blood plasma and can be observed when studying platelet shape. All the platelet membranes appear intact.

The **alpha granules** (A) are present in the granulomere and facilitate platelet aggregation. The granules are present in all the micrographs, but appear to decrease with advancing age (from 40 years onwards). In Figure 5.1 the alpha granules are between 300 and 500 nm in size. In Figure 5.3 the granules are slightly smaller with a range of 275 to 400 nm. The size range appears to stay relatively constant in Figure 5.5 with a size range of 250 to 400 nm. In the 50 to 59 year age group the size range is decreased further to 200 to 300 nm. The size remains stable in the 60 to 69-years and then increases to a range of 250 to 400 nm in size. The alpha granules are relatively distinct in all the micrographs.

Dense bodies (B) are also found in the granulomere and were observed in all the figures except Figure 5.11a and were found to have a relatively constant size of approximately 200 nm. The dense bodies are distinct in all the age groups except in the participants between the ages of 30 and 39 years.

OCS (C) channels are found close to the cell surface. The channels are visible and clearly defined in at least one of the TEM micrographs in each age group. Even though OCS channels may not be present in all the micrographs of a specific age group, it can not be concluded that OCS are absent in all the platelets of that age group. The findings of this study therefore indicate the presence of OCS in all age groups. The OCS functions as an exit route through which substances can be excreted once the platelet is activated.

Mitochondria (D) serve their normal cellular function in platelets and are scattered in the granulomere. Mitochondria further contribute to the granular appearance of the thrombocyte. Mitochondria are visible in all figures except Figure 5.5b and Figure 5.11a where this structure could not clearly be identified. The presence of these organelles in the micrographs of the same age group however, indicates that there is not a complete absence in the entire age group. A complete absence would result in the platelet not being able to produce energy, and this is assumed to not be the case since the platelets are activated when thrombin is added to the plasma.

Dilated channels (E) are bean-shaped openings located within the granulomere. The channels are sometimes formed out of the OCS. Dilated channels are present in the majority of the micrographs but could not be identified in Figures 5.5a and 5.9b.

Microtubules (F) are arranged parallel to each other and form a clear region around the hyalomere. The microtubules are responsible for maintaining the structure of the platelet. Actin and myosin also associate with the microtubules (Gartner & Hiatt 2007). Microtubules could be seen in at least one of the micrographs in each age group.

Glycogen granules (G) are present in platelets and act as the energy source. The granules are arranged in clumps within the granulomere. Glycogen is present in all the micrographs.

5.4.2 Scanning Electron Microscopy

In Figure 5.2, 5.4, 5.6, 5.8, 5.10 and 5.12 the platelets studied with SEM are presented. The platelets in all the age groups are within the normal size range of two to three µm (Meyer *et al*. 2004). The platelets all appear activated since their shape is not discoidal as in the case of inert platelets, but rather spherical which is indicative of activation (Meyer *et al*. 2004). In all the micrographs OCS openings (A), pseudopodia (B) and fibrin fibres (C) were indicated.

The platelet membranes in healthy individuals are thought to be smooth (Pretorius & Oberholzer 2009). Relatively smooth membranes were observed in all the age groups. The texture of the membrane does not appear to change with advancing age. Pseudopodia are formed by the membrane and are visible in all the SEM micrographs. The pseudopodia are present in varying numbers, but no real difference is noted when comparing pseudopodia with an increase in age. Spherical-shaped platelets and other irregular shapes are indicative of thrombocyte activation (Meyer *et al*. 2004). Irregular shapes can be observed in all the SEM micrographs and the spherical shape is clearly illustrated in Figure 5.12a. There is no significant difference in the shape of the platelets with advancing age.

Slight spreading of platelets is usually indicative of hypercoagulability and can be seen in Figure 5.10b and Figure 5.12b. The spreading occurred in individuals in the age group of 60 to 69 years and individuals exceeding 70 years.

The OCS openings are equally distinct in all the age groups with the invaginations clearly visible. No clear distinction can be made between the younger and older individuals with regards to the OCS channels.

Fibrin fibres protruding from the platelet surface are visible in all the age groups in varying quantities. The variation is not necessarily due to a lower frequency of fibrin formation and it would therefore be more useful to study fibre thickness. Morphologically it would appear that the fibrin fibre thickness remains relatively constant with an increase in age up to the age of 60. From 60 onwards it appears that the fibrin fibres become thinner, which is a known characteristic of advancing age.

5.5 Conclusion

Although alpha granules, dense bodies, OCS channels, mitochondria, dilated channels, microtubules and glycogen were observed in all the age groups in different amounts and may not be clearly defined in all the micrographs, there is no actual difference with regards to age or sex when comparing the platelets of healthy individuals. The only difference that can be noted is a decrease in the overall amount of granules within the granulomere of the thrombocyte after the age of 40.

All the platelets were assumed to be activated since the platelets' shape are irregular rather than discoidal which is consistent with the literature (Meyer *et al*. 2004). The texture of the membranes remains relatively smooth with an increase in age. Pseudopodia are present but it is not influenced by age. The same is true for the OCS channels which remain constant with regard to morphology in advancing age. These factors do not indicate a clear alteration when comparing it with an advance in age.

Slight spreading of the platelet and fibrin fibre thickness is more indicative of age-related changes since the spreading occurs in older individuals and fibrin fibre thickness decreases with an advance in age. When looking at the different sexes in the specified age groups, no significant differences could be observed.

Chapter 6

Concluding Discussion

Several research questions regarding the ultrastructure and typical coagulation profile of normal individuals were formulated at the commencement of this study. After analysis of the results, it is suggested here that the research chapters adequately answer all the questions asked, and this will be discussed in detail in this chapter.

Studies of the coagulation cascade and the balance between the procoagulant and anticoagulant pathways have been performed for decades. The main function of the coagulation cascade is to stabilize the primary platelet plug that was formed after initial damage was detected by the endothelium (Meyer *et al*. 2004). It is also known that age-related changes occur with an increase in age and the changes influence various aspects of the coagulation process (Ng 2004; Scarpelini *et al*. 2009; Rivard *et al*. 2005; Roeloffzen *et al*. 2010). The exact age range at which age-related changes occur has, however, not been determined. It was also determined that several characteristics relating to the fibrin networks of individuals need to be present in order to be characterized as a healthy fibrin network. An important finding was that the characteristics vary with an advance in age.

As previously described by Nair and associates (1986), the fibrin networks consist of major (thick) fibres and minor (thin) fibres, also found by Pretorius & Oberholzer in 2009; however the presence of intermediate fibrin diameters were also detected in this study as indicated in Chapter 4 (Nair *et al*. 1986; Pretorius & Oberholzer 2009). The entire thrombus in this research dissertation could be observed as uniformly packaged and evenly distributed fibres which agree with the findings of Sugo and colleagues (2006) (Sugo *et al*. 2006).

The major fibres in all ages should have a regular branching pattern (Pretorius & Oberholzer 2009) and this was found to be true for this study as well.

The thin (minor) fibre quantity appears stable in all age groups with only the diameter of thick fibres that decrease with advancing age, especially after the age of 60. A decrease in diameter of 0.02 µm was observed over the 50 year period (between the age groups starting below 30 years of age and the participants exceeding 70 years of age). Below the age of 60, major, thick fibres appear to be the dominant fibre type with thin fibres arranged in between the thick fibres.

After the age of 50, the major fibres no longer exist as individual fibres only. In some areas the major fibres form fibre bundles consisting of a number of major fibres grouped and twisted together (Chapter 4). The thin, minor fibres form a “net-like” structure which partially covers the formed thrombus from the age of 30 onwards. After the age of 60 the “net-like” structure appears to be the predominant fibre arrangement, covering the largest part of the clot.

With regards to platelet morphology, certain characteristics should be present in order to indicate a normal, healthy platelet. By studying platelets using Transmission Electron Microscopy (TEM) a number of characteristics were observed as can be seen in Chapter 5. Two distinct regions can be observed in the platelet, the outer hyalomere and the inner granulomere (Pretorius *et al*. 2007). The granulomere contains granules that gives it the characteristic granular appearance and can be observed with TEM. The presence of alpha granules, dense bodies, Open Canalicular System (OCS) channels, mitochondria, dilated channels, microtubules and glycogen granules were found to be present in all age groups. The presence of theses structures is therefore indicative of a healthy individual.

The presence of the granules and other structures is consistent with the morphology of the platelets when viewed with Scanning Electron Microscopy (SEM). The surface morphology of the platelets were found to have irregular shapes rather than a discoidal appearance (Meyer *et al*. 2004; Freson *et al*. 2007) which is explained by the activation of the platelets. In all age groups the surface texture of the platelets appears relatively smooth as described by Pretorius & Oberholzer (2009) (Pretorius & Oberholzer 2009). A number of pseudopodia were found to project from the surface as indicated in Chapter 5. OCS openings can be seen as invaginations on the platelet membrane and could be identified in all the age groups. It can subsequently be concluded that all the structures described need to be present in order to be classified as a healthy platelet. The absence of any structures could be an indication of pathology, but can not be included in characteristics of age-related alterations. A number of fibrin fibres were also seen extending from the thrombocyte. The fibrin fibres appeared to decrease in diameter as the age of the volunteers increased, further illustrates the conclusions reached in Chapter 4.

As seen in the previous chapters a number of age-related changes were detected with regards to the coagulation profile, fibrin network and platelet morphology. A decrease in fibrin diameter with regard to age was noted when studying both the fibrin network and platelet SEM micrographs (Chapter 4 and Chapter 5). Fibre bundles were observed in individuals over the age of 50, and are most notable in the age group exceeding 70 years (Chapter 4). A “net-like” structure composed of minor thin fibres was observed in individuals over the age of 30 and increased until the age of 60 where it made up most of the formed thrombus. The amount of granules present in platelets, when using TEM, was found to decrease with an advance in age especially after the age of 40 (Chapter 5). Slight spreading was observed in platelets of individuals over the age of 60 (Chapter 4).

In this study age-related changes were also determined with Thromboelastography® (TEG®) and Fibrinogen Levels. Ng found in 2004 that age-related changes do exist and that the R-Time and K-Time decreases with increased age while the Maximum Amplitude and Alpha Angle increases. A number of age-related alterations could be observed with this method as discussed in Chapter 3. Fibrinogen is the major plasma protein essential in the completion of the final step of blood coagulation (Sugo *et al*. 2006; Wolberg & Campbell 2008). Fibrinogen levels have been found to increase with age (Tracy & Bovill 1992; Meade *et al*. 1977). In this chapter an increase was observed, as would be expected, but the increase was not found to be statistically significant (Chapter 3).

The TEG® Index, however, was found to increase with statistical significance with advancing age. The increase is illustrative of a hypercoagulable state in older individuals.

A number of TEG® variables were also studied:

- The Reaction Time, indicating the initial rate of fibrin formation (O'Shaugnessy *et al*. 2005) was found to decrease with age, but not with statistical significance
- The same is true for the K-Time, or coagulation time representing the time necessary for a clot to form (O'Shaugnessy *et al*. 2005)
- Both the Alpha Angle (indicating the speed at which the thrombus is formed (O'Shaugnessy *et al*. 2005))
- The Maximum Amplitude (indicative of the strength of the formed fibrin clot (O'Shaugnessy *et al*. 2005)) was found to increase with age, but not with statistical significance

The specific age ranges at which the alterations due to age occur were determined. With regards to fibrin network changes a systematic increase in coagulability potential was noted with the most marked alterations after the age of 60 (Chapter 4). Although, some changes could be observed from the age of 50 onwards with the addition of fibre bundles and the appearance of "sticky" fibres. The "net-like" structures were observable from the age of 30 onwards, but were most notable after the age of 60. TEG® variables and fibrinogen levels were found to increase with age, but were most notably different when comparing the older individuals with participants under the age of 30 years.

Hume (1961) found differences between males and females in younger individuals, but found that the differences are absent in older individuals (Hume 1961). Tracy & Bovill (1992) and Wilkerson & Sane (2002) found no differences between males and females (Tracy & Bovill 1992; Wilkerson and Sane 2002). Gorton and co-workers (2000), Scarpelini and colleagues (2009) and Roeloffzen and associates (2010) found that healthy non-pregnant females are more hypercoagulable than men; however, they did not exclude oral contraceptives and other procoagulant factors (Gorton *et al*. 2000; Scarpelini *et al*. 2009; Roeloffzen *et al*. 2010). The influence of the sex of the participants was determined by means of TEG® and Fibrinogen Levels (Chapter 3), as well as platelet studies (Chapter 5) no differences between males and females could be observed. The study of the ultrastructure of the fibrin networks, however, indicated differences that could be linked to the sex of the volunteers (Chapter 4). The difference was absent below the age of 30 years, but a notable difference set in after the age of 30. As age increased, the differences between males and females became more apparent up to the age of 60. After 60 years of age, however, sex-related differences disappeared and the fibrin networks appeared similar in the older individuals.

The results obtained in this research dissertation can be used in the establishment of a normal, healthy database of individuals of different ages with which to compare the coagulation potential (including coagulation profiles, fibrin networks and platelets) of other individuals of varying ages.

The conclusions reached in this study could contribute in future to further comparative studies of coagulopathies, since this study was conducted on control individuals, considered healthy. The results could possibly be used to determine whether alterations are due to normal aging- or sex-related differences or if the changes can be contributed to the coagulopathies being studied. Since coagulation related pathologies are occurring more frequently, this dissertation could aid a rapid, accurate diagnosis when possible patients are encountered.

Chapter 7

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