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ULTRASTRUCTURAL ANALYSIS OF PLATELETS AND FIBRIN NETWORKS IN STROKE PATIENTS

by

ALBE CARINA DE LANGE

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Supervisor: Prof. E. Pretorius

Department: Anatomy

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Abstract

Ischaemic stroke represent more than 80% of the total stroke instances. The location of the occlusion and the amount of brain tissue involved determines the effect of the stroke. Stroke can result in paralysis, memory loss, speech impairment and even a “lock-in” state. The amount of neuronal damage will determine whether these symptoms will be temporary or permanent.

Stroke is deemed the second leading cause of death for individuals over the age of 60. According to the World Stroke Organization (WSO) every six seconds stroke claims a life, regardless of age or gender.

Stroke is a global burden and the medical costs and disability related to stroke in America for 2010 was projected at almost $73.7 billion.

The morphology of platelets, fibrin networks and erythrocytes as well as the differential white blood cell counts of 20 thrombo-embolic ischaemic stroke patients were investigated.

Internal and external alterations were revealed in the platelets of stroke patients when compared to healthy controls. The decreased numbers of alpha granules in the platelets of the

stroke patients indicated these platelets to be activated. Substances released by activated platelets promote fibrin network structure, specifically the formation of fibrin strands and accumulation of additional platelets.

The fibrin network of healthy individuals consists of major, thick fibers with minor, thin fibers distributed between them. The fibrin network of stroke patients exhibited an abnormally layered and matted ultrastructure comprising of mainly thin, minor fibrin fibers packed closely together. An uncharacteristic circular morphology was also observed. These alterations in the fibrin network indicate the activated platelets to be actively involved in the thrombotic event. Neuronal damage related to stroke is also advanced by the vasoactive substances released by activated platelets. It can therefore be deduced that the morphology of the fibrin network is altered long before the concrete thrombotic event transpire.

Large numbers of abnormal erythrocytes were distinguished in the blood of stroke patients. Among these abnormal forms of erythrocytes specifically codocytes, knizocytes, stomatocytes and echinocytes were identified. Abnormal erythrocyte forms were significantly increased in hypertensive patients and females independently. Alterations in the ultrastructure of erythrocytes disturb blood flow in the microcirculation and could possibly augment the ischaemic event.

Inflammation is closely related to ischaemic stroke. An increased monocyte count and a reduced number of neutrophils were a significant feature among all the stroke patients of this study. Patients with hypertension as well as patients consuming aspirin on a daily basis showed the greatest influence on the observed differential white blood cell counts.

These morphological alterations observed in the platelets, fibrin network and erythrocytes as well as the differential white blood cell count could be incorporated in an analysis regime that could probably indicate an impending thrombotic event. Therefore treatment could be initiated before the ischaemic event to possibly prevent the stroke.

For future studies a larger study population, a more refined patient enrolment as well as the analysis of follow-up blood samples from patients could substantiate the above-mentioned findings and provide additional information concerning the thrombotic event and the effectiveness of treatment procedures.

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Declaration

I, Albe Carina de Lange, hereby declare that this research dissertation is my own work and has not been presented for any degree at another University

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List of Tables and Figures

Abbreviations, Symbols and Chemical Formulae

%	Percentage
α-granules	Alpha granules
β-TG	β-thromboglobulin
δ-granules	Delta-granules/ Dense bodies/Dense core granules
λ-granules	Lysosomal granules
µl	Microliter
µm	Micrometer
11-dehydro-TXB2	Thromboxane B2
ADP	Adenosine diphosphate
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
BALB/c	Asthmatic mouse model
Bβ1-42	Cleavage product produced by plasmin
CNS	Central nervous system
CRP	C-reactive protein
D-Dimer	Coagulation fragments indicating thrombin and plasmin activity
Dr.	Doctor

DTS	Dense tubular system
e.g.	Exempli gratia (for example)
ELISA	Enzyme linked immunosorbent assay
ESR	Erythrocyte sedimentation rate
et al.	Et alia (and other)
Factor V, VIII, XI, XIII, VII, XII, XIII	Coagulation factors involved in the coagulation cascade
FEG SEM	Field emission gun scanning electron microscope
FPA	Fibrinopeptide A, cleavage products produced by thrombin
GPIIb-IIIa ($\alpha_{IIb}\beta_3$)	Glycoprotein IIb/IIIa, alpha granule membrane protein
HDLC	High density lipoprotein cholesterol
HIV/AIDS	Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome
HRT	Hormone replacement therapy
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
ISGS	Ischaemic Stroke Genetics Study
LDLC	Low density lipoprotein cholesterol
LM	Light microscope

NCSS	Statistical analysis and graphic software
nm	Nanometer
OCS	Open canalicular system
OsO_4	Osmium tetraoxide
PBS	Phosphate buffered saline
PF-4	Platelet factor 4
PI	Prothrombin index
PMNL	Polymorphonuclear leukocyte
PNS	Peripheral nervous system
PRP	Platelet rich plasma
PTT	Partial Thromboplastin Time
RCC	Renal clear cell adenocarcinoma
SCCS	Surface-connected canalicular system
S-Chol (total)	Total serum cholesterol
SEM	Scanning electron microscope
S-HDLC	Serum high density lipoprotein cholesterol
S-LDLC	Serum low density lipoprotein cholesterol
TEM	Transmission electron microscope

TF	Tissue factor
TIA	Transient ischaemic attack
TNF-α	Tumour necrosis factors alpha
TNF-β	Tumour necrosis factors beta
tPA	Tissue plasminogen activator
TSP	Thrombospondin
t-test	Statistical hypothesis test
TX	Thromboxane
TXA_2	Thromboxane A_2
U/ml	Units per milliliter
vWF	von Willebrand factor
WBC	White blood cell
WSO	World Stroke Organization

CHAPTER 1: INTRODUCTION

Stroke, also referred to as cerebrovascular diseases, is the result of oxygen and nutrient deprivation brought on by blood vessel occlusion or hemorrhage. Neuronal damage is permanent since the affected nerve tissue can not be regenerated. Untreated stroke can lead to the death of millions of neurons. (Stroke, 2011)

Ischaemic stroke represent more than 80% of the total stroke instances. Stroke accounts for roughly 80% of all deaths related to embolism, while the remaining 20% is accredited to other systemic thrombo-embolisms. (Menke et al., 2010) The location of the occlusion and the amount of brain tissue involved determines the effect of the stroke. Paralysis, vision problems, memory loss, speech impairment and even a "lock-in" state where the individual loses the ability to move or speak, are some of the effects of stroke. These symptoms can be temporary or permanent depending on the amount of neuronal damage. (Effects of stroke, 2011)

According to the World Stroke Organization (WSO) every six seconds stroke claims a life, regardless of the gender or age of the individual. (World Stroke Campaign, 2011) The WSO rates stroke as the second leading cause of death for individuals over the age of 60 and the fifth leading cause in individuals between the ages of 15 and 59. It is estimated that almost six million people die from stroke each year, more than the deaths accredited to AIDS, malaria and tuberculosis combined. (World Stroke Campaign, 2011) Stroke is a global burden and the medical costs and disability related to stroke in America were estimated at approximately $73.7 billion for 2010. (Impact of Stroke, 2011) Since stroke has such a detrimental effect on the health as well as economic state, it is important to investigate the possible morphological alterations associated with the thrombotic event.

Various factors can increase the risk of suffering a stroke. Some of these risk factors include gender, age, hypertension, smoking, and hormone replacement therapy. Since there exist uncertainty pertaining to the influence of gender on the risk of stroke, this study will investigate the difference between males and females. Other risk factors of stroke will also be kept in account in the investigations of this study.

Platelets are the most important cellular component in haemostasis since they release constituents responsible for thrombus formation in the event of blood vessel hemorrhage. (Marcucci et al., 2008) The thrombus or gelatinous clot formed consists of a network of fibrin

fibers classified as either thick, major fibers or thin, minor fibers. These fibers form the scaffold important for vessel repair.(Silverthorn, 2007)

Platelet activation can be stimulated as well as be inhibited by leukocytes. (Salvemini et al., 1989; Selak et al., 1988) Since inflammation contributes to cerebral ischaemia, inflammation is considered a trait of the pathophysiology of ischaemic stroke (Emsley and Tyrrell, 2002). Elevated total peripheral white blood cell count is related to poor outcome after stroke (Azzimondi et al., 1995; Chamorro et al., 1995; Di Napoli et al., 2002; Muir et al., 1999; Vila et al., 1999). Leukocytes, as well as erythrocytes, are integrated in platelet aggregates form in whole blood. (Joseph et al., 1989a) Erythrocytes are the biconcave blood cells responsible for gaseous exchange in tissue. It is their specific disk-like shape that enables these cells to effective deliver oxygen to the tissues. (Howard and Hamilton, 1999) The specialized cell membrane of erythrocyte can be influenced by byproducts of activated leukocyte. This can lead to total hemolysis since these products initiate oxidation of haemoglobin and peroxidation of lipids. (Santos-Silva et al., 2002)

The investigation of ultrastructural changes in the platelets, fibrin networks, erythrocytes and also the variations in differential white blood cell counts of stroke patients compared to healthy control individuals will give insight into the possible mechanisms related to stroke and may also indicate specific focus points for treatment.

CHAPTER 2: LITERATURE REVIEW

2.1 CHAPTER OBJECTIVES

In this chapter, the literature will be reviewed for previous research pertaining to the current study.

2.2 INTRODUCTION

In the current thesis the ultrastructure of platelets and fibrin networks as well as the morphology of erythrocytes and leukocytes of stroke patients will be investigated by using scanning and transmission electron microscopy. The total white blood cell count of each patient will also be investigated. This literature review therefore includes a section on the risk factors and prevalence of stroke as well as the ultrastructure of thrombi and the coagulation abnormalities involved in this condition. Furthermore, the role of platelets in thrombosis will be discussed as well as the role of a number of cells involved in the inflammatory process of this disease. This literature review includes a section on previous research done on the ultrastructure of platelets and fibrin networks in different disease conditions.

2.3 WHAT IS STROKE?

In the Steadman's Medical Dictionary, stroke is defined as "any acute clinical event, related to impairment of cerebral circulation that lasts longer than 24 hours. It is also referred to as apoplexy or a brain attack." (Stedman's Medical Dictionary 2006, p. 1849)

It is further explained as an acute neurologic deficit resulting from circulatory impairment that resolves within 24 hours. This type of stroke is called a transient ischaemic attack (TIA) and most TIAs last only 15 – 20 minutes. Stroke causes irreversible brain damage and the type of stroke and the severity of the symptoms are dependant on the location and extent of brain tissue whose circulation has been compromised. The result of a stroke also varies between minimal impairment and rapid onset of coma, and this can be followed by death. Some of the high risk factors of stroke include hypertension, cigarette smoking and estrogen replacement therapy. (Stedman's Medical Dictionary 2006, p. 1849)

Corresponding to the underlying cause, two categories exist for stroke. These include ischaemic stroke and haemorrhagic stroke. (Grotta et al., 2001)

Ischaemic stroke is generally caused by atherothrombosis or embolism of a major cerebral artery and accounts for about 85% of all strokes (Stedman's Medical Dictionary 2006, p. 1849). Mostly it is the middle cerebral artery and its branches that are affected (Durukan and Tatlisumak, 2007).

Nonatheromatous vascular disease and coagulation disorders are two of the less common causes of ischaemic stroke. Severe, acute ischaemia in nerve tissue triggers cellular changes (calcium influx, protease activation) that can swiftly cause irreversible damage (infarction). Around the infarct zone lies a so-called penumbra of ischaemic, electrically silent tissue that may be salvageable by prompt perfusion. The mortality of ischaemic stroke is 15-30% within the first 30 days. (Stedman's Medical Dictionary 2006, p. 1849)

Hemorrhagic stroke occurs when a weakened vessel in the brain severs and blood accumulates into the surrounding tissue causing compression on the brain tissue. As the brain is deprived of blood and oxygen, it will result in a stroke. Two types of hemorrhagic stroke can be identified, according to the location of the hemorrhage, namely intracerebral hemorrhagic stroke and subarachnoid hemorrhagic stroke. (American Heart Association, 2009)

Hemorrhagic stroke, which accounts for the remaining 15% of stroke cases, has a grave prognosis, with a 30-day mortality rate of 40-80%. Steadman's Medical Dictionary states that 30% of ischaemic infarcts, including most of those with severe impairment of cerebral blood flow and extensive tissue death, eventually develop a hemorrhagic component. (Stedman's Medical Dictionary 2006, p. 1849)

2.4 RISK FACTORS

2.4.1 Age, gender and race

Variation in stroke mortality due to age, sex or race differences has been established by several population-based studies. (Gaines and Burke, 1995; Gillum, 1999; Holroyd-Leduc et al., 2000; Modan and Wagener, 1992; Morgenstern et al., 1997; Skeikh et al., 1981)

According to the National Centre for Health Statistics, the age-adjusted stroke mortality rate among nonwhites was nearly twofold that of the rate among whites (National Centre for Health Statistics, 1998). This ethnic difference has been confirmed for nearly all types of stroke (Ayala et al., 2001; Bian et al., 2003; Bravata et al., 2003; Gillum, 1999; Johnston et al., 1998; Morgenstern et al., 1997).

In this study, the focus will specifically rest on the platelet and fibrin ultrastructural differences between male and female patients.

2.4.2 Gender

Some studies have reported that women differ from men with regards to stroke outcomes (Niewada et al., 2005), subtypes of ischaemic stroke (Ayala et al., 2002), as well as their reaction to treatment with tissue plasminogen activator (tPA) (Kent and Hill, 2005).

According to Carandang et al. 2006, the incidence of age-adjusted stroke in women is generally much lower than in men, similar to age-adjusted mortality (Carandang et al., 2006). Other studies have found that mortality after a stroke and the rate of disability, depression and dementia is also higher amongst women than men. (Kapral et al., 2005; Niewada et al., 2005)

However, some studies did not find a noteworthy difference in stroke mortality pertaining to sex or race. (Brown et al., 1996; Di Carlo et al., 2003; Howard et al., 1994; Modan and Wagener, 1992; Sacco et al., 1991)

In 2007, Leslie-Mazwi and co-workers investigated the gender differences in stroke evaluation. They used data from the Ischaemic Stroke Genetics Study (ISGS). They consequently concluded that the diagnoses of ischaemic stroke subtypes could in all probability be analogous among men and women. (Leslie-Mazwi et al., 2007)

A recent study has implicated no gender differences in incident ischaemic stroke concerning the severity of the stroke, subtype of stroke, or the size and location of the infarct. Although weakness was more prominent among women, other traditional stroke symptoms presented to a similar extent in both men and women. (Barrett et al., 2007)

Andersen et al. found stroke to be just as severe in men as in women. They also established that the short-term survival is also the same for both sexes. However, having survived stroke, women live longer than men since they have a decreased possibility of developing successive cardiovascular events. (Andersen et al., 2005)

Gillum amongst others have also indicated a relatively higher risk of mortality among men (Bravata et al., 2003; Gillum, 1999; Holroyd-Leduc et al., 2000).

In a study done by Jiang et al. the authors reviewed gender and race differences in stroke mortality. They found no distinction among gender in hemorrhagic stroke case fatality. On

the other hand, male and female patients with ischaemic, non-specific, and all types of stroke combined, exhibited small differences in case fatality. They established that in cases where all types of stroke are combined, mortality is higher in men than women. (Jiang et al., 2006)

It can therefore be concluded that there is uncertainty pertaining to the effect of gender on not only the outcome and mortality of stroke, but also the ultrastructural components which will be investigated later on in this thesis.

2.5 PREVALENCE OF STROKE

One of the leading causes of mortality and morbidity in the world is stroke (Writing group: Lloyd-Jones et al., 2009), since stroke is responsible for 5% to 10% of all deaths in the Western world (Williams et al., 2003). According to Seshadri et al. approximately 1 out of 6 people will in their lifetime suffer from no less than one stroke (Seshadri et al., 2006).

What is more, it is estimated that more than 30% of patients who live through 6 months will lose their independence, making this disease the foremost cause of adult disability in the Western world (Warlow, 1998; Writing group: Lloyd-Jones et al., 2009).

In 2007, Durukan and Tatlisumak stated that the assumption can be made that stroke will continue, as it already is, to be the most challenging disease. They based this statement firstly on the fact that life expectancy is increasing globally, and secondly the enormous socioeconomic burden stroke has since it consumes as much as 6% of all health care budgets. (Durukan and Tatlisumak, 2007)

The causes, duration, localization and severity of ischaemia of stroke differ between patients and this raises the need for a large sample size when conducting clinical research, thereby avoiding the difficult effects of the diversity (Durukan and Tatlisumak, 2007).

2.6 PATHOPHYSIOLOGY OF STROKE

In 2004, Brey and Coull wrote a book on the pathophysiology, diagnosis and management of stroke in which the authors also addressed coagulation abnormalities in stroke. The authors explained that the coagulation system is responsible for maintaining blood flow in the vessels while ensuring vessel integrity – repairing fissures to prevent seepage from the blood vessel – under normal circumstances. However, if this balance is disturbed it can induce thrombosis. In more or less all cases of ischaemic stroke, initiation of blood coagulation leading to thrombosis is a prerequisite. Thrombus formation usually results from rapid

initiation of haemostasis. This pathological stagnation of blood (Steadman's pg873) occurs when there is injury to the endothelium within an atherosclerotic precerebral artery or within the heart. Hemorrhagic stroke is related to several distinctive defects in haemostasis while the majority of the coagulation disorders that lead to ischaemic stroke are still not well described. A predisposition to thrombosis – called a prothrombotic or hypercoagulable state – may come to pass after blood coagulation is activated, when platelet reactivity is increased or even when fibrinolysis is impaired. (Brey and Coull, 2004)

2.7 STROKE AND IMMUNOLOGY

Inflammation of the central nervous system (CNS) and periphery is not only a significant trait of the pathophysiological response to ischaemic stroke (Emsley and Tyrrell, 2002), but it most probably initiates the development of cerebral ischaemia (Rost et al., 2001).

Ischaemic stroke is associated with some peripheral markers of the response of inflammation with ischaemic stroke, including C-reactive protein (CRP) (Muir et al., 1999; Vila et al., 1999), erythrocyte sedimentation rate (ESR) (Chamorro et al., 1995; Vila et al., 1999), total peripheral white blood cell (WBC) count (Pozzilli et al., 1985), peripheral neutrophil count (Vila et al., 1999) and body temperature (Boysen and Christensen, 2001). The acute phase response of inflammation and the increase of the above mentioned peripheral markers seem to predict poor outcome after stroke (Azzimondi et al., 1995; Chamorro et al., 1995; Di Napoli et al., 2002; Muir et al., 1999; Vila et al., 1999).

The CNS and peripheral responses to stroke have additionally been associated with cytokines like interleukin-6 (IL-6) (Kim et al., 1996; Loddick et al., 1998). In acute stroke patients elevated concentrations of IL-6 have been documented, not only in circulation but also in cerebrospinal fluid. (Beamer et al., 1995; Fassbender et al., 1997; Ferrarese et al., 1999; Kim et al., 1996; Tarkowski et al., 1995; Vila et al., 2000)

Hedley and colleagues did a study on an early and sustained peripheral inflammatory response in acute ischaemic stroke and its relationships with infection and atherosclerosis. They were the first to report the sequential modification of peripheral inflammatory markers in patients with acute ischaemic stroke. They also found that the peripheral markers of inflammation, namely plasma C-reactive protein (CRP), IL-6 and cortisol concentrations, erythrocyte sedimentation rate (ESR), and total white blood cell (WBC) count was elevated early after stroke and sustained for at least 3 months. They offered two possible explanations for this phenomenon. They reasoned that it is either the stroke itself that induces an

inflammatory response which is sustained for a long time and possibly causes further cardiovascular events, or it is a pre-existing inflammatory condition that contributes to the development of stroke. (Emsley et al., 2003)

The likelihood of developing ischaemic stroke is augmented by conditions related to the activation of the immune system, such as systemic infection (Grau et al., 1995).

2.7.1 White blood cells

Patients suffering from ischaemic stroke have been described as having increased peripheral white blood cell counts, even up to three days after onset (D'Erasmo et al., 1991; Pozzilli et al., 1985). In 2003 Emsley et al. reported peaks in peripheral total WBC and neutrophil counts in patients with ischaemic stroke (Emsley et al., 2003). A pattern has been observed concerning the infiltration of WBC to the area affected by ischaemic stroke. Initially neutrophils will accumulate around the core of the infarct (Adams and Sidman, 1968) for up to 24 hours (Akopov et al., 1996), after which they are replaced by mononuclear cells (Adams and Sidman, 1968), accounting for the elevated monocyte count at day 5-7 after the stroke (Emsley et al., 2003). In patients with acute stroke, the cerebrospinal fluid samples have shown a similar outline, where neutrophil outflow reaches its peak on day 4, followed by an increase in macrophages and monocytes until it reaches its' maximum around a week after the stroke (Sörnäs et al., 1972).

The total WBC count is elevated in patients who suffered an ischaemic stroke. Santos-Silva and colleagues found the increase in neutrophils after an ischaemic stroke to be nearly two-fold the amount of neutrophils found in the control group. The assessment of elastase and lactoferrin obtained from the neutrophils of ischaemic stroke patients showed an obvious increase in the activation of leukocytes, over double that of the healthy control group. Thus not only the number of leukocytes increased in patients who suffered an ischaemic stroke, but these leukocytes were also activated. (Santos-Silva et al., 2002)

In brain ischaemia, white blood cells can be damaging in a number of ways. Monocytes express thromboplastin, a substance required for clot formation (Stedman's Medical Dictionary 2006, p. 1985), into the blood (Rivers et al., 1975). In patients with stroke, granulocytes do not only add to the formation of a thrombus but can cause the accumulation of thrombi (Galante et al., 1992).

2.7.1.1 Cytokines

Various cytokines, which include interleukin-1 (IL-1) and tumour necrosis factors alpha (TNF-α) and beta (TNF-β), are expressed by lymphocytes and macrophages. The nervous system can be damaged by these cytokines (Morganti-Kossmann et al., 1992).

Several studies have indicated that the circulating IL-6 concentration in patients with stroke increase considerably for up to 14 days (Beamer et al., 1995; Kim et al., 1996; Perini et al., 2001). Emsley et al. found the there was significant increase for the first 24 hours and after which the IL-6 concentration continued on the plateau reached for almost a week. What interested them was the fact that patients who didn't have any infection exhibited no elevation in the concentration of IL-6. (Emsley et al., 2003)

2.7.1.2 Monocytes

Activated monocytes could play a part in the initial pathogenesis of ischaemic stroke, in addition to being a pathophysiological factor of the subactute stage after a stroke (Kochanek and Hallenbeck, 1992b). In 2001 Grau et al. studied the function of monocytes and the plasma levels of interleukin-8 (IL-8) in acute ischaemic stroke. Grau and his co-workers tested the hypothesis that in acute ischaemic stroke there will be an increase in not only the procoagulant activity of monocytes, but also the products released from monocytes. They measured plasma levels of IL-8 and neopterin by enzyme linked immunosorbent assay (ELISA) in patients 1, 3 and 7 days after ischaemic stroke. They had a group of healthy individuals of the same age and sex, the control group, who were tested on the same days. The release of superoxidanion, activity of procoagulants and transcription of the tissue factor (TF) gene by monocytes were explored.

Their results showed that even though not all activation parameters were elevated, the activity of monocytes increased after ischaemic stroke. However, they could not confirm that tissue factor (TF) was expressed by the monocytes in circulation, or that the activity of procoagulant released by monocytes was elevated. The activation of polymorphonuclear leukocyte (PMNL) by the increased levels of IL-8 early after ischaemia, could possibly contribute to the pathophysiology of stroke. (Grau et al., 2001)

2.7.1.3 Platelets and Leukocytes

Arterial thrombi are often composed of considerable numbers of leukocytes (Henry, 1965). The formation as well as the dissolution of thrombi could most likely be influenced by leukocytes (Silbergleit, 1970). Grau and his colleagues discovered that in the early stages after ischaemic stroke, mononuclear leukocytes have the ability to inhibit the aggregation of platelets. This trait could possibly have antithrombotic consequences. (Grau et al., 1994)

Leukocytes can thus effectively stimulate (Selak et al., 1988) and also inhibit (Salvemini et al., 1989) platelet activation. Leukocytes may therefore play a significant role in managing the ability of platelets to aggregate (Grau et al., 1994). Along with erythrocytes, leukocytes are integrated in the platelet aggregates formed in whole blood (Joseph et al., 1989b).

Studies done by Cha et al. and Marquardt et al. independently, have confirmed that increased platelet activation markers are present during acute ischaemic stroke and even in the chronic phase (Cha et al., 2003; Marquardt et al., 2002). Likewise, McCabe et al in 2004 and Htun in 2006 stated that patients with acute ischaemic stroke have high levels of interaction between platelets and leukocytes, among platelet-monocyte and platelet-neutrophil aggregation in particular. (Htun et al., 2006; McCabe et al., 2004)

2.7.1.4 White blood cell count

An increased white blood cell count is related to the incidence of cardiovascular disease and is considered to be an indicator of an inflammatory response. In 2005, Koren-Morag and his co-workers investigated the relationship between the incidence of ischaemic cerebrovascular disease and white blood cell count. Patients of which the lipid boundaries were demarcated and suffered from pre-existing atherothrombotic disease where employed for the study. Their findings confirmed that elevated white blood cell counts are related to an increased threat for ischaemic cerebrovascular disease. (Koren-Morag et al., 2005)

Inflammation is implicated in the pathogenesis of atherosclerosis and ischaemic stroke. Cardiovascular disease has in numerous studies been connected to a high leukocyte count, which is a marker of inflammation and infection. (Koren-Morag et al., 2005)

Koren-Morag and his associates confirmed the statement that elevated white blood cell count is linked with a greater possibility of suffering ischaemic cerebrovascular disease, independent of other conventional risk factors for stroke like age, sex, prior myocardial

infarction, hypertension, diabetes mellitus, current smoking, peripheral vascular disease or blood lipids. An additional indicator of an increased risk of ischaemic cerebrovascular disease is plasma fibrinogen. It is also a notable marker of inflammation. In the same study, Koren-Morag et al. also found that the plasma fibrinogen diminished the association of elevated white blood cell count with ischaemic cerebrovascular disease but did not eliminate it completely. (Koren-Morag et al., 2005)

A high number of leukocytes might probably increase the possibility of the rupture of an atheromatic plaque. Acute thrombosis can result from this or even chronic atherosclerosis, which can initiate ischaemic cerebrovascular disease. Thus an increased number of leukocytes can set ischaemic cerebrovascular disease in motion. (Grau et al., 1996)

Results obtained from *in vitro* as well as animal studies have implicated leukocyte count as a valuable marker for the initial formation of an atheroma (Ross, 1999). Brain damage in hypertensive intracerebral hemorrhage is directly proportional to the number of leukocytes present in peripheral blood (Suzuki et al., 1995). A greater danger of developing stroke is linked to an elevated, or even normal, leukocyte count. (Ernst et al., 1987)

According to Harrison and Marshall the leukocyte count of patients who recently suffered from a transient ischaemic attack (TIA), are directly proportional to their chance of having another transient ischaemic attack or even stroke (Harrison and Marshall, 1987).

Balestrino and his colleagues did a study in 1998 on the effect of white blood cell count and erythrocyte sedimentation rate on the outcome of patients with acute ischaemic stroke. They found that an increased white blood cell count is not a consequence of stroke, as the greater part of patients had the normal amount of white blood cells. They did find elevated erythrocyte sedimentation rate in more than half of the patients, and that the ESR is associated with the size of lesions. They concluded that it is not ischaemic stroke that is responsible for the elevated WBC count and ESR, but rather an infection that brings about the procoagulant condition in patients with stroke. (Balestrino et al., 1998)

We can therefore assume that there is a greater risk for patients with elevated numbers of white blood cells in their blood to suffer from these harmful consequences. (Balestrino et al., 1998)

2.7.2 Erythrocytes

Red blood cells can be altered by oxygen metabolites and proteases released by activated white blood cells (WBC). These alterations may be oxidative or proteolytic in nature. (Santos-Silva et al., 2002)

The erythrocyte membrane can be changed by leukocyte activation products. When there is an alteration on its membrane, this will also change the antigenicity of the erythrocyte. These by-products from the activated leukocytes are also able to trigger the oxidation of haemoglobin and peroxidation of lipids. The ultimate consequence of all these alterations is hemolysis of the red blood cell membrane. (Santos-Silva et al., 2002)

The ability of erythrocytes to biosynthesise is very restricted. It also has a feeble repair system, thus whenever it experiences stress, either physical and/or chemical, it will obtain physical damage or even molecular damage. (Santos-Silva et al., 2002)

As the WBC numbers increase in ischaemic stroke patients, so will the activation products released from the activated leukocytes. Adjacent cells, including RBC, can be affected by these activation products. And since they are almost unable to bend, they most likely accumulate in narrow blood vessels in circulation. This may count for an extended contact period between these accumulated activation product and the neighbouring cell because of the slow-moving blood in the vessel. (Santos-Silva et al., 2002)

An elevated rate of erythrocyte sedimentation, which is closely related to systemic inflammation or infection, is implicated in the critical damage caused by stroke (Chamorro et al., 1995).

2.8 ROLE OF PLATELETS AND FIBRIN IN STROKE

2.8.1 Platelets

It is thought that platelets contribute significantly to the formation of atheromas as well as the thrombotic complications associated with atheromas (Fuster et al., 1992; Ross, 1993).

Van Kooten et al. in 1999 investigated the increased platelet activation in the chronic phase after cerebral ischaemia and intracerebral hemorrhage. In previous studies van Kooten and his co-workers have reported enhanced thromboxane (TX) biosynthesis in the acute phase after ischaemic stroke. They studied excessive excretion of 11-dehydro-TXB2, which is a non-invasive index of platelet activation, in the urine to see if it was present in the chronic

phase after a transient ischaemic attack (TIA) or stroke, as well as intracerebral hemorrhage. They concluded that platelet activation is frequently present in patients in the chronic phase after stroke, as well as those with intracerebral hemorrhage. Aspirin treatment can substantially suppress continuing platelet activation associated with atrial fibrillation and poor stroke outcome. (van Kooten et al., 1999)

Grau and co-workers investigated the increased fraction of circulating activated platelets in acute and previous cerebrovascular ischaemia. They stated the importance of establishing the circulating activated platelets as to help assess the prognosis of arterial vascular disorders such as stroke, as well as the therapeutic regimes to be followed. Their results indicated an increased expression of platelet neoantigens in acute cerebrovascular ischaemia and a smaller quantity in previous cerebrovascular ischaemia. New anti-platelet drugs may be of benefit as, after cerebrovascular ischaemia, platelet activation was ongoing even after aspirin and phenprocoumon therapy. (Grau et al., 1998)

In 2000, Kurabayashi et al. studied the possible existence of platelet activation before the onset of cerebral infarction. To accomplish this, they examined the ultrastructural features of platelets and coagulation-fibrinolytic markers for the acute, subacute and chronic phase of cerebral infarction. Ultrastructural study of the circulating platelets indicated no difference between the acute and chronic phases and not much difference between cerebral infarction and artherosclerosis. Even though the plasma coagulation-fibrinolytic markers were elevated at the acute phase of cerebral infarction, there was no variation among the chronic phase of cerebral infarction, atherosclerosis and healthy individuals. Although coagulation-fibrinolytic markers were derived from the thrombotic event of cerebral infarction, the shape of circulating platelets is more likely altered by pre-existing atherosclerosis than by the thrombotic event itself. (Kurabayashi et al., 2000)

In 1989, Joseph et al. made use of transmission electron microscopy (TEM) to calculate the organelles (dense bodies, alpha granules, and mitochondria) enclosed within platelets from acute ischaemic stroke patients and healthy controls. Analysing the morphology of the platelets from patients who had recently suffered cerebral infarction, Joseph and his co-workers discovered similar as well as dissimilar features compared to the platelets from controls. In both the experimental group and the healthy control group all components of the organellar zone were present. These include the dense bodies and alpha granules as well as mitochondria. However, upon quantification of the organelles present, they discovered the platelets from acute ischaemic stroke patients were changed. The authors also revealed that platelets of patients who suffered an acute ischaemic stroke contained significantly less alpha

granules. As alpha granules are the source of β-thromboglobulin (β-TG) and platelet factor 4 (PF-4), this morphological finding of Joseph et al. may show a relationship between the decreased levels of alpha granules in the platelets and the increased levels of β-thromboglobulin and PF-4 in the plasma as previously described by Shah et al. (Shah et al., 1985). This study also confirmed their previous observations of increased dense body secretion from platelets in whole blood of patients who suffered acute stroke (Joseph et al., 1989a). They also noted that the platelets of stroke patients contained fewer mitochondria. This could possibly be ascribed to mitochondria being released or consumed to a larger degree in the activated platelets of stroke patients, but the significance is still indeterminate. Their observations thus indicate an association between acute cerebral infarction and the increased platelet secretion. It also indicates that platelet secretion may possibly be of separate significance to the mechanical occlusion of blood vessels by platelet aggregates in the pathogenesis of cerebral infarction. (Joseph et al., 1989b)

2.8.2 Fibrin

Systems responsible for protecting the body against blood loss and exsanguination include cascades of coagulation as well as fibrinolysis. These mechanisms can also join with the immune system to serve as protection against microbial assault and systemic infection. The specific patterns found in disease have changed over time as the human body has been exposed to transformation in the environment. In like manner, the pathological progression of thrombotic disorders now has a strong association with the formation of fibrin as well as inflammation (Standeven et al., 2005).

A clot has to be stable as to be capable of withstanding the pressure in a blood vessel. This is not only important for the prevention of a bleed, but also to endorse the healing of wounds.

The coagulation cascade is set in motion as soon as there is injury in the vascular system, which initiates the production of thrombin. Thrombin will consequently convert fibrinogen to polymerizing fibrin. The new fibrin fibres form cross-links between one another. An expanding clot is formed by the integration of a range of proteins into this meshwork of fibres. These proteins will strengthen the clot and make it resistant to termination. Fibrinolysis is started at the same time as the formation of fibrin, as to ensure the healthy balance between the formation and breakdown of a clot (Standeven et al., 2005). Serving as both a co-factor with plasmin and the substrate that it disintegrates, fibrin and particularly its structure, has a great influence on the effectiveness of fibrinolysis. (Collet et al., 1993; Collet et al., 1996; Collet et al., 2000)

Plaques are very fragile. When a plaque ruptures, platelets accumulate on the surface of the plaque. A fibrin mesh is formed by this platelet aggregation (Standeven et al., 2005).

Fibrinogen and factor XIII, two proteins involved in the production of fibrin, have been implicated as playing a vital part in the pathogenesis of coronary artery disease and myocardial infarction. Genetic variance in these two proteins has also been associated with change in the structure of fibrin. Alterations in the configuration of the fibrin clot may influence the likelihood of suffering thrombotic vascular disease. (Standeven et al., 2005)

A healthy individual's risk of suffering from a stroke is correlated to the levels of plasma fibrinogen. (Danesh et al., 2005)

Although fibrinogen concentration has a great effect on the structure of the fibrin clot (Glover et al., 1975; Scott et al., 2004; Undas and Zeglin, 2006), various studies have indicated the concentration of thrombin to have the greatest effect on the formation of the fibrin clot structure (Blombäck et al., 1989; Blombäck et al., 1994; Carr and Hermans, 1978). Diminished levels of thrombin produce thick, loosely-woven fibrin fibers while thin, tightly-packed fibrin fibers are the result of elevated levels of thrombin during clot formation. (Wolberg, 2007)

Thick fibrin fibers arranged in a loose, woven manner will be lysed more effectively (Collet et al., 2000), while thin, closely-packed fibrin fibers are more resilient to fibrinolysis (Wolberg, 2007).

2.8.2.1 Thrombin and Plasmin

Fibrinopeptide A (FPA) and Bβ1-42 are cleavage products produced by thrombin and plasmin respectively when the blood coagulation and fibrinolytic systems are activated. Elevated thrombin and plasmin activities in plasma are indicated by increased plasma concentrations of FPA and Bβ1-42 (Lane et al., 1983).

Lane et al. determined the plasma concentrations of FPA and Bβ1-42 in patients who have had thrombotic stroke. They found that patients initially exhibited greatly elevated levels of FPA and Bβ1-42 in the plasma, but that the levels decreased to normal levels 1 month after the infarct. On the other hand, the concentrations of platelet release product β-thromboglobulin (β-TG) in the plasma were elevated directly after the stroke, and did not change with time after the infarct. They deduced from these findings that following thrombotic stroke thrombin and plasmin activities in the plasma are increased. They added that these increased protease activities are most likely not directly linked to an increased in vivo platelet

release reaction and that it may be useful in detecting the risk of another infarction or the progression of the stroke. (Lane et al., 1983)

2.8.2.2 Vasoactive substances

In 1987, Holmes commented on the large amounts of vasoactive substances contained in platelet secretory products which include thromboxane, adenosine diphosphate (ADP), adenosine triphosphate (ATP), calcium and serotonin. (Holmsen, 1987)

Two studies concluded that vascular and neural injury can result from the liberation of these vasoactive substances, predominantly thromboxane and serotonin (De Clerck et al., 1984; De Clerck et al., 1985).

Various studies conducted on both rats and rabbits show platelet activating agents, ADP and arachidonic acid in particular, bring about functionally noteworthy structural damage in the ipsilateral middle cerebral artery area when injected directly into the carotid artery (Fieschi et al., 1975; Fujimoto et al., 1985; Furlow and Bass, 1975). Joseph and co-workers stated that this trend is most likely mediated by platelet release products and not related to occlusive platelet aggregates (Joseph et al., 1989b)

2.8.3 Ultrastructure of platelets and fibrin

Up to 80% of acute stroke is caused by precerebral or cerebral artery occlusions (Williams et al., 1999).

In 2006, Marder et al. analysed the thrombi retracted from cerebral arteries of patients with acute ischaemic stroke. Upon examination of the thromboemboli, they discovered that 75% had the same structural characteristics. These thromboemboli contained random fibrin:platelet sediments integrated with linear clusters of nucleated cells, particularly monocytes and neutrophils, and confined areas of erythrocyte accumulation. This was a dominant histological trend in emboli retrieved from both cardioembolic and atherosclerotic sources. Overall, unique composites of erythrocytes called “red” clots were only found with incomplete extractions and no cholesterol crystals were present. Marder and co-workers concluded that thromboemboli retrieved from patients with acute ischaemic stroke have interrelated characteristics concerning their histology, whether originating from cardiac or arterial sources. They added that the dominant fibrin:platelet configuration from the thromboemboli, lays the foundation for both antiplatelet and anticoagulant treatment strategies in the prevention of stroke (Marder et al., 2006).

2.9 PREVIOUS RESEARCH DONE ON ULTRASTRUCTURE OF PLATELETS IN DIFFERENT DISEASE CONDITIONS

2.9.1 Asthma

Pretorius and Oberholzer compared the platelet and fibrin ultrastructure of the BALB/c asthmatic mouse model with that of human asthmatics. They found that platelets of patients suffering from uncontrolled asthma had the same morphology as that of the control BALB/c mice. Both presented with a mesh of minor thin fibres covering the major thick fibres. Granular platelet aggregates presenting with finger-like projections, connected loosely to the network of fibres (Pretorius and Oberholzer, 2009).

Pitchford established in 2007 that the function of platelets differs in asthma as compared to their established actions exerted during thrombosis and hemostasis. In asthma, platelets not only release adhesion molecules but they are also associated with the immune system as they are responsible for the leukocyte activation, the liberation of an excessive amount of inflammatory mediators, setting the adaptive immunity cells in action and consequently enabling the immunity cells to undergo chemotaxis. Pitchford also refers to clinical information obtained from patients presenting with asthma, allergic rhinitis and allergic dermatitis. After exposure to allergen, the behaviour as well as the function of the platelets changed in these patients. These findings emphasize the importance of studying platelets and fibrin in disease. (Pitchford, 2007)

As fibrinogen is deemed an acute phase protein and it is generally accepted as an indicator of systemic inflammation. It is suggested that platelets take part in various facets of asthma; they are employed to the lungs of asthmatic patients after the exposure to allergens (Pitchford et al., 2004) and may contribute to airway remodelling when activated (Morley et al., 1984).

Extravascular thrombin is common among asthma patients. A possible connection is implicated between fibrin web coating the major fibres and the occurrence of both fibrinogen as well as fibrin in the mucus of asthma patients. (Pretorius and Oberholzer, 2009)

2.9.2 HIV

The changes in the haematological parameters of patients suffering from HIV/AIDS are well established (Bamberg and Johnson, 2002). Cytopenias are a predominant result of

HIV/AIDS which influences the erythrocytes and the thrombocytes (Bamberg and Johnson, 2002), but principally the leukocytes (Izmailova et al., 2003; Smith et al., 2000).

The disease also brings about instability in the thrombocytic facet of hemostasis, leading to thrombocytopenia. This diminishes the capacity of the patient's blood platelets to aggregate. (Leissinger, 2001; Poliakova et al., 1995)

Usually, the changes in platelet ultrastructure is directly caused by the virus itself or induced by antibody activity. In contrast, there is evidence of changes in platelet aggregation before thrombocytopenia occurs. Some studies implicate HIV-infected megakaryocytes as the cause of thrombocytopenia, as they produce damaged platelets (Cole et al., 1998; Kravchenko et al., 1992).

The thrombocytopenia that occurs in approximately 40% of HIV infected patients may thus be caused by either the increased destruction of peripheral platelets, or HIV-infected megakaryocytes that produce defective platelets. It may also be as a result of a combination of the two. (Pretorius et al., 2008)

In 2008, Pretorius and colleagues studied the ultrastructural changes of platelets in HIV patients using scanning electron microscopy (SEM). They found aggregation of platelets as well as morphological changes including membrane blebbing and ruptured cellular membranes. Blebbing of the membrane is usually an indication of apoptosis. They concluded that there may be a relationship between thrombocytopenia and the distorted morphology of the accumulated platelets in HIV patients, possibly on account of the destruction of the peripheral platelets. (Pretorius et al., 2008)

2.9.3 Macrothrombocytopenia

Normal platelet size usually ranges from 1.5 µm to 2.5 µm. Macrothrombocytopenia is a rare disease that presents with enlarged circulating platelets (ranging between 5 µm and 20 µm). Other traits of this condition include thrombocytopenia, bleeding, short circulating times in blood and abnormal platelet destruction (Pretorius et al., 2009b).

Pretorius and her team used scanning electron microscopy (SEM) and transmission electron microscopy (TEM) to examine the network of fibrin fibres as well as the platelet aggregates of a family with established macrothrombocytopenia. They identified two variants of giant platelets: rounded, giant aggregate resembling platelets from the control group, and a giant flattened aggregate, with a dense outer border and a a bulbous pseudopodia-like central part.

According to TEM micrographs, control platelets contain alpha granules and dense bodies that transport, along with other substances, fibrinogen. However, in the platelets of persons with macrothrombocytopenia the TEM micrographs illustrated large vacuoles and regions predominantly not containing any dense bodies or alpha granules (Pretorius et al., 2009b).

2.9.4 Renal clear cell adenocarcinoma

Morphological alterations have been indicated by scanning electron microscopy (SEM) photography of platelets from patients suffering from renal clear cell adenocarcinoma (RCC). Compared to the controls exhibiting smooth rounded globular membranes, the aggregates in RCC had torn membranes with a spotted, crenated, prune-like shape. This disruption in the architecture of the cytoskeleton, resemble the apoptotic changes of programmed cell death Pretorius et al. described in 2008. (Pretorius et al., 2009a)

2.10 RESEARCH OBJECTIVES

This literature review clearly indicates that stroke, in particular thrombo-embolic ischaemic stroke, is associated with changes in fibrin, platelet and blood cell architecture. Therefore, the following research objectives will be investigated in the current thesis:

1) Using Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) to determine the morphology of platelets in stroke patients
2) To utilize TEM and SEM for the investigation of the ultrastructure of fibrin network in stroke patients.
3) To use SEM and TEM to examine the morphology of erythrocytes in stroke patients.
4) Using Light Microscopy (LM) to establish the total white blood cell (WBC) count in stroke patients.

CHAPTER 3: MATERIALS AND METHODS

3.1 CHAPTER OBJECTIVE

This chapter provides all methods and procedures employed in this thesis.

3.2 HOSPITAL PROCEDURES

This study was done in collaboration with Dr. W Duim, a neurologist from the Department of Neurology, University of Pretoria and the Little Company of Mary hospital in Pretoria.

Dr. W Duim interacted with the patients and informed consent was obtained from either the patient or a family member before blood was drawn. A nurse at the abovementioned hospital drew the blood after informed consent had been acquired. All patient information was handled anonymously.

A total number of 20 thrombo-embolic ischaemic stroke patients were included in this study, consisting of men and women. All patients underwent magnetic resonance (MR) brain scanning to exclude all other causes and confirm the thrombo-embolic ischaemic stroke.

On day of admission, 5ml of blood was drawn from each stroke patient in a citrate tube. All research was done on this single vile of blood.

3.3 LABORATORY PROCEDURES

The laboratory procedures were performed on the single collected citrated tube of blood, in the following manner:

1. Blood smears were made for the differential white blood cell count under light microscope
2. Platelet-rich plasma (PRP) obtained were used to prepare
    a. Scanning electron microscopy (SEM) samples of the fibrin networks
    b. SEM samples of the activated platelets
    c. Transmission electron microscopy (TEM) samples of the activated platelets
3. The remaining blood pellet obtained were used to prepare
    a. SEM samples of erythrocytes
    b. TEM samples of erythrocytes

3.3.1 Light microscopy

Blood smears

On the day of collection of the blood three blood smears were made of each patient in the following manner: A drop of blood was put onto glass slides and distributed with another glass slide to form a smear. These blood smears were then left to air dry.

Differential white blood cell count

The blood smears on the glass slides were stained with Rapid Hematological Stain. The following procedure was followed: Each slide was dipped 5 times in Methanol to ensure fixture of the blood smear to the slide, after which it was dipped 15 times in Eosin and 25 times in Methylene Blue. The Eosin stained the acidic cells while the Methylene Blue was used to stain the basic cells in the blood smear. These stained slides were investigated histologically with a Nikon Optiphod transmitted light microscope (Nikon Instech Co., Kanagawa, Japan) to establish differences in white blood cell count. Up to a 100 leukocytes were counted in each slide and the average number of each leukocyte was used in the statistical analysis.

3.3.2 Electron microscopy

3.3.2.1 Fibrin network

Preparation of fibrin clot with thrombin to exhibit the fibrin network

After making the three blood smears, the remaining blood was left for 45 minutes to obtain platelet rich plasma (PRP).

Fibrin clots were prepared by using human thrombin (provided by The South African National Blood Services). The thrombin was 20 U/ml and was prepared in biological buffer containing 0.2% human serum albumin. When thrombin was added to PRP, fibrinogen was converted to fibrin.

10 μl of human PRP was mixed with 10 μl of human thrombin. The PRP and thrombin mix were immediately transferred with a pipette tip to a 0.2 μm millipore membrane to form the fibrin clot (coagulum) on the membrane. Samples were made in duplicate for SEM investigation and 1 sample was made for TEM investigation. The millipore membranes were

immediately placed in a Petri dish on filter paper dampened with PBS to create a humid environment and placed at 37°C for 10 minutes. Following incubation the millipore membranes with the coagula were placed in PBS and magnetically stirred for 20 minutes. This washing process was done to remove any blood proteins trapped within the fibrin network.

The millipore membranes with the PRP and thrombin mix were fixed in a mixture of phosphate buffered saline (PBS), distilled water, 2.5% gluteraldehyde and formaldehyde in the ratio of 5:3:1:1 respectively for 30 minutes. After 30 minutes the millipore membranes with the PRP and thrombin mix were rinsed three times in 0.075M sodium potassium phosphate buffer (pH=7.4) and distilled water in the ratio of 1:1 for 5 minutes. After rinsing, the millipore membranes were placed in secondary fixative, 1% osmium tetraoxide (OsO_4) solution for 30 minutes. Following fixation, the samples were rinsed again as described above. The samples were then dehydrated in 30%, 50%, 70%, 90% and three changes of 100% ethanol.

Preparation of the washed fibrin clot for scanning electron microscopy (SEM)

Following dehydration, the SEM procedures were completed by critical point drying of the material, mounting and coating the sample with carbon and examining the tissue with a Zeiss Ultra plus FEG scanning electron microscope.

Preparation of the washed fibrin clot for transmission electron microscopy (TEM)

Following dehydration, the sample was infiltrated with Quetol Epoxy resin and 100% ethanol mixture to the ratio of 1:1 for 30 minutes. After 30 minutes in the Quetol Epoxy resin and ethanol mixture followed a change of Quetol Epoxy resin for 1 hour. After an hour, the sample was embedded in Quetol Epoxy resin in rubber moulds. The sample embedded in the Quetol Epoxy resin was oven dried at 60°C for three days overnight to polymerise the Quetol Epoxy resin for optimal cutting with the ultramicrotome.

Ultra-thin sections (80-100 nm) were cut with a diamond knife using an ultramicrotome, after which the sections were contrasted with uranyl acetate for 7 minutes followed by 5 minutes of contrasting with lead citrate. The samples were allowed to dry for a few minutes before examination with the JEOL transmission electron microscope (JEM 2100F). Photomicrographs were taken to reveal the morphology of the platelet cell membrane and cell organelles.

3.3.2.2 Platelets

Preparation of only PRP on the millipore membrane to exhibit the activated platelets under scanning electron microscope (SEM)

Fibrin clots were prepared by using only platelet-rich plasma (PRP). Different surfaces, namely a millipore membrane versus a glass cover slip, were used to determine which surface would give the best results.

a. Millipore membrane

20 μl of human platelet-rich plasma (PRP) was transferred directly to a 0.2 μm millipore membrane using a pipette tip. Samples were made in duplicate. The millipore membranes were placed promptly in a Petri dish on filter paper dampened with PBS to create a humid environment and placed at 37°C for 10 minutes. Following incubation the millipore membranes with the coagula were placed in PBS and magnetically stirred for 20 minutes. This washing process was done to remove any blood proteins trapped within the fibrin network.

The millipore membranes with the PRP and thrombin mix were fixed in a mixture of phosphate buffered saline (PBS), distilled water, 2.5% gluteraldehyde and formaldehyde in the ratio of 5:3:1:1 respectively for 30 minutes. After 30 minutes the millipore membranes with the PRP and thrombin mix were rinsed three times in 0.075M sodium potassium phosphate buffer (pH=7.4) and distilled water in the ratio of 1:1 for 5 minutes. After rinsing, the millipore membranes were placed in secondary fixative, 1% osmium tetraoxide (OsO4) solution for 30 minutes. Following fixation, the samples were rinsed again as described above. The samples were then dehydrated in 30%, 50%, 70%, 90% and three changes of 100% ethanol.

Following dehydration, the SEM procedures were completed by critical point drying of the material, mounting and coating the sample with carbon and examining the tissue with a Zeiss Ultra plus FEG scanning electron microscope.

b. Glass cover slip

The same procedures were followed as described above except that the 20 μl sample of human platelet-rich plasma (PRP) was transferred directly to a glass cover slip using a pipette tip. Samples were also made in duplicate.

Preparation of sample on the millipore membrane to exhibit the activated platelets under transmission electron microscopy (TEM)

For the preparation of the transmission electron microscopy (TEM) samples, only millipore membranes were used and not glass cover slips. TEM samples should preferably be prepared on a flexible surface - this does not only ensure optimum embedding, but also a supple sample for optimum slicing with the ultramicrotome.

Human thrombin was used to prepare a fibrin clot (human thrombin was provided by The South African National Blood Services). The thrombin was prepared in biological buffer containing 0.2% human serum albumin to be 20 U/ml. When thrombin was added to PRP, intracellular platelet components e.g. transforming growth factor, platelet derived growth factor and fibroblastic growth factor were liberated into the coagulum.

10 μl of human PRP was mixed with 10 μl of human thrombin. To form the fibrin clot (coagulum) on the membrane, the mixture of PRP and thrombin was immediately transferred with a pipette tip to a 0.2 μm millipore membrane. Only 1 sample was made for TEM investigation. The millipore membrane was immediately placed in a Petri dish on filter paper dampened with PBS to create a humid environment and placed at 37°C for 10 minutes. Following incubation the millipore membrane with the coagula was placed in PBS and magnetically stirred for 20 minutes. This washing process was done to remove any blood proteins trapped within the coagulum.

The millipore membrane with the PRP was fixed in the same manner as discussed for samples on millipore membrane prepared for SEM, followed also by washing, secondary fixation, a second series of washing and then dehydration to a 100% ethanol as described above.

Following dehydration of the sample to a 100% ethanol, the sample was cut in thin strips and infiltrated with Quetol Epoxy resin and 100% ethanol mixture to the ratio of 1:1 for 30 minutes. After 30 minutes in the Quetol Epoxy resin and ethanol mixture followed a change of Quetol

Epoxy resin for 1 hour. After an hour, the sample was embedded in Quetol Epoxy resin in rubber moulds. The sample embedded in the Quetol Epoxy resin was oven dried at 60°C for three days overnight to polymerise the Quetol Epoxy resin for optimal cutting with the ultramicrotome.

Ultra-thin sections (80-100 nm) were cut with a diamond knife using an ultramicrotome, after which the sections were contrasted with uranyl acetate for 7 minutes followed by 5 minutes of contrasting with lead citrate. The samples were allowed to dry for a few minutes before examination with the JEOL transmission electron microscope (JEM 2100F). Photomicrographs were taken to reveal the morphology of the platelet cell membrane and cell organelles.

3.3.2.3 Erythrocytes

Preparation of blood pellet

400 µl of the thick blood pellet was transferred to a glass vial and immediately fixed in a mixture of 5 parts phosphate buffered saline (PBS), 3 parts distilled water, 1 part 2.5% gluteraldehyde and 1 part formaldehyde. After 30 minutes of fixation, the sample was rinsed three times in 0.075M sodium potassium phosphate buffer (pH=7.4) for 5 minutes before being placed in secondary fixative, a 1% osmium tetraoxide solution, for 30 minutes. Following fixation, the sample was again rinsed three times in 0.075M sodium potassium phosphate buffer (pH=7.4) for 5 minutes. The sample was then dehydrated in 30%, 50%, 70%, 90% and three changes of 100% ethanol. The sample remained in the glass vial for the whole procedure. After each step, the sample was centrifuged and the particular chemical drawn off and discarded. The sample was distributed to 2 test tubes for the following preparatory steps for TEM and SEM separately.

Preparation of blood pellet for TEM

Following dehydration, the sample in the test tube for TEM analysis was infiltrated with a Quetol Epoxy resin and 100% ethanol mixture to the ratio of 1:1 for 30 minutes. After 30 minutes, the sample was centrifuged and pure Quetol Epoxy resin was added for 1 hour. After an hour, the sample was centrifuged for the last time before being embedded with Quetol Epoxy resin in rubber moulds. The samples embedded in the Quetol Epoxy resin was oven dried at 60°C for three days overnight to polymerise the Quetol Epoxy resin for optimal cutting with the ultramicrotome.

Ultra-thin sections (80-100 nm) were cut with a diamond knife using an ultramicrotome. These sections were contrasted with uranyl acetate for 15 minutes followed by 10 minutes of contrasting with lead citrate, after which samples were allowed to dry for a few minutes before examination with the JEOL transmission electron microscope (JEM 2100F). Photomicrographs revealing the morphology of the erythrocytes' cell membrane and cell organelles were taken.

Preparation of blood pellet for SEM

The SEM procedures were completed by drying the samples with hexamethyldisilazane (HMDS) for 30 minutes. After the 30 minutes, 200 μl of the sample and HMDS were placed on a cover slip. The cover slip was left to air dry and then coated with carbon. The sample was examined with a Zeiss Ultra plus FEG scanning electron microscope.

CHAPTER 4: PATIENT INFORMATION

4.1 CHAPTER OBJECTIVES

Chapter 4 supplies demographic and health status information of the patients participating in this study. The results of specific blood screening will also be discussed.

4.2 INTRODUCTION

Worldwide, acute stroke is one of the foremost causes of mortality and morbidity. In the industrialized countries acute stroke is the third leading cause of death. Every year an estimated 795 000 Americans suffer a first time stroke or a recurring stroke. (American Stroke Association, 2009) Stroke is the most important cause of morbidity and long-term disability in Europe. In South Africa it also imposes an enormous economic burden. (European Stroke Organisation, 2011) Approximately 87% of all cases of stroke are considered to be ischaemic stroke. About 80% of deaths related to embolism can be attributed to stroke while the remaining 20% is caused by other systemic thrombo-embolism. (Menke et al., 2010)

4.3 BLOOD PROFILES

Blood profiling involves the testing of blood drawn from patients and then comparing the results with established normal ranges. Blood testing thus gives a clear indication of abnormalities concerning various blood proteins. Standard thrombophilia screening including the prothrombin time, prothrombin index, partial thromboplastin time (PTT) and D-Dimer is used to establish the presence of several factors involved in the thrombotic event. Also the cholesterol levels are tested, since elevated cholesterol is a risk factor for stroke. The different standard thrombophilia screening factors as well as the other factors tested for are as follows:

4.3.1 Standard thrombophilia screening factors

4.3.1.1 Prothrombin time

This test is used to evaluate the adequacy of the extrinsic system and common pathway in the clotting mechanism. (Blood Testing Protocols, 2008) Measurement of clotting time of plasma recalcified in the presence of excess tissue thromboplastin. Factors measured are fibrinogen, prothrombin, and factors V, VII, and X. It is used for monitoring anticoagulant therapy. (Online Medical Dictionary, 2010)

4.3.1.2 Prothrombin index (PI)

The result for the prothrombin time is expressed as a ratio (clotting time for patient plasma divided by time for control plasma); a correction factor (International Sensitivity Index) is applied to the prothrombin ratio (as the sensitivity of commercial thromboplastin reagents is variable). (International normalized ration (INR), 2001)

4.3.1.3 Partial Thromboplastin Time (PTT)

PTT is the test of the intrinsic (factors VIII, IX, XI, and XII) and common (fibrinogen, prothrombin, factors V and X) pathways of coagulation. A mixture of plasma and phospholipid platelet substitute is recalcified and the time required for the appearance of fibrin strands measured. Activation may be provided by contact with the glass tube or exposure to activators before addition of the calcium chloride. It is used as a screening test and to monitor heparin therapy. (Online Medical Dictionary, 2010)

4.3.1.4 D-Dimer

This test is a very specific confirmatory test for disseminated intravascular coagulation (DIC). It is also used for the detection of deep vein thrombosis, acute myocardial infarction, and unstable angina. The Fragment D-Dimer assesses both thrombin and plasmin activity. (Blood Testing Protocols, 2008)

4.3.2 Other blood testing factors

4.3.2.1 S-Chol (total)

This test is used to determine the risk of developing coronary heart disease and hyperlipidemias. (Blood Testing Protocols, 2008)

4.3.2.2 Serum low density lipoprotein cholesterol (S-LDLC)

This test measures beta lipoproteins and is also used to predict heart disease. (Blood Testing Protocols, 2008)

4.3.2.3 Serum high density lipoprotein cholesterol (S-HDLC)

This test measures alpha lipoprotein and is used to predict heart disease. (Blood Testing Protocols, 2008)

4.4 RISK FACTORS

Since several factors are involved in the pathophysiology of stroke, the above-mentioned blood profiling should be considered with stroke risk factors to establish a comprehensive overview of each patient.

4.4.1 Non-modifiable risk factors

Risk factors that can not be regulated include age, race, gender, genetic origin and prior stroke.

4.4.1.1 Age

Stroke can occur at any age. However, approximately 75% of all stroke cases occur in individuals over the age of 65. (The Internet Stroke Centre, 2010) In men and women the risk of having a stroke more than doubles each successive 10 years after 55 (Brown et al., 1996; Wolf et al., 1992).

4.4.1.2 Sex

Stroke occurs more often in men than in women. In spite of this, more than 50% of the total deaths attributed to stroke occur in women. The risk of stroke for women is increased by the use of birth control pills, hormone replacement treatment (HRT) and pregnancy. (Stroke risk factors, 2010)

4.4.2 Modifiable risk factors

These are factors that can be controlled by medication or changes in lifestyle. Hypertension, hypercholesterolemia, cigarette smoking and oral contraceptives fall in this category.

4.4.2.1 Hypertension

Hypertension is the most dominantly recognized risk factor for ischaemic stroke at all ages. (Casper et al., 1992; Kuller, 1978; Palmer et al., 1992)

4.4.2.2 Hypercholestrolemia

Hypercholesterolemia (elevated blood cholesterol) increases the risk of having a stroke. Decreased high density lipoprotein cholesterol (HDLC) levels may be a stroke risk factor for men. (Stroke risk factors, 2010)

4.4.2.3 Cigarette smoking

The risk of suffering an ischaemic stroke is nearly double for smokers compared to non-smokers. A clear dose-response relationship has been noted. The major risk of stroke is only reduced after two to four years of smoking cessation, for all ages and in heavy as well as moderate smokers. (Kawachi et al., 1992; Shinton and Beevers, 1989; Wolf et al., 1988)

4.4.2.4 Oral contraceptives

Estrogen containing oral contraceptives are strongly related with stroke risk. It appears that only high estrogen content of greater than 50 µg increases the risk for stroke since Petitti et al. found low-dose oral contraceptive (less than 50 µg estrogen) showed no increased risk. (Petitti et al., 1996)

4.5 MATERIALS AND METHODS

A questionnaire was used to collect data regarding certain demographics and the general health status of each patient participating in the study. The information collected from the questionnaire is summarized in Table 4.1 and Table 4.2. Standard thrombophilia screening and other blood profiling performed on day of submission are summarized in Table 4.3.

4.6 RESULTS AND DISCUSSION

Table 4.1. Demographic and health status information of stroke patients.

Patient number	Gender	Age	Blood pressure	Cholesterol	Smoking	Aspirin	Previous stroke
1	Female	20	Normal	Normal	Yes	No	No
2	Female	38	Normal	Normal	Previous	No	No
3	Male	44	Hypotension	Normal	No	Yes	No
4	Female	46	Hypertension	Normal	No	No	No
5	Female	49	Normal	Normal	No	No	No
6	Male	50	Hypertension	Normal	No	No	No
7	Male	55	Hypertension	Elevated	No	No	No
8	Male	56	Normal	Normal	No	No	No
9	Female	62	Normal	Normal	No	No	No
10	Female	64	Hypertension	Normal	Yes	No	No
11	Male	67	Hypertension	Normal	Yes	No	No
12	Female	71	Normal	Normal	No	No	No
13	Female	74	Hypertension	Normal	No	No	No
14	Female	75	Normal	Normal	No	No	No
15	Female	81	Hypertension	Elevated	Previous	Yes	Yes
16	Female	82	Normal	Elevated	No	No	No
17	Female	82	Hypertension	Elevated	No	Yes	Yes
18	Female	84	Hypertension	Elevated	No	Yes	No
19	Female	84	Hypertension	Normal	No	No	No
20	Female	86	Normal	Elevated	No	Yes	Yes

Table 4.2. Mean values of the demographic and health status information of stroke patients given in Table 4.1.

	Percentage (%)
Gender	
Male	25.00
Female	75.00
Blood pressure	
Normal	45.00
Hypotension	5.00
Hypertension	50.00
Cholesterol	
Normal	70.00
Elevated	30.00
Smoking	
Current smokers	15.00
Previous smokers (more than 6 months)	15.00
Previous stroke	
More than 1 year ago	15.00

Table 4.3. Mean values of the blood profiling results of the stroke patients.

	Percentage Decreased (%)	Percentage Normal (%)	*Percentage Elevated (%)*
Prothrombin time	7.14	92.86	*0.00*
Prothrombin Index (PI)	0.00	100.00	*0.00*
PTT	42.85	57.15	*0.00*
D-Dimer	0.00	50.00	*50.00*
S-Chol (total)	0.00	60.00	*40.00*
S-LDLC	0.00	46.67	*53.33*
S-HDLC	80.00	20.00	*0.00*

Three-quarters of the patients were women and the remaining quarter was men. None of the women were on oral contraceptives or hormone replacement therapy (HRT). One patient was twenty and one patient was in her thirties. The age groups forty, fifty, sixty and seventy each had three patients. Six patients were older than 80 years of age.

Two of the patients were active smokers, while two other patients were previous smokers (more than 6 months in cessation).

Three of the patients have suffered a previous stroke. These patients were on aspirin-based medication before the last stroke. One other patient, who had no history of a previous stroke, was also on aspirin-based medication. He was the only hypotensive patient in the sample group.

Half of the stroke patients had pre-existing high blood pressure, while 40% of these patients also had pre-existing elevated cholesterol.

The blood profiles showed that more than half of the patients had elevated LDLC; more than 80% of the patients had decreased HDLC, while only approximately 40% of the patients had increased total cholesterol.

Lipoproteins, a combination of lipids and proteins, are the form in which lipids are transported in the blood. The low-density lipoproteins (LDL) transport cholesterol from the liver to the tissues of the body. LDL cholesterol is therefore considered the “dangerous” cholesterol. The high-density lipoproteins (HDL) transport cholesterol from the tissues of the body to the liver so it can be disposed of in the bile. HDLC is therefore considered the "beneficial" cholesterol. The higher the HDLC level, the lower the risk of coronary artery disease. (MedicineNet.com, 2010)

All patients had normal Prothrombin Time (PI). In 7.14% of patients the prothrombin time was decreased while 92.86% of the patients had normal prothrombin time. Almost 60% of the patients had normal PTT, while the remaining patients’ PTT was decreased.

D-Dimer was only elevated in 40% of the patients. This indicates increased activity of thrombin, and plasmin. Since thrombin is involved in fibrin formation and plasmin is associated with fibrinolysis, we can assume both these processes are activated.

4.7 CONCLUSION

In this study, more women were admitted to Little Company of Mary as stroke patients of Dr Duim. The twenty patients were of different age groups, ranging from 18 to 86. Half of the patients were above the age of 65, which is considered as the specific age of increased risk for stroke. Some of the patients were current or previous smokers, which is also a risk factor for stroke. Certain patients have previously suffered a stroke. Previous stroke increases the risk factor for subsequent strokes. Hypertension and hyperlipidemia, as pre-existing conditions and in the blood profiles, confirmed these conditions as significant risk factors for thrombotic ischaemic stroke. Elevated D-dimer in half the patients could be a possible indication of the activated coagulation cascade in these patients.

CHAPTER 5: MORPHOLOGICAL ANALYSIS OF PLATELETS IN STROKE

5.1 CHAPTER OBJECTIVES

This chapter aims to examine the morphology of the platelets found in the blood of stroke patients. These platelets will be compared to that of healthy individuals. Probable cause and effect of changed morphology will be discussed.

5.2 INTRODUCTION

Platelets are unique cells. Although they appear to be very simple, anucleated cells with a translucent cytoplasm, they contain various organelle involved in metabolic as well as secretory processes. Upon stimulation, granules are liberated from the platelets into the external medium. Yet, since platelets do not synthesize the constituents they secrete, they cannot be regarded as secretory cells. (Rendu and Brohard-Bohn, 2002)

The processes of platelet adhesion and coagulation are greatly dependent on the platelet membrane since the glycoproteins of the surface membrane are essential for attachment to the vessel endothelium. Platelets are joined together by the platelet membrane glycoprotein (GPIIb/IIIa) receptors. Phospholipids present in the platelet membrane are imperative to the intrinsic coagulation pathway, since they supply locations for essential attachment of calcium and coagulation factors. (Gaspard, 2009)

5.2.1 Platelet morphology

Within a platelet, several organelles are arbitrarily scattered throughout the cytoplasm. Skeletal components, consisting of microtubules and actin filaments, a complex membranous system and an abundance of glycogen serving as an energy resource are also present. The membranous system includes two prominent systems, the open canalicular system (OCS) which connects the cytosol to the surrounding medium, and the dense tubular system (DTS) responsible for the storage of important metabolic enzymes. (Rendu and Brohard-Bohn, 2002)

The distinctive size, quantity and function of each of these components in a typical platelet, will provide a means of comparing platelets of stroke patients to platelets from healthy control individuals.

5.2.1.1 Alpha-granules

Alpha granules are large, single membrane spherical organelles with a diameter ranging between 200-400 nm. They are referred to as the alpha granules (α-granules) since they are the largest and most numerous granules found in human platelets. Alpha granules store a collection of plasma proteins involved in hemostasis, wound healing and cell-matrix interactions. The alpha granules can then release these stored proteins at the location of vessel injury when required. (Rendu and Brohard-Bohn, 2002)

5.2.1.2 Delta-granules (Dense bodies)

Delta granules (δ-granules) are also referred to as dense bodies or dense core granules since they are both hefty in structure and very electron dense. With a mean diameter of 150 nm, delta granules are the smallest granules found in the cytoplasm of platelets. Dense core granules can, as their name implies, be identified by their solid, dense core enclosed within a translucent area and bordered by a single membrane. On average 5-6 dense bodies can be contained within a single platelet. Dense bodies hold small non-protein molecules, like ADP, ATP, calcium and serotonin. These molecules play a fundamental part in enabling the aggregation of platelets to be enlarged and initiating the alterations in the vascular endothelium as well as the function of leukocytes. (Zarbock et al., 2007)

5.2.1.3 Lysosomes

Lysosomes (λ-granules) are the third type op granule found within platelets and are involved in the release reaction of platelets. The diameter of lysosomes is 175-250 nm, making these granules intermediate between delta granules and alpha granules pertaining to their size. The distinctive feature of lysosomes is that they contain exclusively acid hydrolases such as glycosidases, acid proteases and cationic proteins with bacterial activity. Only upon strong stimuli will these enzymes be secreted from the lysosomes. The liberated hydrolytic enzymes will, through the action of hydrolytic degradation, assimilate substances in platelet aggregates and break these substrates down to their most basic units, i.e. glycoproteins and glycolipids will be broken down to sugars, amino acids and simple lipids. (Rendu and Brohard-Bohn, 2002; Zarbock et al., 2007)

5.2.1.4 Mitochondria

The diameter of mitochondria can range from 0.5 µm (500 nm) to 10 µm. Only a few mitochondria are found within a platelet. Although they are structurally simple, they play a meaningful role in the cell specifically relating to its energy metabolism. Mitochondria can solely support all the energy requirements of the platelet since anaerobic glycolysis does not influence the level of ATP in the platelet or the normal platelet function. Mitochondria also contain calcium. This mitochondrial calcium may possibly be just as important as the DTS and extracellular calcium in the activation of platelets. (Michelson and Coller, 2007)

5.2.1.5 Glycogen

Glucose is the principal energy supply for platelets and is absorbed from the plasma fairly quickly. Approximately half of the assimilated glucose is either involved in supplying energy for synthetic purposes or converted into glycogen for storage, under basal settings. (Calverley and Thiehelt, 2009) Glycogen can be found in isolation but also in large clusters of particles within the platelet. (Rendu and Brohard-Bohn, 2002)

5.2.1.6 Open canalicular system (OCS)

The demarcated openings to a complex arrangement of internal membrane channels form the open canalicular system (OCS), also referred to as the surface-connected canalicular system (SCCS). The cytoplasmic space is permeated by dilated channels. Two major functions are performed by the OCS. In the first place, plasma and membrane receptors are collected in the OCS. In the resting platelet, the OCS contain approximately a third of the thrombin receptors. When the platelets are activated, these receptors are transported to the surface. The OCS not only contains receptors, they can also accumulate specific membrane receptors from the plasma after cell activation, by the process of downregulation which is its second function. The vWF receptor is an example of a receptor transported into the OCS by downregulation upon platelet activation. (Joseph and Italiano, 2008)

5.2.2 Platelets and haemostasis

Haemostasis is a multifaceted process which involves the blood vessel wall, cellular components and soluble factors in circulation. Platelets are the most important cellular components in this process, especially pertaining to primary hemostasis, while coagulation factors are essential for secondary hemostasis. (Marcucci et al., 2008)

Primary hemostasis is initiated upon damage to a blood vessel. At the location of injury to the vascular endothelium, the exposed collagen will interact with platelets through the following processes: firstly the platelets will adhere to the collagen (adhesion), the platelets will then be activated (activation), resulting in the liberation of the contents of their granules (secretion) and finally the platelets will collect around the area (aggregation). Platelet aggregation is not only supported by the exposed collagen, but also by factors like fibrinogen, von Willebrand factor (vWF) and thromboxane. (Ramasamy, 2004)

After primary haemostasis, a series of events is initiated by coagulation factors called the coagulation cascade. The end product of this cascade is fibrin fibers. This process of fibrin formation is referred to as secondary haemostasis, and is responsible for the development of a stable clot. (Marcucci et al., 2008)

'Platelet release reaction' is controlled by the secretions of particular granules, such as the alpha granules, delta granules and lysosomes, while the metabolic processes of the platelet are controlled by mitochondria and the dense tubular system (DTS). It is the collaborative actions of the mitochondria and DTS that supply the metabolic energy and manage the cytosolic calcium essential for the secretion of the assorted granule components. (Rendu and Brohard-Bohn, 2002)

A great quantity of vasoactive substances is contained within the secretory products of platelets. Thromboxane (TXA_2), calcium, serotonin, adenosine diphosphate (ADP) and adenosine triphosphate (ATP) are some of these vasoactive substances. (Holmsen, 1987)

5.2.3 Platelets and stroke

Joseph et al. stated that platelet secretion and ischaemic stroke occur concurrently to each other.(Joseph et al., 1989b)

Joseph and fellow researchers studied platelet morphology, specifically the secretory organelles, in patients who suffered an acute ischemic stroke. Transmission electron microscopy was used to count the organelles found in both the platelets of ischaemic stroke patients as well as healthy controls. The organelles quantified were alpha granules, dense bodies and also mitochondria. (Joseph et al., 1989b)

Upon examination of platelets from both healthy individuals and patient who had recently suffered an acute ischaemic stroke variation but also shared characteristics were found. The

similarities included the presence of a circumferential band of microtubules that appeared intact, a large amount of glycogen particles, sporadic Golgi complexes, and the dense tubular system. Alpha granules, dense bodies and also mitochondria, all components of the organellar zone, were present in both groups. However, variation was observed in the quantification of these organelles. Observations indicated alterations in the number of alpha granules, dense bodies and mitochondria in the platelets of the acute ischaemic stroke patients when compared to those of healthy control individuals. Platelets from the acute ischaemic stroke patients contained significantly fewer alpha granules than their healthy counterparts. An increase in dense body secretion in whole blood confirmed their earlier findings (Joseph et al., 1989a). Fewer mitochondria might possibly indicate that the activated platelets of stroke patients release or consume mitochondria more readily than healthy individuals' platelets. (Joseph et al., 1989b)

Several animal studies substantiate the hypothesis that stroke may be exacerbated by vasoactive substances released from platelet organelles, since these substances can pass through the disrupted bloodbrain barrier and come in direct contact with cerebral tissue. (Fieschi et al., 1975; Fujimoto et al., 1985; Furlow and Bass, 1975) Consequently, neuronal as well as vascular injury can arise from these released substances. Thromboxane and serotonin in particular can cause the greatest damage. (De Clerck et al., 1984; De Clerck et al., 1985)

5.3 MATERIALS AND METHODS

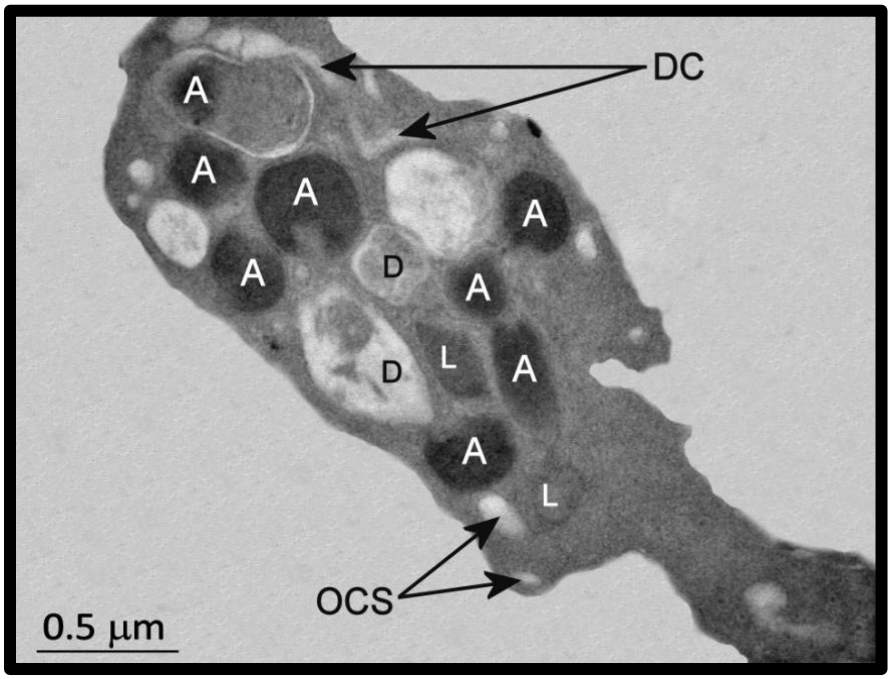
To investigate the morphological changes in platelets from platelet-rich plasma (PRP) from stroke patients, samples were prepared for scanning electron microscopy (SEM) as well as transmission electron microscopy (TEM) as explained in Laboratory procedures in Chapter 3.

5.4 RESULTS AND DISCUSSION

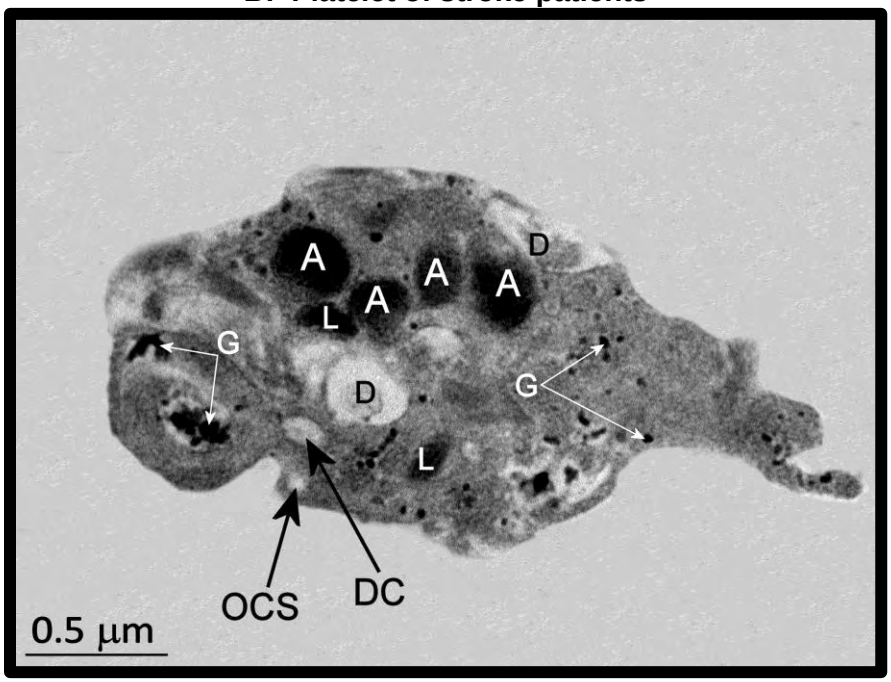
Both the external structure and the internal morphology of platelets were investigated. SEM was used to analyze the external surface while the internal structures were investigated using TEM. Firstly the TEM results will be discussed, followed by a discussion of the SEM findings.

5.4.1 Transmission electron microscopic investigation

A: Control Platelet

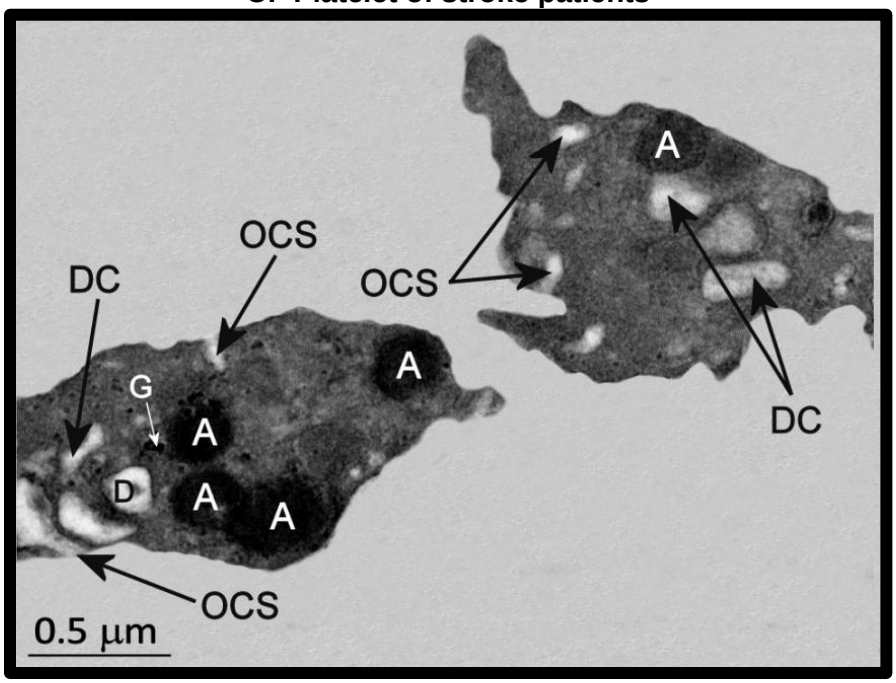


B: Platelet of stroke patients

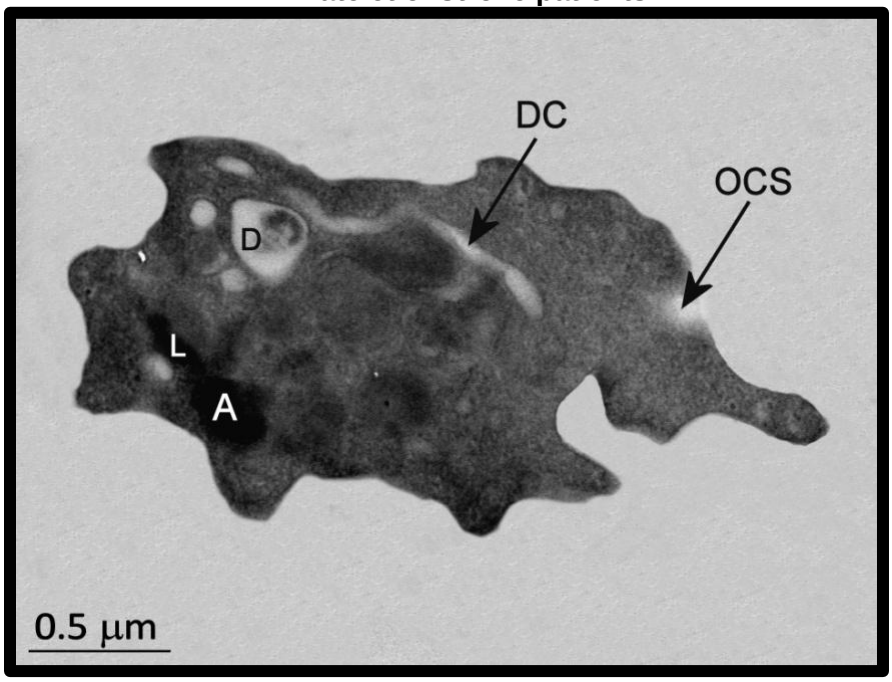


A – Alpha granule	**D** – Dense body with dense granule
L – Lysosome	**G** – Glycogen
OCS – Open canalicular system	**DC** – Dilated channel

C: Platelet of stroke patients

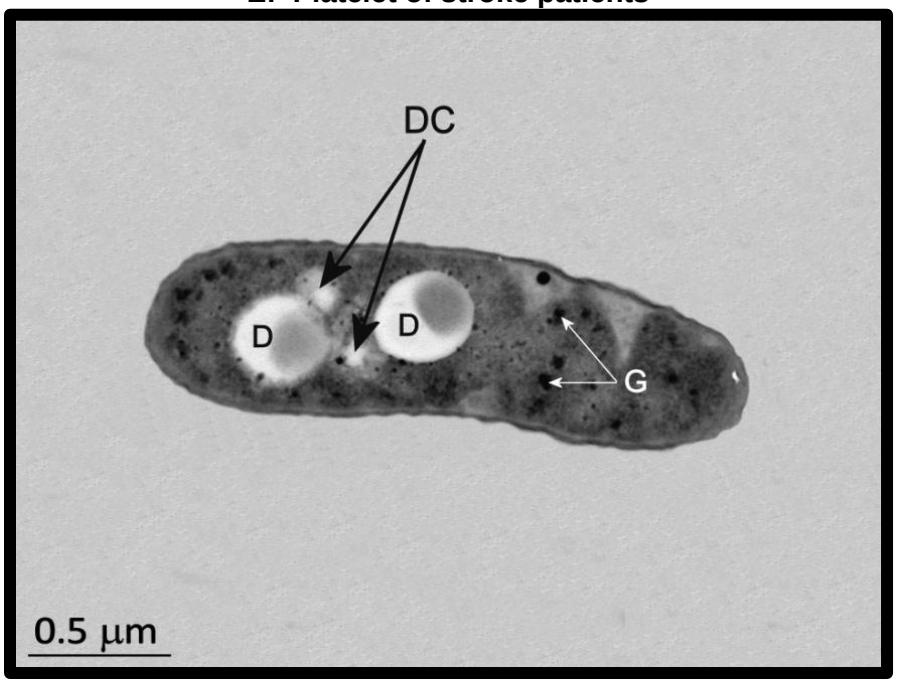


D: Platelet of stroke patients

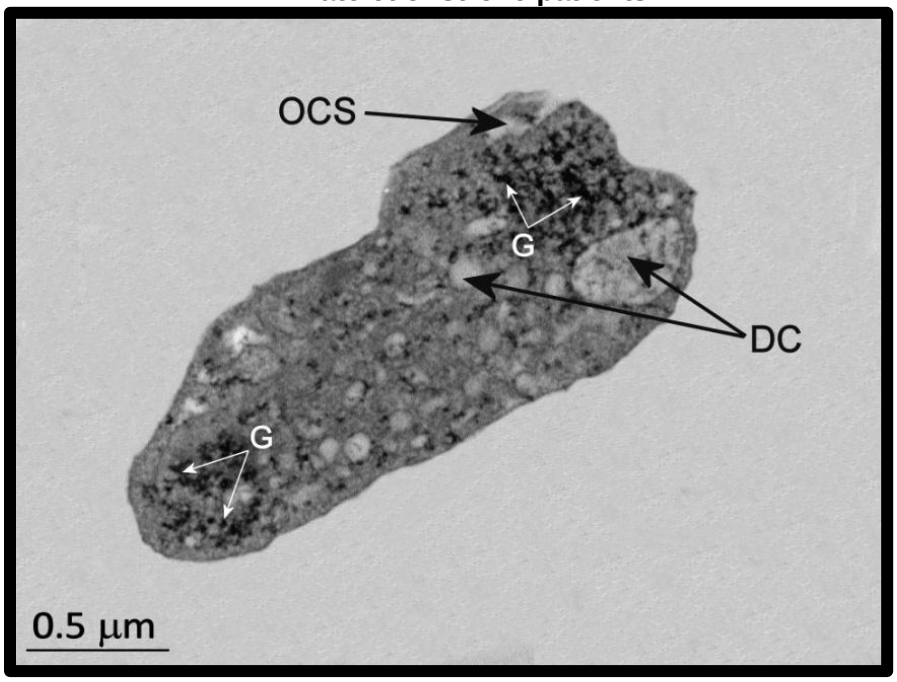


A – Alpha granule	**D** – Dense body with dense granule
L – Lysosome	**G** – Glycogen
OCS – Open canalicular system	**DC** – Dilated channel

E: Platelet of stroke patients



F: Platelet of stroke patients



A – Alpha granule	**D** – Dense body with dense granule
L – Lysosome	**G** – Glycogen
OCS – Open canalicular system	**DC** – Dilated channel

Figure 5.1. Transmission electron micrographs of platelets.

A = Control; **B** to **F** = Platelets found in stroke patients**.**

Figure 5.1.A. is a TEM micrograph of the cellular organelles of a typical platelet. Figures 5.1.B to H are representative micrographs of platelets found in the plasma of stroke patients. Less to no alpha granules (A) can be seen in the platelets of stroke patients. Dense granules within dense bodies (D), dilated channels and pores of the OCS can also be identified. In some of these micrographs glycogen (G) can be identified.

Platelets release the contents of their granules upon stimulation (Ramasamy, 2004). Thus, the decreased number of granules in the stroke patient platelets, when compared to that of the healthy control, indicates the liberation of the contents of these granules.

Figure 5.1.B and C show platelets from stroke patients that contain half the amount of alpha granules (A) than that of the control platelet. Figure 5.1.D. contains only one alpha granule, while the remaining platelets (E and F) contain no alpha granules. These findings are similar to the findings of Joseph et al. that acute ischaemic stroke patients contain considerably less platelet alpha granule compared to control individuals (Joseph et al., 1989b).

Alpha granules can be qualified as typical secretory vesicles since they transport their enclosed proteins to be liberated on the cell surface. These soluble intragranular proteins released can either be disperse into the extracellular matrix, become integrated in the membrane or adhere to the surface of the platelet where they are converted into peripheral proteins of the plasma membrane. (Rendu and Brohard-Bohn, 2002)

Table 5.1. Constituents found in the alpha granule matrix

Category	Examples
Haemostasis factors and cofactors	Fibrinogen
	Factor V, VIII, XI, XIII
	Plasminogen
Adhesive glycoproteins	Thrombospondin (TSP)
	von Willebrand factor (vWF)
Proteoglycans	β-thromboglobulin (β-TG)
	Platelet factor 4 (PF-4)
Miscellaneous	Immunoglobulins
	Albumin
	GPIa/multimerin

Alpha-granules store various haemostatic factors and co-factors involved in the coagulation cascade. A fibrin clot is produced by thrombin-mediated conversion of fibrinogen to fibrin. Fibrinogen is stored in the alpha granules. The inactive thrombin precursor, prothrombin, factor V, XI and XI, all involved in the intrinsic clotting cascade, are also secreted upon platelet activation. Additionally, alpha granules contain proteases which reduce fibrinolysis by inhibiting plasmin activity. However, alpha granules also contain antithrombin which mediates fibrinolysis. (Blair and Flaumenhaft, 2009)

The dominant protein found in the platelet alpha granules is thrombospondin (TSP), an adhesive glycoprotein. TSP is important in thrombus formation and is associated with subendothelial basement membranes of arteries. (Chevill, 1994)

The adhesive glycoprotein Von Willebrand Factor (vWF) serves as the link between activated platelets and the blood vessel subendothelium in the case of haemostatic crisis. vWF is also concerned with platelet plug development. vWF perform its function as the primary liaison of

platelet attachment to the blood vessel wall and mediator of platelet aggregation in blood vessels that possess a high shear rate. (Baronciani and Mannucci, 2010)

After platelet activation, large amounts of β-thromboglobulin is released from the alpha granules. β-TG, a heparin-binding proteoglycan, is involved in the stimulation of plasminogen activator synthesis, which converts plasminogen to the fibrinolytic enzyme plasmin. Thus β-TG is indirectly involved in fibrinolysis. (Bokan and Orahovec, 2004)

Platelet factor 4 (PF-4) neutralizes the anticoagulant consequence of heparin (Beckstead et al. 1986; Hayward et al. 1993; Sander et al. 1984).

For the formation of a haemostatic platelet plug upon vessel injury, adhesive proteins need to attach to receptors on the surface of the activated platelet (Ugarova et al., 1993). Several receptors have been identified in the unit membrane of alpha granules. Glycoprotein IIb/IIIa (GPIIb-IIIa ($\alpha_{IIb}\beta_3$)) is one of these receptors found in the inner lining of the unit membrane. (Harrison and Cramer, 1993) This calcium-dependent membrane glycoprotein functions as a receptor for fibrinogen, fibronectin and vWF on activated platelets. The adhesion, spreading and aggregation of platelets are dependent on the interaction of GPIIb-IIIa with these substrates. (Andrieux et al., 1989)

Thromboxane A_2 (TXA_2) originates from arachidonic acid liberated from the platelet plasma membrane upon activation by thrombin, collagen or other activating agonists. TXA_2 assists in the process of activating and recruiting additional platelets to the location of the haemostatic plug formed. (Paul et al., 1999)

The reduced numbers or total absence of alpha granule in the platelets of stroke patients could possibly indicate that components of these granules have been released into the plasma. Most of the released components of alpha granules, including fibrinogen, vWF and thrombospondin, are involved in platelet activation, spreading and aggregation along with fibrin formation. It can therefore be assumed that these processes have been initiated and also amplified in stroke patient platelets by the liberated factors mentioned above.

Figure 5.1.B and E show two dense bodies containing delta granules like the control platelet. Figure 5.1.C and D show only one dense body. Figure 5.1.F appears to contain no dense body. Increased platelet dense body secretion in acute ischaemic stroke patients have also been reported by Joseph et al. (Joseph et al., 1989b)

Secretion of dense body contents is exceptionally essential since the coagulation factors of hemostasis necessitate calcium and platelet aggregation is mediated by ADP. The released ADP triggers additional platelet delta granules to release their ADP stores, thus augmenting the development of platelet aggregation. ADP, together with TXA_2, mediates the development of the primary haemostatic plug since it causes the platelet aggregate to expand. (Gaspard, 2009)

Although ADP is the most important platelet activator, it is proposed that the serotonin derived from dense bodies can also activate additional platelets and consequently employ them into the aggregate. (Zarbock et al., 2007)

King et al. found in atherosclerotic mice that diminished secretion of platelet dense body components correlates with a noticeable decrease in the progression of arterial thrombosis and inflammation. (King et al., 2009) It may therefore possibly suggest that increased release of dense granule components could be associated with increased development of arterial thrombosis and inflammation.

The decreased amount of dense bodies present in the platelets of stroke patients therefore indicates the increased secretion of dense body constituents. These liberated components probably include ADP, serotonin and calcium, all of which are involved in the aggregation of platelets to form the primary platelet plug. Thus we can assume the increased dense body secretion is associated with the thrombotic event.

Figure 5.1.B, C, D and F seem to display pores of the open canalicular system (OCS). Escolar and White found the OCS of human platelets activated by thrombin can simultaneously operate as the transport channel for elements into the platelet and a pathway for the emancipation of alpha granule constituents released during the platelet release reaction. They referred to the OCS as the “final common pathway”. (Escolar and White, 1991) Figure 5.1.C, D, E and F seem to reveal channels and cavities of the dilated channels (DC). These dilated channels appear especially enlarged in Figure 5.1.C and F. Morgenstern et al. found that it is most likely the residual membranes of the alpha granules after platelet degranulation that gives rise to the dilated system of membranes. (Morgenstern et al., 1990) The presence of the enlarged OCS pores and the dilated channels confirm the assumption that alpha granule secretion is increased in the platelets of stroke patients, thus promoting coagulation formation.

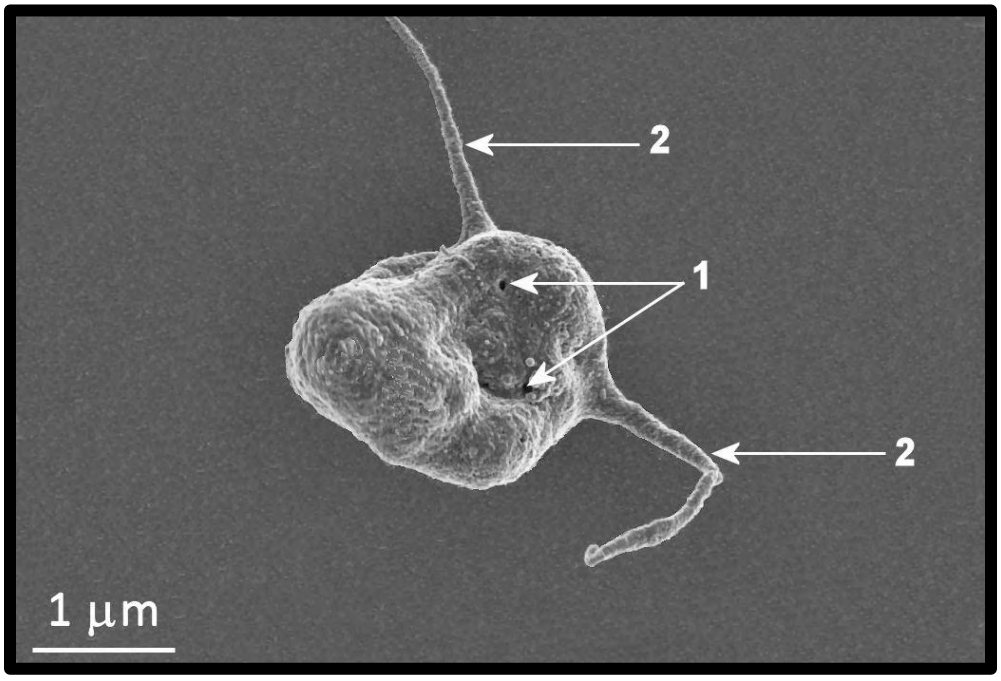
No mitochondria seem to be present in either the control platelet (Figure 5.1.A) or any of the platelets from the stroke patients (Figure 5.1.B to F). Joseph et al. discovered that acute

ischaemic stroke patients contain not as many mitochondria as seen in control platelets. They stated that the decreased numbers of platelet mitochondria could be as a result of platelet activation that leads to increased mitochondrial release or consumption (Joseph et al., 1989b).

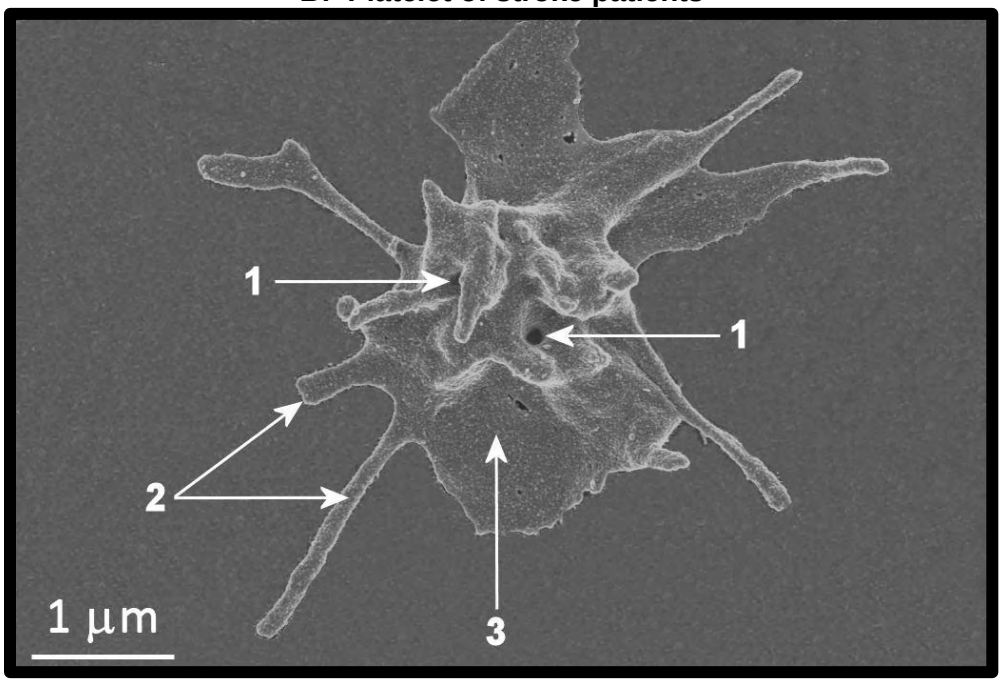
Figure 5.1.B appears to have the same amount of lysosomes as is seen in the control platelet. Figure 5.1.D appears to have one lysosome, while the remaining micrographs appear to contain no lysosomes. Glycogen appear to be present in Figure 5.1.B, C, E and F but not in the control platelet (Figure 5.1.A).

5.4.2 Scanning electron microscopic investigation

A: Control Platelet

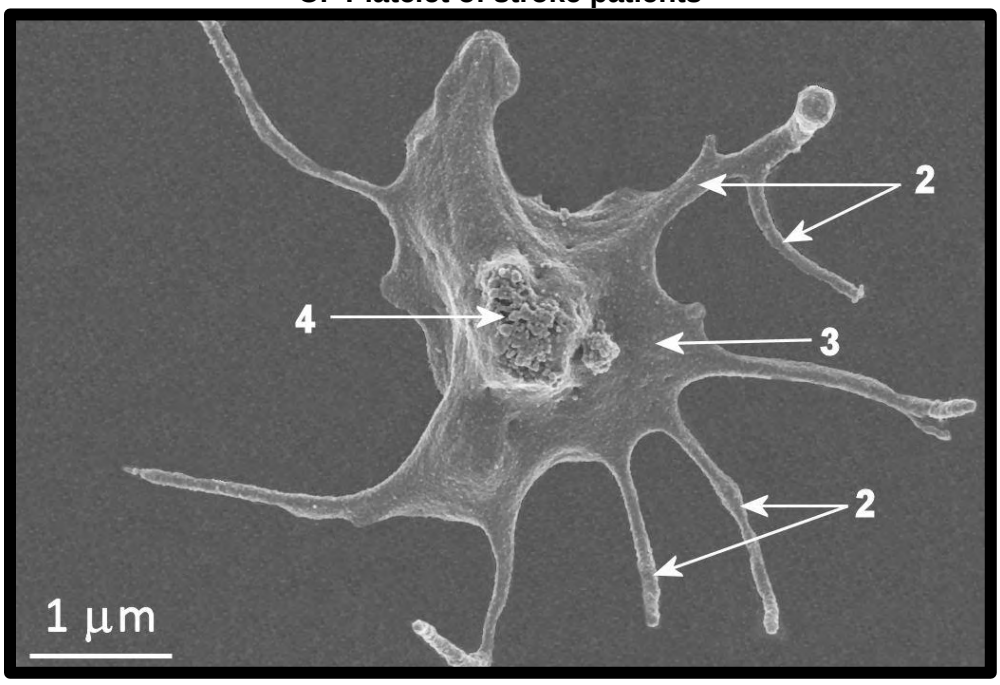


B: Platelet of stroke patients

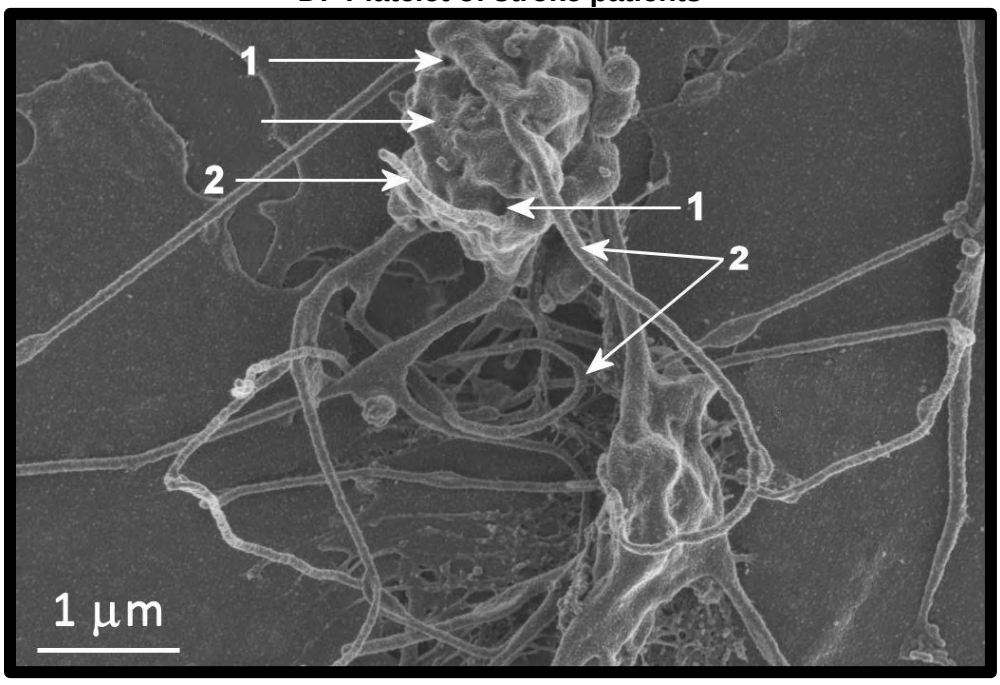


1 – Open canalicular system	**2** – Platelet-associated fibrin fibers
3 – Uncharacteristic flattened area	**4** – Apoptotic membrane blebbing
5 – Uncoagulated proteins	

C: Platelet of stroke patients

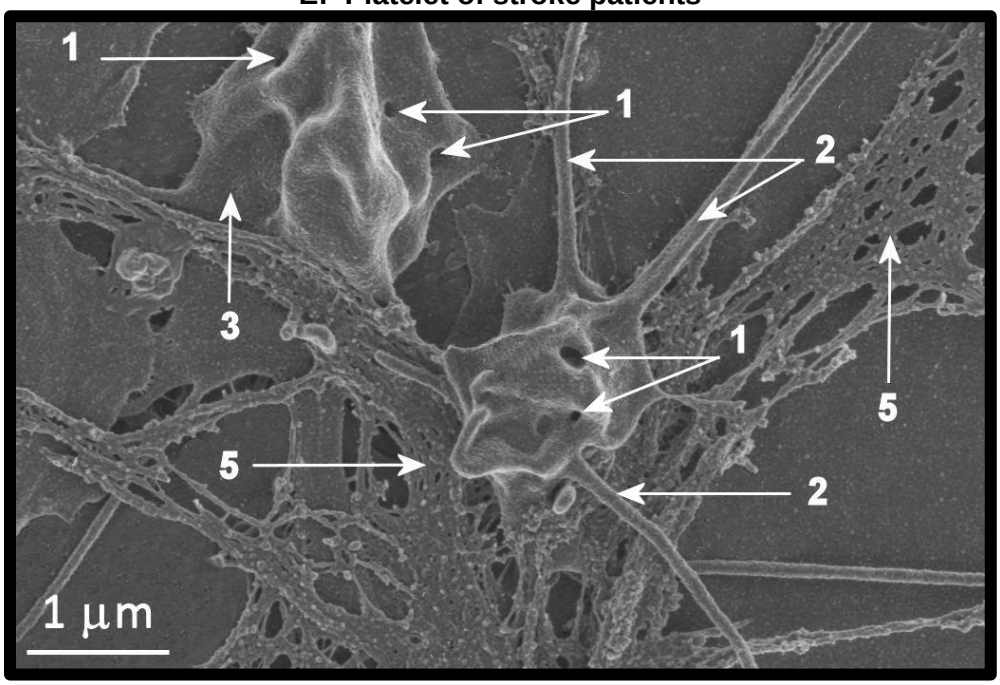


D: Platelet of stroke patients



1 – Open canalicular system	**2** – Platelet-associated fibrin fibers
3 – Uncharacteristic flattened area	**4** – Apoptotic membrane blebbing
5 – Uncoagulated proteins	

E: Platelet of stroke patients



1 – Open canalicular system	**2** – Platelet-associated fibrin fibers
3 – Uncharacteristic flattened area	**4** – Apoptotic membrane blebbing
5 – Uncoagulated proteins	

Figure 5.2. Scanning electron micrographs of platelets.

A = Control; **B, C** = Platelets found in stroke patients;

D, E = Platelet aggregates found in stroke patients.

Figure 5.2. A is the SEM micrograph of a typical control platelet. The platelet has a compact, rounded shape and a few thick fibrin fibers associated with the platelet extend from the body. Small pores of the open canalicular system (OCS) can also be seen on the body of the platelet.

Figure 5.2. B and C are SEM micrographs of single platelets representative of the activated platelets found in the platelet rich plasma from stroke patients. The aggregates of platelets from the stroke patients' PRP are represented by the SEM micrographs of Figure 5.2. D and E.

The bodies of these platelets appear to be not as rounded as that of the control platelets and apoptotic membrane blebbing appeared on some of the platelets (Figure 5.2. C).

When coagulation factors gather on the surface of the activated platelets an enzyme cascade is initiated. Thrombin, a protein-cleaving enzyme, is produced by this cascade. Thrombin will attach to a large protein, fibrinogen, and convert it to a large threadlike polymer called fibrin. A fibrin network will be formed in the area surrounding the activated platelets, providing stability to the clot. (Chevill, 1994)

Thrombin is a haemostatic agent that converts fibrinogen to fibrin. It is also involved in the activation of platelets. Activation of platelets by thrombin results in events analogous to apoptosis which includes platelet shrinkage and apoptotic membrane blebbing amongst others. (Fox, 1996; Heemskerk et al., 1997; Shattil et al., 1998; Vanags et al., 1997) Wolf and co-workers added that, by stimulating the aggregation of platelets, facilitating the adhesion of platelets to injured blood vessels and activating the coagulation cascade, hemostasis is endorsed by these apoptotic events. (Wolf et al., 1999) Since these platelet samples were prepared without the addition of thrombin, it can be assumed that thrombin was possibly present in the platelet-rich plasma (PRP) of the stroke patients.

Brown and colleagues first reported on the direct activation of platelets by shear stress in the absence of exogenous chemical agonists (Brown et al., 1975). Kroll et al. subsequently confirmed that several platelet responses, like shear-induced platelet aggregation and von Willebrand factor binding, are initiated by shear stresses in whole blood or platelet-rich plasma (PRP) (Kroll et al., 1996). Leytin and co-workers found that platelet apoptosis is not only induced by chemical stimuli, but can also be initiated by mechanical rheological forces, also referred to as pathological high shear stresses (Leytin et al., 2004). They added that the reticuloendothelial system could recognize and eliminate these shear-damaged platelets from circulation (Leytin et al., 2004).

Open canalicular system pores can be identified on these platelets, however they seem to be larger than that of the control platelet (Figure 5.2. B and D). White and Clawson defined the open canalicular system (OCS) as a unique intricate system of tunneling invaginations of the platelet cell membrane (White and Clawson, 1980). The OCS is closely associated to alpha granules. Firstly, the OCS assist alpha granule contents release. To accomplish their physiological function, the contents of the alpha granules must be liberated from their intracellular store into the external medium. This is done when the alpha granule membrane fuses with the plasma membrane or the OCS's membranes that are connected to the surface. (Flaumenhaft, 2003) Secondly, the OCS and alpha granules provide extra membrane surface

that enables the platelet, upon stimulation, to more than double its surface area (Blair and Flaumenhaft, 2009). Since platelet aggregation is facilitated by alpha granules, the enlarged OCS pores could thus indicate increased release of alpha granule contents into the external medium that could lead to the aggregation of platelets.

A significantly greater number of the platelet-associated thick fibrin fibers can also be identified in these micrographs. These thick fibrin fibers appear to have either a very extended (Figure 5.2. B, C and E) or coiled (Figure 5.2. D) morphology. The coiled fibers seem to connect platelets with each other (Figure 5.2. D). Even without the addition of thrombin, a network of thin fibrin fibers appears to have formed in some cases (Figure 5.2. D). This may possibly confirm earlier assumption that thrombin was present in the PRP of the stroke patients. Within this thin fibrin fiber network uncoagulated proteins appear to be trapped.

There seems to be areas of flattened morphology, referred to as spreading, found directly around the body of the platelet and between the platelet-associated thick fibrin fibers (Figure 5.2. B, C and E). Upon vessel injury, platelets need to rapidly change their morphology from their resting discoid shape to its active form. As a platelet adheres to subendothelial ligands of a damaged vessel wall, a multitude of signaling events will activate the platelet. The platelet will then be converted from its tightly-packed discoid, resting shape to a more flattened shape. This process is called platelet spreading. Spreading enables the platelet to flatten over the damaged area while the activated platelet releases its components to facilitate fibrin formation and the recruitment of additional platelets. (Hartwig, 2007; Hoylaerts, 2002)

5.5 CONCLUSION

The observed changed morphology of stroke patient platelets when compared to healthy control platelets, are affirmative for both SEM and TEM.

TEM micrographs revealed a number of changes in the stroke platelets compared to the control. Decreased amounts of alpha granules indicated possible increased secretion of their constituents. Several constituents of the alpha granules are involved in thrombus formation (like fibrinogen, factor V and TSP), while others are associated with platelet aggregation (like vWF and TXA_2). The likely increased dense body secretion (including ADP and serotonin) also contributes to the aggregation of platelets. Furthermore, the dilated channels and pores of the OCS substantiate possible increased release of alpha granule components. Observations thus support the implication of increased formation of the platelet plug as well as fibrin fibers.

SEM micrographs also exhibited changes in the external morphology. OCS pores associated with alpha granule release, were observed along with thick major and some thin minor fibers. Some platelets exhibited spreading associated with platelet activation. It can therefore be assumed that these platelets where activated, and has already released some of their granule components to promote platelet aggregation and also initiate the formation of fibrin strands for the developing clot.

In view of these observations, we can assume that platelets of stroke patients are activated and actively involved in the thrombotic event. Not only do platelets play a role in primary and secondary haemostasis which leads to thrombus formation, but their released vasoactive substances could bring about the neuronal damage associated with stroke.

CHAPTER 6: ULTRASTRUCTURAL ANALYSIS OF FIBRIN NETWORKS IN STROKE

6.1 CHAPTER OBJECTIVES

The fibrin networks of stroke patients will be analyzed in this chapter to determine if the morphology is similar to that of healthy control individuals. Possible factors responsible for any altered morphology will be discussed.

6.2 INTRODUCTION

6.2.1 Coagulation cascade

The formation of a gelatinous clot from liquid blood is referred to as blood coagulation. Two pathways can initiate this complex process, namely the intrinsic pathway and the extrinsic pathway. The intrinsic pathway commences upon the exposure of collagen, which activates the enzyme factor XII to initiate the cascade. Upon the exposure of tissue thromboplastin, a tissue factor exposed when tissue is damaged, the extrinsic pathway is set in motion and factor VII is activated by tissue factor. The common pathway is where the extrinsic pathway and intrinsic pathway merge to create the enzyme, thrombin. Thrombin is responsible for converting fibrinogen to fibrin polymers. The clot mainly consists of these insoluble fibrin fibers. (Silverthorn, 2007)

Wolberg suggested in 2007 that structurally altered fibrin clots can be produced by abnormal thrombin generation. These structural changes lead to an increased risk of bleeding or thrombus. (Wolberg, 2007)

The properties of the fibrin clot are therefore greatly influenced by fibrinogen (Scott et al., 2004). A compact network of thick fibrin fibers results from elevated levels of fibrinogen (Blombäck et al., 1989; Undas and Zeglin, 2006). High levels of fibrinogen also increase the rate of activation of the fibers (Scott et al., 2004).

Elevated fibrinogen is recognized as a risk factor for cardiovascular incidence. A relationship has been shown between coronary artery disease and abnormal fibrin clot structure, strength

and stability. (Lord, 2007) A correlation has been found between plasma fibrinogen levels and a healthy individual's risk of suffering a stroke (Danesh et al., 2005).

6.2.2 Fibrin networks in stroke

Weisel summarized the impact of fibrin structure on clot stability as follows. Weisel said that clot stability refers to both viscoelastic and fibrinolytic properties. The clot should possess properties that enable it to be firm and strong enough to fulfill its mechanical functions (viscoelastic), but it should also possess properties that will ensure that it is dissolved effectively in a timely manner (fibrinolytic). (Weisel, 2007)

Cryptogenic stroke is the term used for stroke that occurs without clear aetiology. Approximately 30-40% of all strokes are classified as cryptogenic stroke (Guercini et al., 2008). This includes cases of acute ischaemic stroke. Ischaemic and cryptogenic stroke is associated with alterations in the structure of the fibrin clot as well as fibrinolysis resistance. (Undas et al., 2009)

6.2.2.1 Fibrin clot structure

Altered clot morphology, which significantly contributes to the thrombotic event, results form changed fibrin fiber formation. A collection of plasma proteases and cofactors, under strict regulation, control fibrin clot formation. Although hemostasis is mediated by this system, as it reduces the loss of blood from injured blood vessels, it can also cause pathological thrombus formation when fibrin formation and platelet activation lead to vessel occlusion. (Gailani and Renne, 2007)

In 2009 Undas et al. investigated alterations in the structure and function of fibrin clots in patients with cryptogenic ischaemic stroke. Their results showed that not only was the clot made from plasma obtained from stroke patients much more compact than those of control patients, but also that the formation of the clot was also much faster. They also stated that the compact clot, comprising of thicker fibers, influences fibrinolysis since proteins cannot move freely through the dense network. Undas et al. added that the processes involved in clot structure alterations in stroke patients are uncertain. They implicated fibrinogen as a major predictor of the properties of the fibrin clot (Blombäck et al., 1989; Scott et al., 2004), but added that similar fibrinogen levels were observed in both stroke patients and healthy controls. (Undas et al., 2009)

Fibrin configuration can also possibly be influenced by components released by platelets.

Thrombospondin (TSP) is a protein found in the plasma, but only in trace amounts. High concentrations of TSP are only released during hemostasis. It is released by the alpha granules of the activated platelets and it has been suggested that TSP could initiate the acceleration of the growth rate of fiber by interrelating with fibrin intermediates. (Bale and Mosher, 1986)

Platelet factor 4 (PF-4) is a low molecular weight protein localized in the alpha granules of platelets. Upon platelet aggregation, PF-4 is secreted into the surrounding medium where it neutralizes heparin by inhibiting heparin to bind to antithrombin. In this way PF-4 counteracts the anticoagulant effect of heparin. (Handin and Cohen, 1976) PF-4 therefore mediates coagulation by inhibiting the anticoagulant function of heparin.

6.2.2.2 Fibrinolysis

A clot of fibrin fibers can be disintegrated by the process of fibrinolysis. Steadman's Medical Dictionary describes fibrinolysis as "the hydrolysis of fibrin" or "the process of dissolution of fibrin in blood clots". (Stedman's Medical Dictionary 2006, p. 723) To maintain a healthy balance between clot formation (coagulation cascade) and the disintegration of the clot (fibrinolysis), these two processes are simultaneously activated. (Standeven et al., 2005)

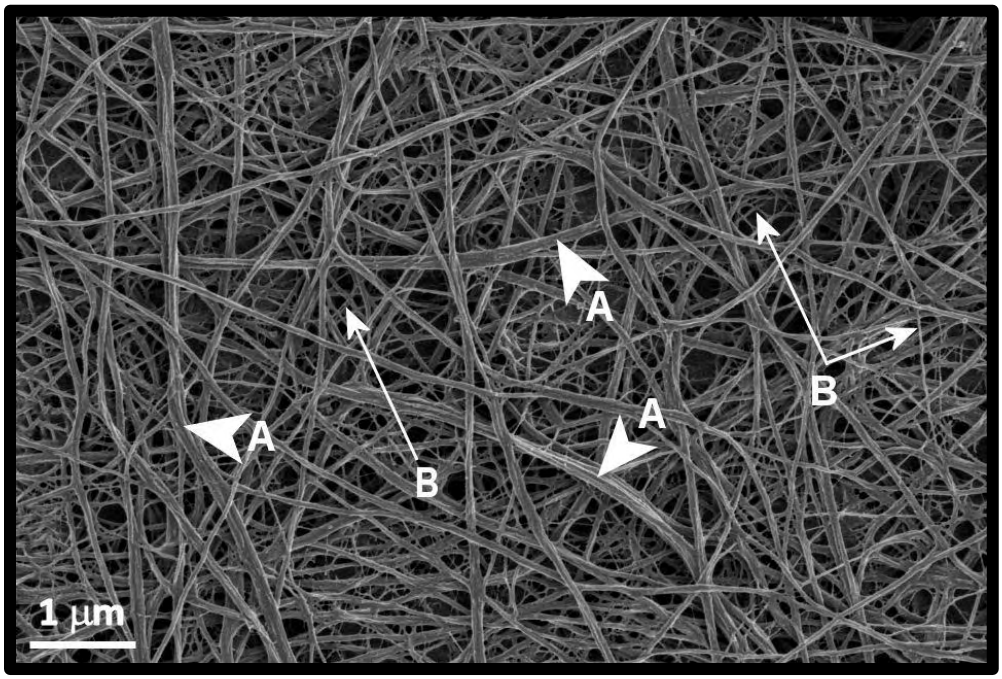
The physical properties of the fibrin scaffold and the combination of regulated enzymatic activity influence the effectiveness of fibrinolysis. In 2008, Weisel and Litvinov researched the processes of fibrinolysis and how the lysis rate is influenced by the structure and stability of the clot. They found that, rather than being digested from the outside by erosion of the surface, fibrinolysis advance by lateral transection of the fibers. Plasmin, the fibrinolytic enzyme, progresses laterally across the fibers and attach to locations established by its own proteolytic activity. The conditions under which a clot is formed will influence various properties of that specific clot, leading to great structural, biological, physical as well as chemical variance between clots. The nature and rate of fibrinolysis will thus be influenced by these properties. Weisel and Litvinov stated that generally the thinner fibers appear to be lysed at a much slower rate than thicker fibers, but added that lysis rate is also dependant on other physical properties of the clot and not only on the diameter of the fibers lysed. Fibrinolysis is also influenced by platelet aggregation since platelet aggregates influences the structure of fibrin. (Weisel and Litvinov, 2008)

6.3 MATERIALS AND METHODS

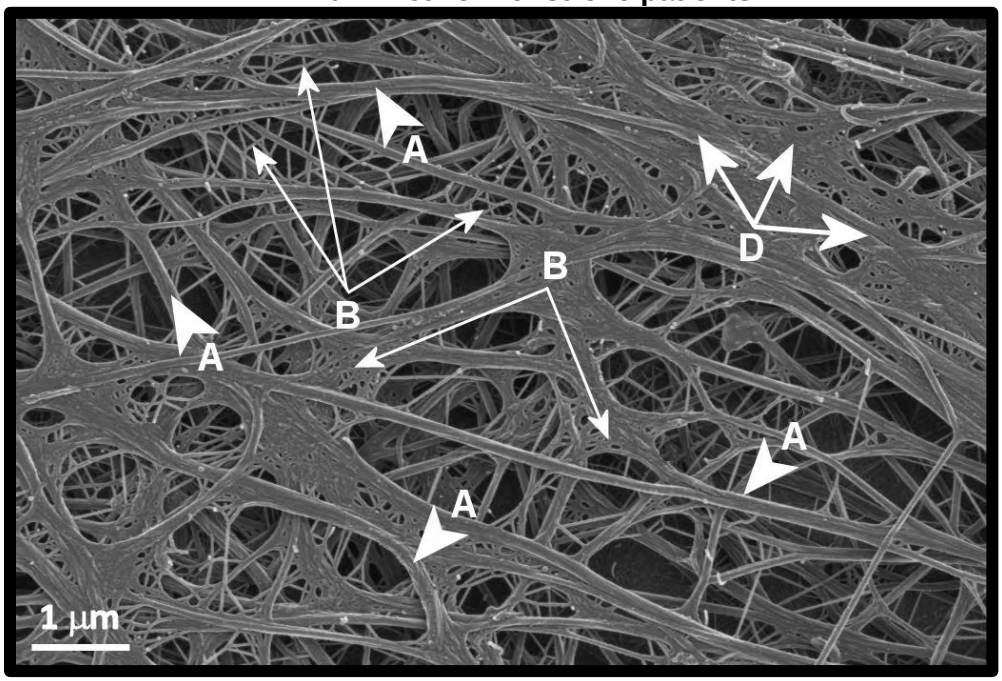
The external, structural alterations in the fibrin networks of plasma from stroke patients were analyzed by preparing the samples for scanning electron microscopy (SEM) as described in Laboratory procedures in Chapter 3.

6.4 RESULTS AND DISCUSSION

A: Control Fibrin network

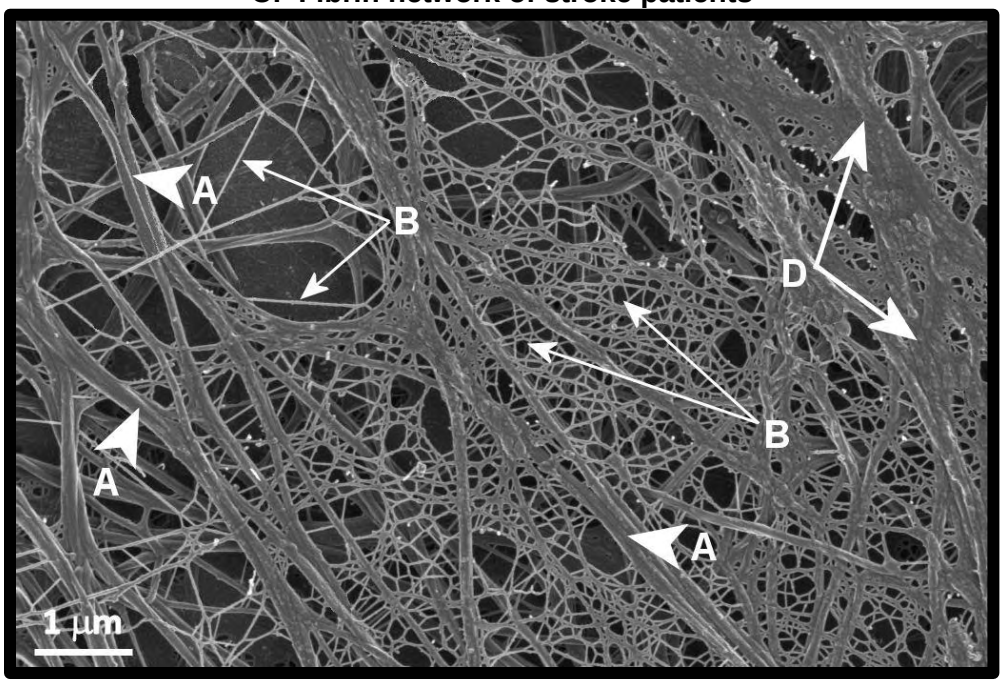


B: Fibrin network of stroke patients

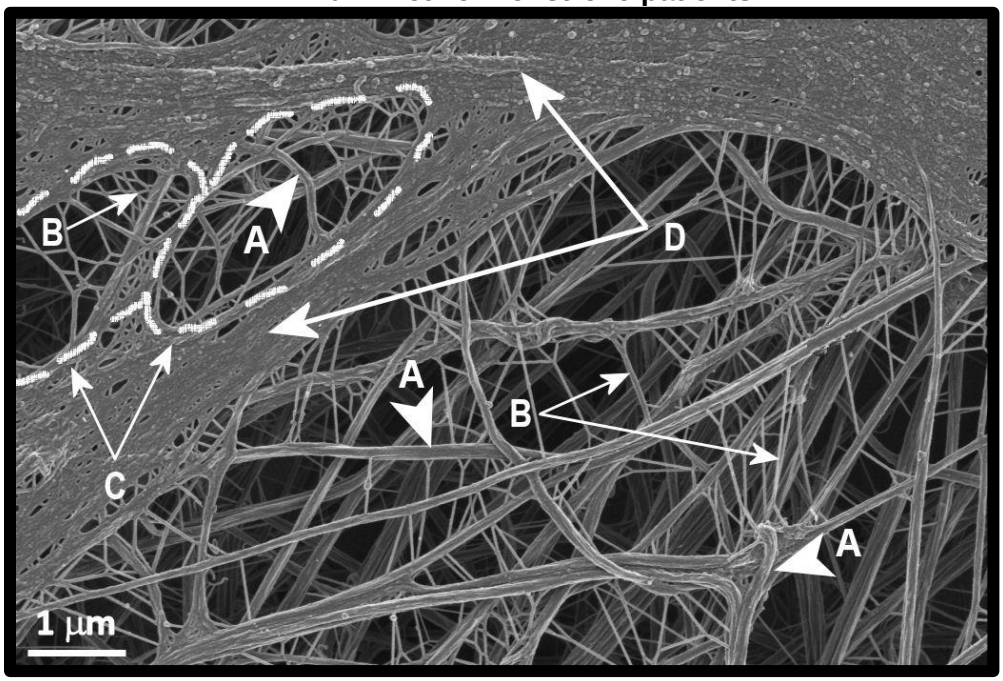


A – Thick, major fibers	**B** – Thin, minor fibers
C – Circular formations	**D** – Coagulant formation

C: Fibrin network of stroke patients

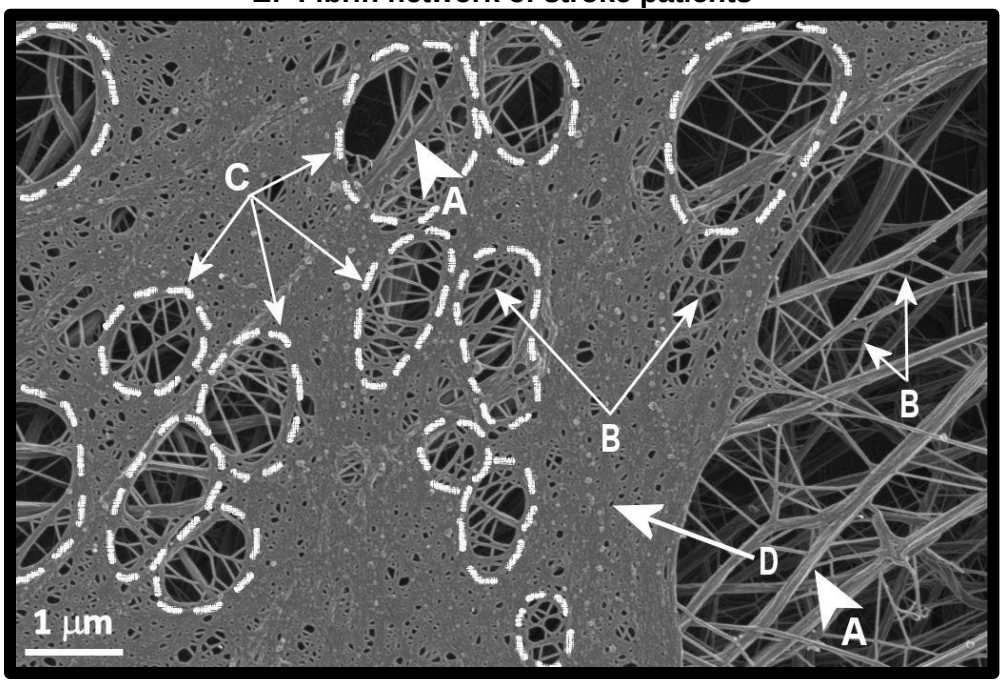


D: Fibrin network of stroke patients

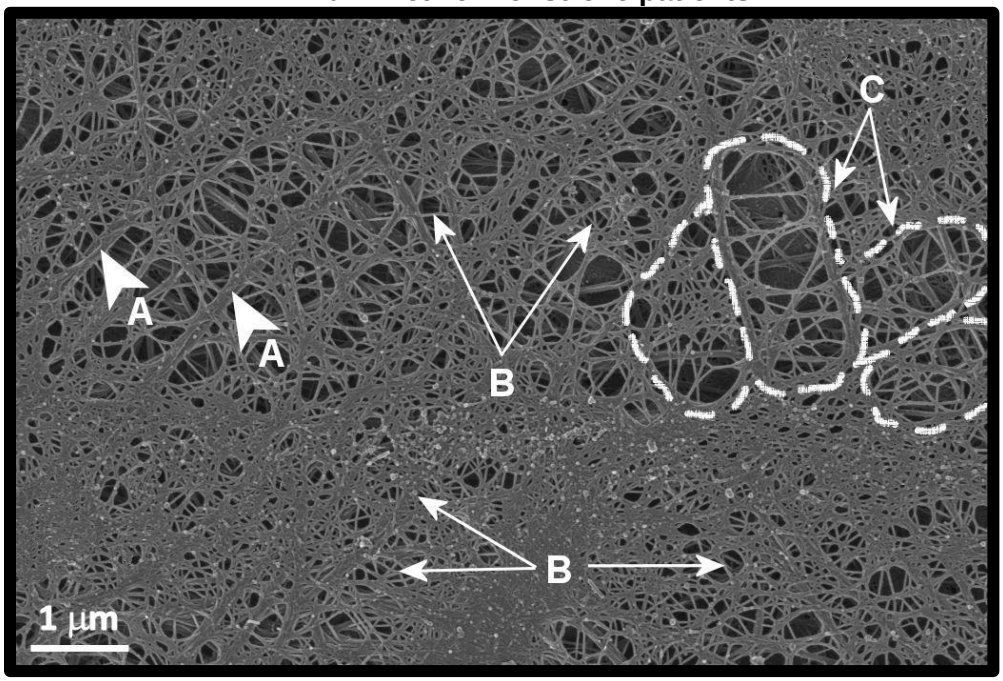


A – Thick, major fibers	**B** – Thin, minor fibers
C – Circular formations	**D** – Coagulant formation

E: Fibrin network of stroke patients

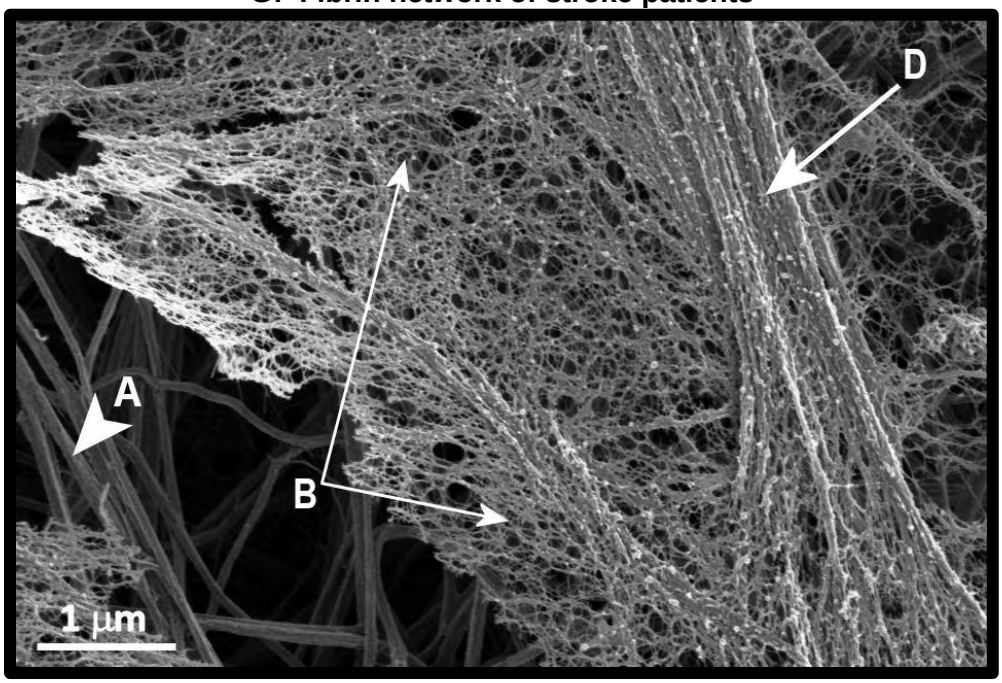


F: Fibrin network of stroke patients

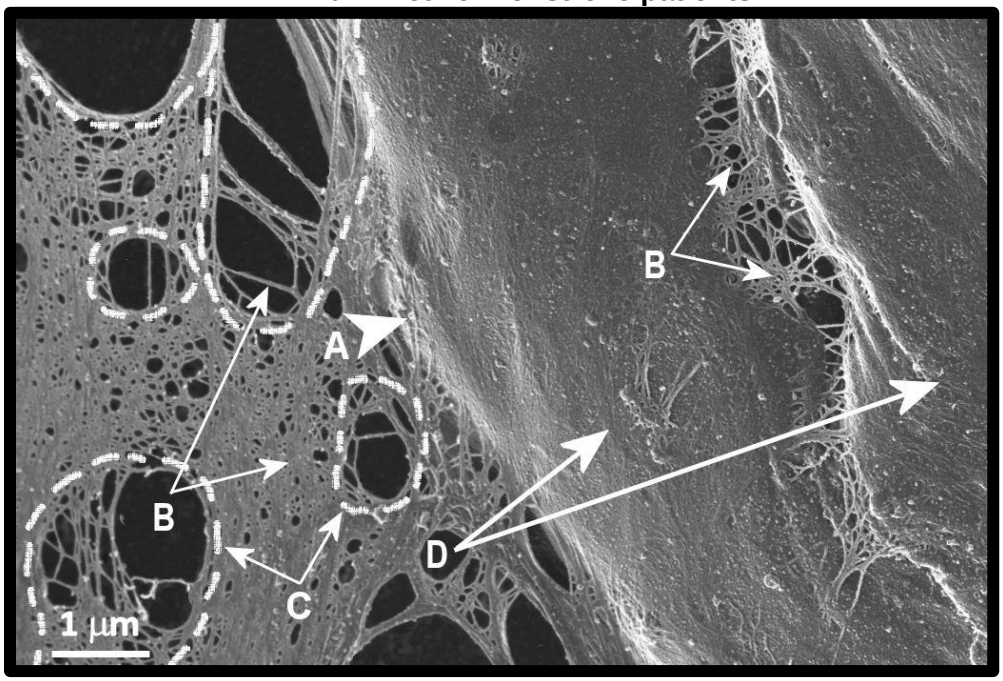


A – Thick, major fibers	**B** – Thin, minor fibers
C – Circular formations	**D** – Coagulant formation

G: Fibrin network of stroke patients

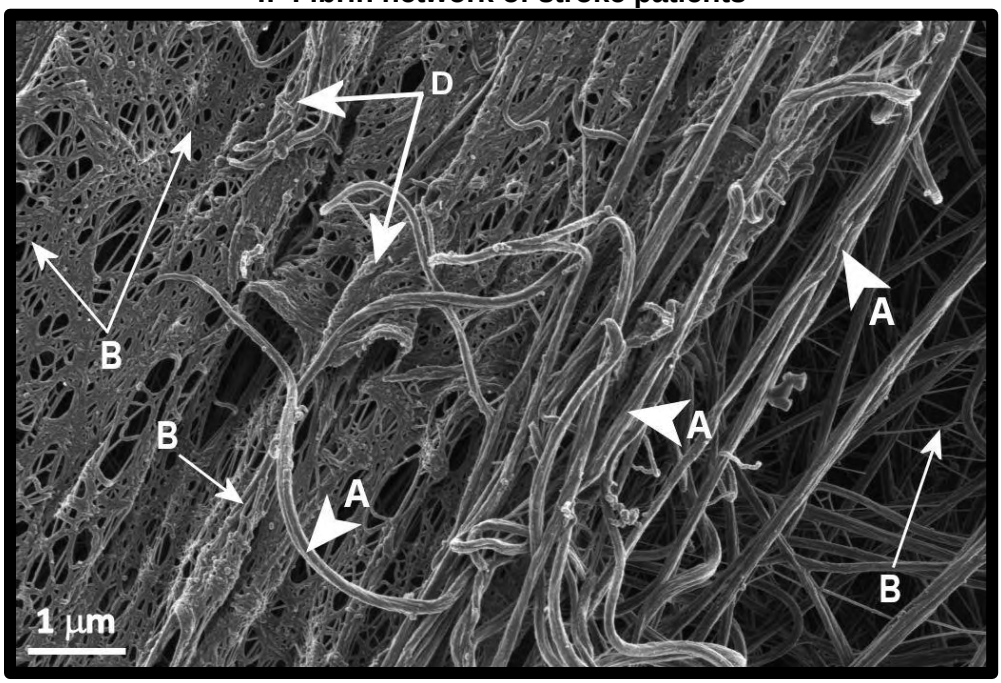


H: Fibrin network of stroke patients

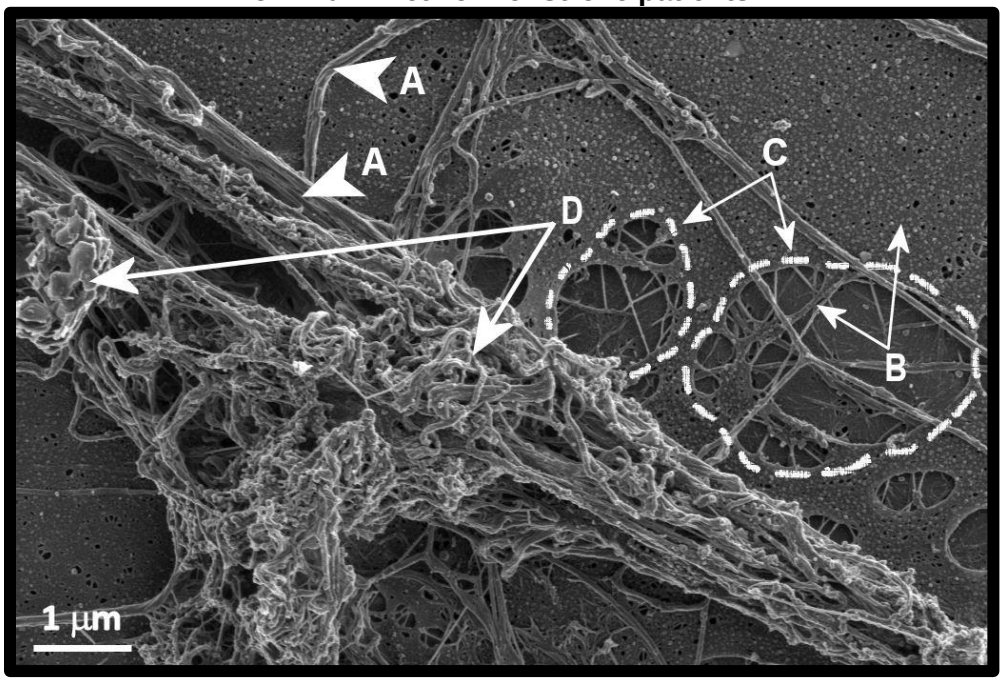


A – Thick, major fibers	**B** – Thin, minor fibers
C – Circular formations	**D** – Coagulant formation

I: Fibrin network of stroke patients



J: Fibrin network of stroke patients



A – Thick, major fibers	**B** – Thin, minor fibers
C – Circular formations	**D** – Coagulant formation

Figure 6.1. Scanning electron micrographs of fibrin networks.

A = Control; **B** to **J** = Fibrin networks found in stroke patients.

Figure 4.1.A shows a typical fibrin network found in a healthy individual. Various thick, major fibers (A) can be seen with some thin, minor fibers (B) dispersed between them. Figure 4.1.B to J is representative of fibrin morphology found in the plasma of stroke patients. Several alterations can be identified from these SEM micrographs.

In Figure 4.1.B the fibrin network is thickened giving it a layered and matted appearance. It seems that most of the thin, minor fibers become so closely packed between the thick, major fibers that the morphology becomes considerably denser than that of the control network. A significant increase in the minor, thin fibers can be identified in Figure 4.1.C. The dispersal of the minor, thin fibers is also changed and now seems like a net between the thick, major fibers. The thick, major fibers also have a dissimilar, linear appearance compared to the control. In Figure 4.1.G the minor, thin fibers are also increased, but the fibers seem thinner and more fragile. They are also more closely-packed.

Weisel discovered a unique and remarkable trait among polymers: a fibrin clot's properties pertaining to its viscoelasticity and fibrinolysis are directly affected by the branched network structure of the fibrin clot. (Weisel, 2007) This means that the strength and stability of the clot is influenced by the specific arrangement of the fibrin fibers. This also means that the rate of fibrinolysis will be affected by this same specific arrangement of the fibers.

Although the arrangement of the a fibrin clot can be affected by various entities, including fibrinogen concentration (Glover et al., 1975; Scott et al., 2004; Undas and Zeglin, 2006), several studies found that it is the thrombin concentration that has the greatest influence on the structure of the fibrin clot (Blombäck et al., 1989; Blombäck et al., 1994; Carr and Hermans, 1978).

Thick, loosely-woven fibrin fibers result from a low thrombin concentration at the time of clot formation while elevated levels of thrombin produce thinner, more tightly-packed fibrin fibers (Wolberg, 2007).

Alisa S. Wolberg reviewed thrombin generation and fibrin clot structure in 2007. She explained the influence of the fibrin clot structure on fibrinolysis in the following manner. She said that during fibrinolysis, fibrin serves as both the substrate that plasmin disintegrates as well as the co-factor enabling plasmin to perform this function optimally (Wolberg, 2007). She mentioned the various studies of Collet et al. that indicated that it is therefore evident that if the structure of fibrin is changed, the fibrin will not be as susceptible to fibrinolysis as it ought to be. Collet et al.

particularly stated that if the fibrin clot consists of loosely packed fibers, it will be lysed more effectively. (Collet et al., 1993; Collet et al., 1996; Collet et al., 2000)

In 2003 Collet and partners confirmed that the rate of fibrinolysis is impacted more by the arrangement of the fibrin network than by thickness of the fibers. (Collet et al., 2003)

Plasmin production and activity is influenced by the fibrin fiber arrangement. Studies of Collet et al. show that thick, loosely-woven fibrin fibers resulting from a low thrombin concentration consist of fewer individual fibers while thin, tightly-packed fibrin fibers consist of more individual fibers per fibrin(ogen) content. (Collet et al., 1993; Collet et al., 1996) Gabriel et al. indicated that the thick, loosely-woven fibrin fibers enhance the overall fibrinolytic activity since it advances the rate of plasmin production; whereas the thin, thickly-packed fibrin fibers cause a slower rate of plasmin production. (Gabriel et al., 1992) Since the compact three-dimensional fibrin morphology of thin, thickly-packed fibers inhibits the free movement of plasmin, thus limiting its activity, as well as the transport of proteins involved in the process of fibrinolysis (Blinc et al., 1991) the susceptibility of these clots to undergo fibrinolysis and the rate of lysis is influenced. (Carr and Alving, 1995; Collet et al., 1993; Collet et al., 1996; Gabriel et al., 1992)

This may explain the arrangement of the fibrin network found in the plasma of stroke patients. Elevated thrombin concentration could be the reason for the thin, tightly-packed morphology observed. And since the rate of production and activity of plasmin is inhibited by the compact yet fewer individual fibers, these clots will possibly not be lysed as efficiently or as quickly as necessary.

It appears that the minor, thin fibers are not only increased, but that they form circular arrangements (Figure 4.1. D, E, F, H, J).

Weisel and Nagaswami found increased thrombin and thrombospondin (TSP) concentrations independently can result in large quantities of thin fibrin fibers arranged in bundles. They also found that elevated platelet factor 4 (PF-4) concentrations result in more compact bundles of thin fibrin fibers with large pores dispersed throughout. (Weisel and Nagaswami, 1992)

Ryan et al. investigated the effect of different concentrations of thrombin on the formation of the fibrin network. They found that fiber length was decreased as the concentration of thrombin was increased. They added that at higher concentrations of thrombin, numerous short oligomers are formed prior to the occurrence of lateral expansions, giving rise to a meshwork of thin, short fibers. (Ryan et al., 1999)

There therefore exists an inverse relationship between the concentration of thrombin and the final thickness of the fibrin fibers (Bale and Mosher, 1986).

Thrombin not only catalyzes the conversion of fibrinogen to fibrin, it also initiate the aggregation and activation of platelets (Coughlin, 2001; Mann et al., 2003). Upon platelet aggregation during hemostasis, the activated platelets liberate the contents of their dense bodies and alpha granules (Kaplan, 1981). Throughout this degranulation process, a fibrin network is formed around these activated platelets since fibrinogen activation leads to polymerization (Sixma and Wester, 1977). Proteins secreted by platelets during aggregation can modify the arrangement of fibrin fibers formed in the presence of those platelets (Dhall et al., 1983).

Thrombospondin (TSP) is a one of the main proteins released from the alpha granules of activated platelets (Baenziger et al., 1971). Various studies have indicated that TSP can interact with fibrinogen (Dixit et al., 1984; Lahav et al., 1984; Leung and Nachman, 1982). During the polymerization of fibrin, TSP can become integrated in the fibrin network which results in the formation of a more fragile coagulate.

Upon their activation, platelets also release large amounts of another key protein, platelet factor 4 (PF-4), into the medium surrounding the expanding blood coagulants (Bikfalvi, 2004; Zucker and Katz, 1991). Although it has been reported that PF-4 posses both pro-coagulant as well as anti-coagulant properties, PF-4 have been demonstrated to contribute to thrombus formation (Dudek et al., 1997; Eslin et al., 2004; Slungaard et al., 2003).

The circular arrangement of fibrin fibers seen in the stroke patients could therefore probably be brought about by either increased thrombin concentration, the liberation of TSP from the activated platelets or the release of PF-4.

The circular morphology could also possibly be ascribed to inflammation, since it seems to have an almost similar appearance than that of asthmatic mice (Pretorius et al., 2007).

It appears that the altered morphology is closely associated with the thrombotic event. It appears that the condensed morphology of the thick, major fibers (Figure 4.1.H), the thin, minor fibers (Figure 4.1. C, D and E) or both the thick and thin fibers (Figure 4.1. G, I and J) form such a dense connection that it seems to form coagulants. This may be as a result of slower and ineffective lysis of the clot due to the plasmin's decreased production and hindered movement through the compact structure as described above (Carr and Alving, 1995; Collet et al., 1993; Collet et al., 1996; Gabriel et al., 1992).

6.5 CONCLUSION

Clots consisting of thin, thickly-packed fibrin fibers are more resilient to fibrinolysis than clots composed of thick, loosely-woven fibers (Wolberg, 2007).

The SEM micrographs of the stroke patients showed altered morphology compared to the control fibrin network seen in Figure 4.1.A. The overall appearance of the fibrin networks appear to be layered and matted. A greater amount of thin fibers appear to be present. This changed morphology could be the result of either elevated levels of fibrinogen or an increased concentration of thrombin present in the plasma of the stroke patients. The increased thrombin concentration will not only results in the mentioned morphology, but can also complicate and even inhibit the process of fibrinolysis of the network formed. An uncharacteristic circular morphology is also present in some of the micrographs. These altered morphologies could be ascribed to elevated concentrations of thrombin, TSP or even PF-4 present in the plasma upon platelet aggregation and activation. It is most likely that this altered morphology is present long before the occurrence of the actual thrombotic event. In conclusion, it is suggested that these ultrastructural analysis could possibly be employed as an identification procedure for imminent stroke or as an approach for monitoring the effects of different treatments.

CHAPTER 7: ULTRASTRUCTURAL ANALYSIS OF ERYTHROCYTES IN STROKE

7.1 CHAPTER OBJECTIVES

The erythrocyte morphology of stroke patients will be investigated in this chapter. Erythrocyte morphology from stroke patients will firstly be compared to normal ranges, after which the effect of selected factors on the morphology will be considered.

7.2 INTRODUCTION

Erythrocytes, also referred to as red blood cells, are responsible for the transport of respiratory gases. In the 120 days of their lifespan they travel approximately 480km around the body to ensure gaseous exchange between the lungs and the tissues. The mature erythrocyte is approximately 7.8 μm in diameter and contains no nucleus. This characteristic aids in the reduction of weight and contributes to the biconcave disk-like shape for optimal function. As erythrocytes function in the large blood vessels as well as the microcirculation of small capillaries, flexibility is an important feature.(Howard and Hamilton, 1999)

The flexibility of the erythrocyte results from the specific configuration of its cell membrane. The erythrocyte membrane consists of a membrane skeleton, integral proteins and a lipid bilayer. Proteins constitute 50% of the membrane, while fats and carbohydrates represent 40% and 10% of the membrane respectively. (Hoffbrand et al., 2003a) Abnormalities in the shape of the erythrocyte and even untimely destruction can be brought on by defects of the membrane proteins and lipids.(Howard and Hamilton, 1999) Target cells and echinocytes are examples of erythrocyte abnormalities caused by elevated levels of cholesterol and phospholipids. (Hoffbrand et al., 2003a)

7.2.1 Rheology

The discipline of the flow as well as the deformation of blood is referred to as blood rheology. The importance of blood rheology for the clinical application concern the two major components of circulatory resistance namely the vascular component and the rheological component. (Stuart and Nash, 1990)

In the large blood vessels, it is bulk flow and the viscosity of the blood (consisting of the concentration of erythrocytes as well as the viscosity of the plasma) that add to the rheology of the blood. The deformability and aggregation of the erythrocytes don't play such a big role in macrocirculation. However, when the red blood cells travel through narrow capillaries, these cells need to deform to enable them to pass through. Thus, in microcirculation, the resistance of bloodflow is mainly influenced by the ability of individual cell to deform, also referred to as cellular rheology. Cellular rheology also has an effect on the survival time of the cell in circulation. The structure of the cell, which includes the geometry of the cell, membrane properties and the viscosity of the cytoplasm, is directly associated to cell's ability to deform. Since certain haematological illnesses are connected to structural abnormalities, the blood flow in the microcirculation, as well as the lifespan of the erythrocyte, will in all probability be involved. (Stuart and Nash, 1990)

Numerous studies have confirmed the erythrocyte's reduced ability to deform in ischaemic pathological events. (Forconi et al., 1983; Forconi, 1988; Forconi et al., 1979; Hung et al., 1991)

7.2.2 Abnormal erythrocyte morphologies

Sheetz and Singer (Sheetz and Singer, 1974) as well as Evans (Evans, 1974) proposed a model to possibly explain the transformation of discocytes to either stomatocytes or echinocytes. According to their bilayer-coupled hypothesis, convex structures, such as the distinguished spicules of echinocytes, form when any factor causes the outer membrane leaflet to expand relative to the inner leaflet. In contrast cavities are formed when inner membrane leaflet expand relative to the outer leaflet, and the extra area has to be accommodated, leading to the formation of stomatocytes. (Evans, 1974; Sheetz and Singer, 1974) From a membrane mechanics viewpoint, it is the variation between the two membrane leaflets, concerning area as well as tension, which instigate the advance of a considerable spontaneous curvature of the lipid bilayer (Petrov, 1999).

The rheological characteristics of red blood cells can be negatively influenced by various factors. Extracellular factors include the concentration of cholesterol, fibrinogen and gamma-globulins found in the plasma. Factors connected with the structural conformation of the cell membrane as well as the intracellular levels of ATP, which are inversely proportional to the cytosolic concentration of calcium, also impair the rheology of the erythrocyte. (Forconi et al., 1990; Forconi et al., 1992)

The altered erythrocyte morphology can thus be the result of either elevated levels of coagulation factors (like fibrinogen) as implicated in chapter 5 or the influence of leukocyte activation as described in chapter 7.

White blood cells (WBC) can indirectly cause alterations in the cell membrane. When white blood cells (WBC) or leukocytes are activated they release proteases and oxygen metabolites which in turn have proteolytic and oxidative effects on the membrane of the erythrocytes. These membrane alterations lead to destruction of the erythrocyte membrane, called hemolysis. (Santos-Silva et al., 2002)

7.2.3 Erythrocytes and stroke

Santos- Silva and colleagues found that not only the number of white blood cells is elevated in ischaemic stroke patients, but also that these white blood cells are activated. (Santos-Silva et al., 2002) When these large numbers of leukocytes are activated, they all release activation products. These activation products then affect adjacent cells like the erythrocytes. As the erythrocytes undergo oxidative and proteolytic changes resulting from their contact with the activation products, they loose their flexibility and build up in the narrow blood vessels of the microcirculation. Blood flow will be slowed resulting in a prolonged period of contact between the activation products accumulating at the blockage and the adjoining cells. (Santos-Silva et al., 2002)

According to Steadman's Medical Dictionary erythrocyte sedimentation rate (ESR) refers to "the rate of settling of red blood cells in anticoagulated blood". (Stedman's Medical Dictionary 2006, p. 1639) They continue to say that an inflammatory state is implicated by an increased erythrocyte sedimentation rate. (Stedman's Medical Dictionary 2006, p. 1639) Chamorro et al. implicated an elevated erythrocyte sedimentation rate in the critical damage caused by stroke. (Chamorro et al., 1995)

7.3 MATERIALS AND METHODS

Samples of the erythrocytes from the blood pellet of stroke patients were prepared for both scanning electron microscopy (SEM) and transmission electron microscopy (TEM) investigation. The specific procedures are explained in Chapter 3 under Laboratory procedures.

7.4 RESULTS AND DISCUSSION

Changes of the external surface of the erythrocytes were examined using scanning electron microscopy (SEM) while the internal structures were investigated using transmission electron microscopy (TEM). A discussion of the SEM and TEM results will firstly be explained followed by the statistical analysis.

7.4.1 Scanning electron microscopic investigation

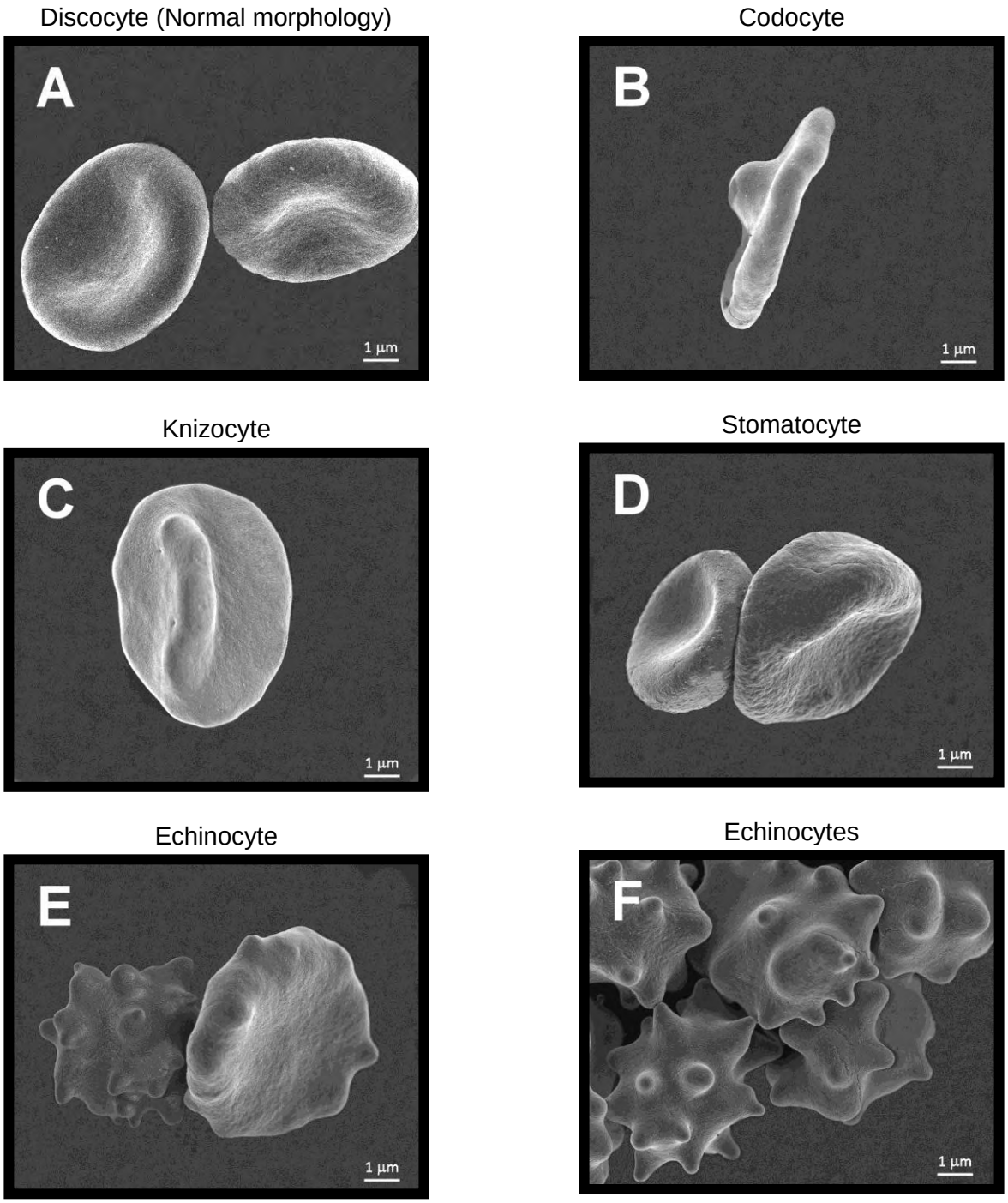


Figure 7.1. Scanning electron micrographs of erythrocyte morphologies.

A = Discocytes (normal morphology), **B** = Codocyte, **C** = Knizocyte, **D** =Stomatocyte, **E, F** = Echinocytes

The SEM micrographs revealed abnormal erythrocyte morphology. Table 7.1 summarizes the different types of erythrocyte abnormalities as seen in the blood samples of the stroke patients (Faculty of Health Sciences, Stellenbosch University, 2010).

Table 7.1. Erythrocyte morphological abnormalities.

Description	Characteristic Shape	Criteria: Scanning electron microscopy
Discocyte (Normal)	Discoid Biconcave	Shallow but visible round depression in central portion of cell
Codocyte (Target cell)	Bell-shaped with considerable reduction in thickness	Single concavity with extruded opposite side or flattened ring around elevated central portion of cell
Knizocyte	Pinched Appear to contain a central stick Presence of 2 or more invaginations on the membrane	Triconcave depression or cell with pinched area in centre
Stomatocyte	Cup-shaped with slot-like central pale area (central linear slit or stoma)	Swollen cell periphery with smaller concavity or concavity flattened on one side, indicating the beginning of sphering
Echinocyte	Red cell covered with 10-30 short Fairly regularly spaced projections or spicules of regular form	Deformed and angular cell periphery with spicule formation

(Faculty of Health Sciences, Stellenbosch University, 2010)

7.4.2 Transmission electron microscopic investigation

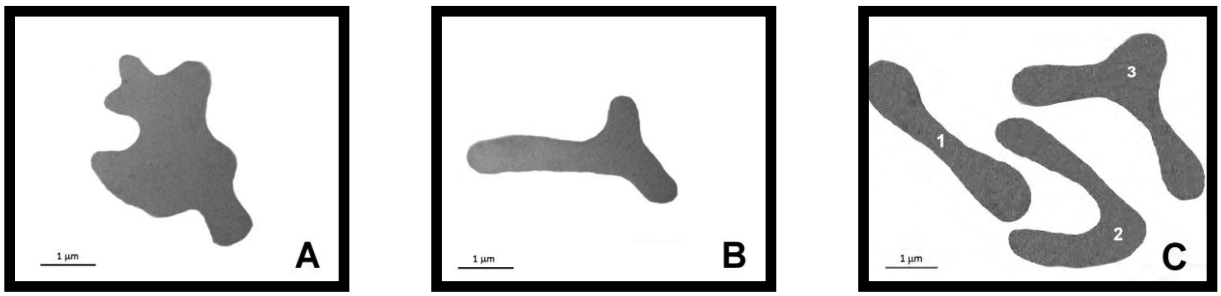


Figure 7.2. Transmission electron micrographs of erythrocyte morphologies.

A = Echinocyte, **B** = Knizocyte,

$\mathbf{C_1}$ = Discocyte (normal morphology), $\mathbf{C_2}$ = Stomatocyte, $\mathbf{C_3}$ = Codocyte

TEM micrographs did not show any particular internal structures within the erythrocytes. The micrographs did however confirm the different abnormal erythrocyte shapes as were seen in the SEM micrographs.

7.4.3 Statistical analysis

Turchetti and co-workers studied the variations of erythrocyte morphology in different pathologies. They found that in healthy control individuals, the mean blood proportions were as follows: 55% of the erythrocytes were that of the most deformable, bowl-shaped morphology; 44% were that of the more rigid discocytes; and only 1% of erythrocytes had altered morphology, mainly that of echinocytes and knizocytes. (Turchetti, 1997)

For statistical analysis of the amount of abnormal erythrocytes found in the blood of stroke patients, 10 SEM micrographs of each patient were taken on low magnification. In these micrographs up to a 100 erythrocytes were counted. Morphology was categorized as either normal or abnormal. The abnormal morphology included codocytes (target cells), knizocytes, stomatocytes and echinocytes. Percentages were calculated and analysis was done using the statistical program NCSS with the level of significance set at 0.05.

Firstly a t-test was done to compare the data from the erythrocyte counts of stroke patients were to normal ranges as described by Turchetti et al in 1997. The expected standard abnormal

percentage of erythrocytes found in the blood of healthy individuals (1%) was compared to the average percentage of abnormal erythrocytes found in the blood of the stroke patients (92%). The t-test showed that the stroke patients had a significantly larger percentage of abnormal erythrocytes compared to the normal levels found in healthy control individuals.

Secondly, certain factors were assessed to determine their effect on the erythrocyte counts in stroke patients. Some factors investigated had a significant influence on the percentage of abnormal erythrocytes while others had no significant effect.

7.4.3.1 Hypertension vs. Normal blood pressure

The parametric two-sample t-test with a significance of 0.05 was used to compare hypertensive patients (n = 10) with non-hypertensive patients (n = 9); the hypotensive patient was not included in this assessment. Nine hypertension patients were randomly selected and compared with the nine normal blood pressure patients of the study to overcome the lack of equal variance in this set of data. Normotensive patients showed a significantly increased amount of abnormal erythrocytes compared to patients with hypertension.

Shear stress refers to the force of friction from a fluid acting on a body in the path of that fluid (Shear Stress, 2006). Shear stress can thus be interpreted as the effect that plasma friction force has on blood cell, including erythrocytes.

In macrocirculation as well as microcirculation there exists a significant rheological occurrence – the erythrocytes membrane continuously rotates around the liquid content of the cell. This causes the external shear stress to be transferred to the cytoplasm through the cell membrane. The cytoplasm surges in a circular motion within the cell as the membranes' lipid-protein planes alternate sliding forward and backward. The rheological behaviour of red blood cells is thus controlled by the microviscous attributes of the cell membrane and cytoplasm. (Dormandy, 1983; Forconi et al., 1987; Stoltz, 1983)

Leverett et al. found that the variation in the force and exposure time of shear stress significantly influence the amount of damage to the erythrocytes in circulation. They indicated that little damage occurs when both the force and exposure time of shear stress is low since the damage is subject to the effects of solid surface interaction. Hemolysis dominantly occurs when the force of the shear stress is very high over an extended period of time. (Leverett et al., 1972)

This occurrence of a greater percentage of abnormal erythrocytes in the non-hypertensive patients could possibly be ascribed to their erythrocytes' "naivety" to external shear pressure. It is possible that the erythrocytes in the blood of hypertensive patients are exposed to relatively low shear force pressure over an extended period of time while the erythrocytes of normotensive patients experience almost no shear force pressure. When the stroke occurs, the force of the shear pressure is suddenly drastically increased. It is possible that the erythrocytes of normotensive patients are not accustomed to the sudden shear force leading to greater damage to the erythrocytes. Since the hypertensive patients' erythrocytes are probably accustomed to some force, even though it is not as great as the force accompanied by the stroke, they are not as damaged as those of non-hypertensive patients.

7.4.3.2 Gender

Since the group of 20 stroke patients consisted of 15 females and only 5 males, equal variance between the two gender groups of unequal size was accomplished by using a two-sample t-test with a significance set at 0.05. This test indicated that females have a greater percentage of abnormal erythrocytes than males. However, these results should be taken with caution since the sample size of the male patients was much smaller than that of the female patients.

Androgens have a two-fold effect on the hematopoietic system. These male hormones stimulate erythrocyte production through androgen-dependent, receptor-mediated synthesis of erythropoietin. Androgens are also involved in the increased synthesis of hemoglobin by directly influencing hematopoietic stem cells (Weinbauer et al., 2010). Androgenic hormones are therefore considered as a specific stimulating factor of erythropoiesis (Kennedy and Gilbertsen, 1957).

It is therefore possible that male patients exhibited less abnormal erythrocytes than females since androgens instigate the production of erythrocytes resulting in more rapid production of new, healthy erythrocytes after the stroke occurrence.

7.4.3.3 Other factors

The other factors investigated included the effect of age, gender, hypertension in conjunction with elevated cholesterol, the daily use of aspirin, a previous stroke as well as smoking. All of these factors did not have a significant effect on the percentage of erythrocyte abnormality.

Table 7.2. is a summary of the statistical analysis of the two factors that had a significant effect on the erythrocyte morphology while Table 7.3. shows the factors that did not affect the morphology of the erythrocytes.

Table 7.2. Factors with a significant effect on the erythrocyte counts of stroke patients.

Factor	Groups	Statistical test employed	P value
Hypertension	Hypertensive vs. normal blood pressure	Two-Sample T-test[1]	0.0169
Gender	Male vs. Female	Two-Sample T-test[2]	0.858

[1] The null hypothesis used was that no difference existed in terms of erythrocyte abnormalities between the two compared groups – hypertension (n = 10) and normal blood pressure (n = 9).

[2] The null hypothesis used was that no difference existed in terms of erythrocyte abnormalities between males (n = 5) and females (n = 15).

Table 7.3. Factors with no significant effect on the erythrocyte count of stroke patients.

Factor	Groups	Statistical test employed	P value
Age	Under 65 years of age vs. over 65 years of age	Two-Sample T-test	0.858
Hypertension and cholesterol	Hypertension and cholesterol vs. hypertension alone	Two-Sample T-test	0.485
Aspirin[1]	Using aspirin vs. not using aspirin	Two-Sample T-test	0.548
Previous stroke[2]	Previous stroke vs. first time stroke	Two-Sample T-test	0.776
Smoking[3]	Smoking vs. previous smoking vs. non-smoking	One-way ANOVA	0.182

[1] Results should be taken with caution due to the small aspirin consuming population size (n = 4) in comparison to the much larger non-aspirin consuming population size (n = 16).

[2] Results should be taken with caution due to the small population size of patients who suffered a previous stroke (n = 3) in comparison to patients who suffered their first stroke (n = 17).

[3] Results should be taken with caution due to the small population size of patients who are current smokers (n = 3) and previous smokers (n = 2) in comparison to the non-smoking patients (n = 15).

7.5 CONCLUSION

In several pathologies, the morphology of the erythrocyte is changed, resulting in serious impairment of the flow of erythrocytes in the microcirculation (Forconi et al., 1987; Stoltz, 1983).

Stroke patients presented with a large amount of erythrocytes with abnormal morphology which include codocytes, knizocytes, stomatocytes and echinocytes. Furthermore it appears that high blood pressure and gender play a role in the development of these abnormal erythrocytes while other stroke risk factors like age, smoking and a previous stroke do not influence the formation of abnormal erythrocytes.

CHAPTER 8: DIFFERENTIAL WHITE BLOOD CELL COUNT IN STROKE

8.1 CHAPTER OBJECTIVES

This chapter will focus on the differential white blood cell counts of stroke patients. Firstly, the differential white blood cell counts of the stroke patients will be compared to standard ranges. Secondly, selected factors will be compared to investigate their effect on the differential white blood cell counts.

8.2 INTRODUCTION

The term 'white blood cells' is used to describe all the cells of the blood that are nucleated. This group of blood cells, which are also called leukocytes, can be further divided into granulocytes and agranulocytes based on their morphology.(Howard and Hamilton, 1999) Three types of granulocytes can be identified namely, the neutrophils, eosinophils and the basophils. These cells together with the monocytes (which are agranular in structure) all play a defending role in the body. (Hoffbrand et al., 2003b) Cells that are damaged or any foreign particles are enclosed and eliminated by the granulocytes and monocytes in the process of phagocytosis. (Howard and Hamilton, 1999) The other type of agranulocyte found in the blood is the lymphocytes which are involved in the immune reactions of the body. In normal peripheral blood will only contain mature granulocytes, monocytes and lymphocytes. (Hoffbrand et al., 2003b)

8.2.1 Morphological classification of leukocytes

Under light microscope, the different types of leukocytes can by identified according to specific morphological traits.

Neutrophils are the most abundant of the blood leukocytes. They are characterized by multi-lobed dense nucleus enveloped by pale cytoplasm. Fine azurophillic (pink-blue) or grey-blue granules found in the cytoplasm, can be classified as either secondary of specific depending on their function. These cells circulate in the blood for approximately 10 hours where they will remain until they are summoned to the location of tissue injury or infection to terminate foreign material. These processes of migration and destruction are referred to as chemotaxis and phagocytosis respectively.

Monocytes have clumped chromatin arranged in either a large central oval or indented nucleus. The cytoplasm has the appearance of ground glass from the many fine vacuoles in the blue staining cytoplasm. Granules can also be found in the cytoplasm. Monocytes circulate for 20-40 hours in the blood before migrating into the tissue where they differentiate into macrophages.

Eosinophils contain coarse granules which stain deep red. Their nuclei possess no more than three lobes. Like neutrophils, they are involved in chemotaxis and phagocytosis. In the tissue they target foreign material too large for normal phagocytosis by secreting their cytotoxic enzymes. They also play a special role in allergic responses, defend the body against parasites and can also assist in the removal of fibrin formed during inflammation.

Basophils are the least numerous of all the blood leukocytes. Their main feature is the large numbers of dark purple granules in the cytoplasm overlying the nucleus. These granules contain histamine and heparin, both mediators of acute inflammation. These cells and their tissue equivalent, mast cells, are partakers in reactions of immediate hypersensitivity.

Lymphocytes appear as small round cells, with a round nucleus enclosed in an agranular thin border of cytoplasm. They are responsible for resistance against infection and other foreign invasion.

8.2.2 White blood cells and stroke

Cerebral ischaemia is in all probability brought on by inflammation of not only the central nervous system (CNS) but also the peripheral nervous system (PNS) (Rost et al., 2001). Inflammation of the nervous system as a whole is characteristic of the pathophysiology of ischaemic stroke (Emsley and Tyrrell, 2002).

Several studies have indicated that an elevated total peripheral white blood cell (WBC) count is one of many peripheral markers associated with poor outcome after stroke (Azzimondi et al., 1995; Chamorro et al., 1995; Di Napoli et al., 2002; Muir et al., 1999; Vila et al., 1999).

According to both Pozzilli et al. and D'Erasmo et al. for up to three days after a stroke, the peripheral white blood cell (WBC) count can be elevated (D'Erasmo et al., 1991; Pozzilli et al., 1985). Not only the overall peripheral WBC count but specifically also the neutrophil count have been reported to reach extreme highs, up to a two-fold increase compared to healthy control subjects (Santos-Silva et al., 2002), in ischaemic stroke patients (Emsley et al., 2003).

Monocytes can, upon activation, initiate the early pathogenic processes of ischaemic stroke and then continue to play a pathophysiological role in the subacute stage after stroke. (Kochanek and Hallenbeck, 1992a)

Galante and associates warned against the effects of granulocytes, which include neutrophils, on thrombus formation. They stated that granulocytes are implicated in thrombus formation as well as the aggregation of the thrombi. (Galante et al., 1992)

Marder et al.,found in 2006 that thrombi obtained from patients who suffered an acute ischaemic stroke, contained specifically neutrophils and monocytes in linear clusters. (Marder et al., 2006) As leukocytes interact with platelets to form aggregates, strong associations form between specifically platelets-and-monocytes and platelets-and-neutrophils (Htun et al., 2006; McCabe et al., 2004).

8.3 MATERIALS AND METHODS

Blood smears were prepared for the differential white blood cell count under light microscope as described in the Laboratory procedure section in Chapter 3.

8.4 RESULTS AND DISCUSSION

Firstly the morphological classification as seen under Light microscope (LM) will be shown followed by the statistical analysis of the differential white blood cell (WBC) counts.

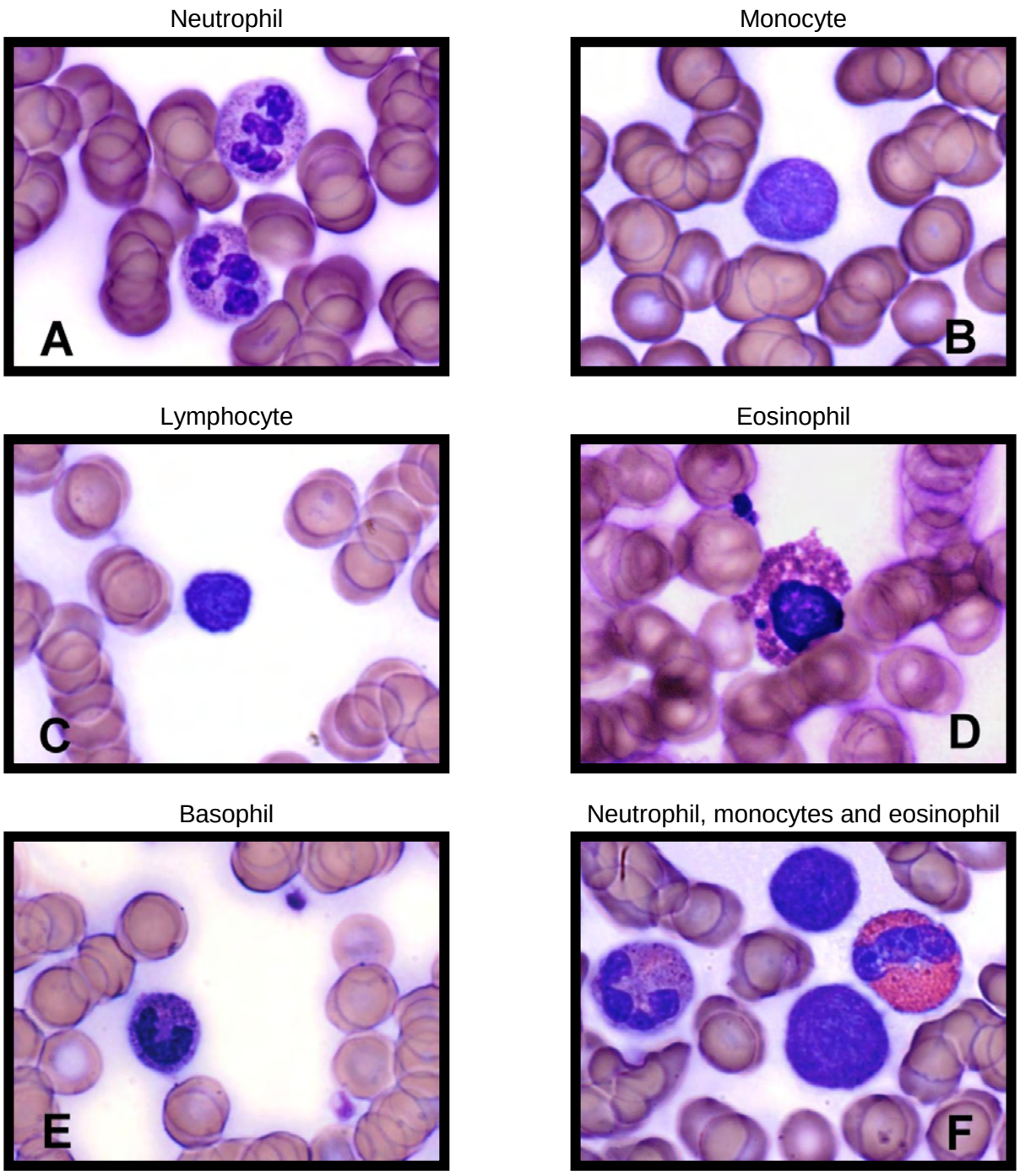


Figure 8.1. Different WBC morphologies.

A = Neutrophils, **B** = Monocyte, **C** = Lymphocyte, **D** = Eosinophil, **E** = Basophil,
F = (from the left) a Neutrophil, two Monocytes and one Eosinophil

8.4.1 Statistical analysis

According to the Schilling classification of the differential white cell count, normal ranges can be assigned to each type of white blood cell. Table 8.1 is a summary of these ranges.

Table 8.1. Schilling Classification of the Differential White Cell Count.

Cell	Normal %
Neutrophilic segmented cells	55-75
Lymphocytes	20-35
Monocytes	2-6
Eosinophilic segmented cells	1-3
Basophilic segmented cells	0-1

(Hospital Corpsman Revised Edition, 2010)

To determine the percentages for each type of white blood cell in each of the stroke patient, up to a hundred white blood cells were counted on each of the three blood smears made for each patient. White blood cells were categorized as neutrophils, monocytes, lymphocyte, eosinophils or basophils. Percentages were calculated and analysis was done using the statistical program NCSS with a significance level of 0.05.

Firstly a parametric one-sample t-test (for neutrophils, monocytes, lymphocytes and basophils) as well as the non-parametric Wilcoxon signed rank test (for the eosinophils with a normal distribution pattern) was done to compare the data from the differential white blood cell counts of stroke patients to normal ranges as described in Table 8.1. The analysis showed that the stroke patients had a significantly larger percentage of monocytes and also a significantly smaller percentage of neutrophils when compared to the normal ranges assigned in Table 8.1. The monocyte counts (Figure 8.2.) and neutrophil counts (Figure 8.3) of the stroke patients are represented in the graphs below. The patient numbers are in accordance to the numbers assigned to the patients in Table 3.1. in Chapter 3.

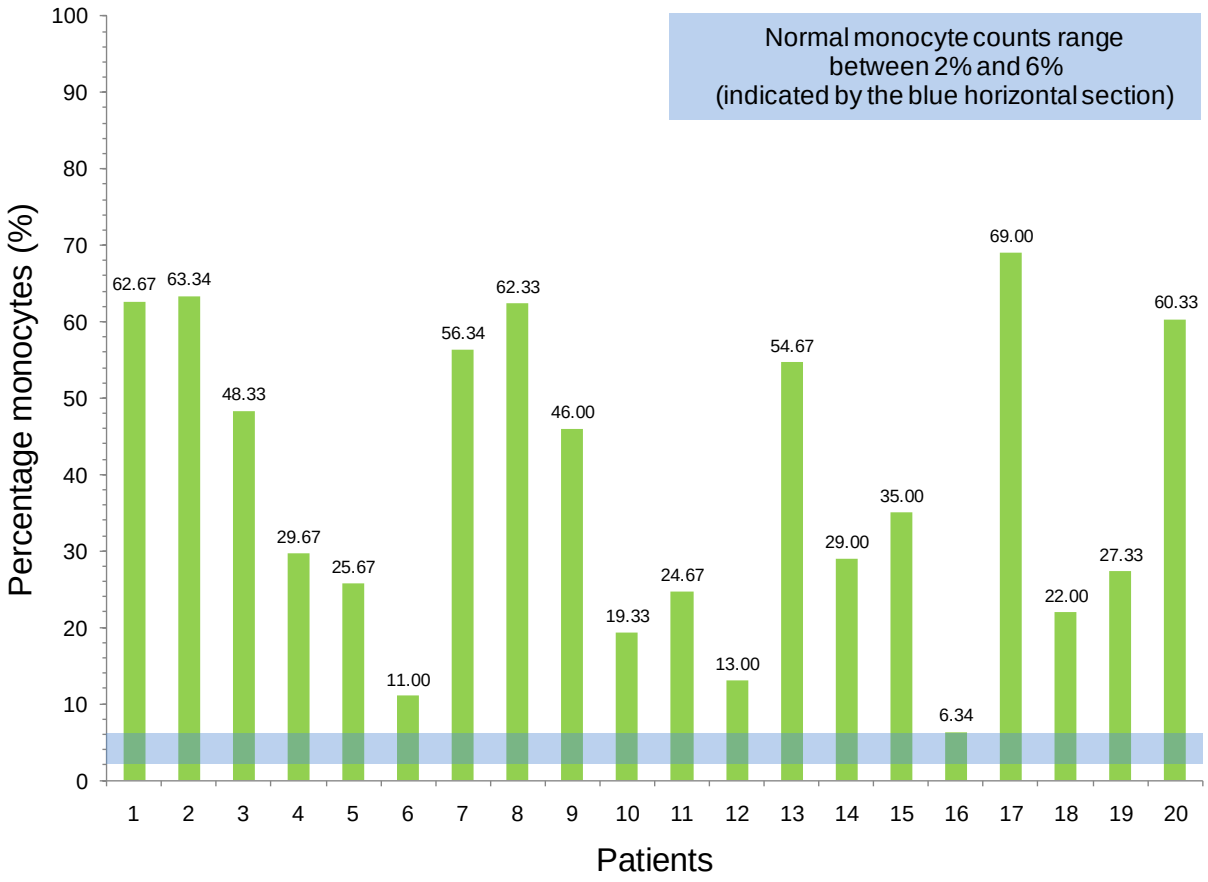


Figure 8.2. Monocyte counts of stroke patients.

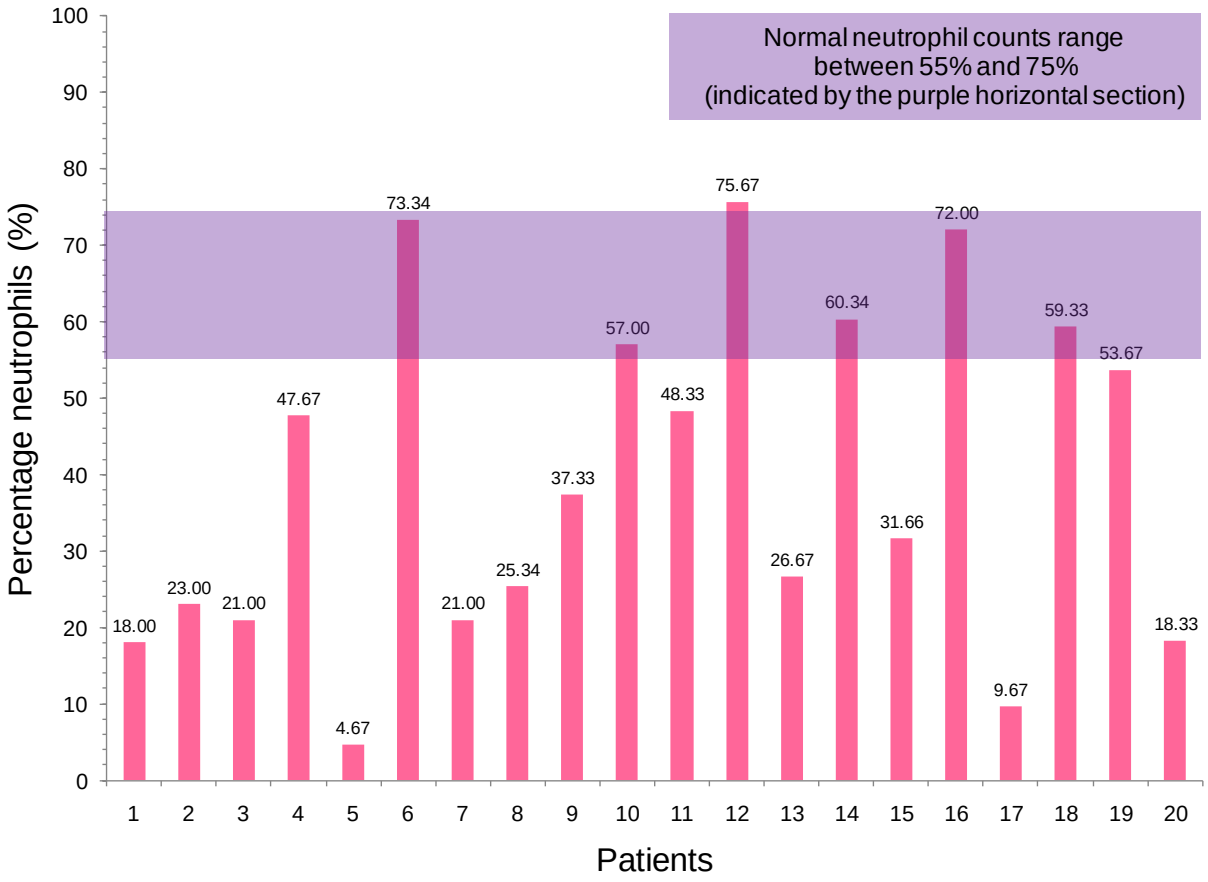


Figure 8.3. Neutrophil counts of stroke patients.

Monocytosis is defined as an abnormal increase in the number of monocytes in the circulating blood. (Stedman's Medical Dictionary 2006, p. 1223) Monocytes are closely related to the immune system since they are the precursor cells of macrophages. Macrophages are involved in the break down of erythrocytes in the spleen as well as extravascular hemolysis. Often hemolytic states are associated with monocytosis. Macrophages also play a role in granulomatous inflammation. (Beck, 2009) Therefore monocytes are closely associated with the immune system, inflammation as well as hemolysis.

Stedman's Medical Dictionary defines neutropenia as the presence of abnormally small numbers of neutrophils in the circulating blood. (Stedman's Medical Dictionary 2006, p. 1317) The chemotactic activity of neutrophils is specifically inhibited by elevated fibrinogen levels. Fibrinogen is also implicated in inflammatory response regulation. (Higazi et al., 1994) Neutropenia can therefore be brought on by an increased fibrinogen concentration that is associated with stroke.

After analyzing the different white blood cells separately, selected factors were evaluated to establish their effect on the differential white blood cell counts in stroke patients. Some factors significantly influenced the percentages of the differential white blood cell counts while others had no noteworthy effect on the percentages.

8.4.1.1 Hypertension vs. Normal blood pressure

Hypertensive patients (n = 10) were compared with non-hypertensive patients (n = 9) with the use of the parametric two-sample t-test with a significance of 0.05. This assessment did not include the hypotensive patient. To overcome the lack of equal variance in the set data, nine hypertension patients were randomly selected to be compared with the nine normal blood pressure patients of the study. The test indicated that normotensive patients had significantly more monocytes compared to hypertensive patients, while other types of white blood cells were not significantly increased of decreased.

Hypertension is associated with inflammation. Since inflammation influences endothelial function and also alters rheological properties an increased WBC count may augment the hypertensive state. (Nakanishi et al., 2002)

Cozel et al. found that hypertension causes subendothelial accumulation of monocytes. (Clozel et al., 1991) Hypertension could possibly also increase monocyte adhesion by augmenting the sensitivity of the endothelium for adhesion. (Dorffel et al., 1999)

The lower monocyte count in the hypertensive patients when compared to the normotensive patients could therefore be attributed to a possible accumulation of monocytes in the subendothelium resulting in fewer monocytes in the peripheral blood.

8.4.1.2 Aspirin

To investigate the effect that aspirin consumption has on the differential white blood cell count in stroke patients, the two-sample t-test (for neutrophils, monocytes, lymphocytes and basophils) and Mann-Whitney U test (for eosinophils that did not meet equal variance) was used. Since the eosinophils did not meet equal variance four random patients from the group that did not consume aspirin were selected; the other leukocyte groups did meet equal variance thus all sixteen patient who do not consume aspirin were consumed. The tests indicated significantly increased numbers of lymphocyte in the patients who do not consume aspirin compared to non-aspirin consuming individuals, while the other types of white blood cells did not show any significant increase or decrease in numbers.

Platelets are not only involved in blood coagulation, they also play a significant role in inflammation. Activated platelets attach to lymphocytes circulating in the blood and acts as a bridge for these attached lymphocytes to enter the lymphoid system. (Wang and Niu, 2008)

Aspirin can diminish the probability of cardiovascular patients to suffer an ischaemic event. Aspirin binds irreversibly to cyclo-oxygenase and blocks TXA_2 synthesis. In this way, aspirin inhibits platelet action. (Wong et al., 2004)

Aspirin-like drugs (ALD) have also been shown to initiate to some extent the activation of particularly T-lymphocytes. (Flescher et al., 1995)

It can therefore be assumed that the platelets of the aspirin-consuming patients are possibly not as active as the platelets of patients not using aspirin. This may influence these platelets' binding to the activated lymphocyte in the blood circulation, leading to probably more lymphocytes remaining in circulation and less lymphocytes entering the lymphoid system.

Table 8.2 is a summary of the statistical analysis of the two factors that had a significant effect on the erythrocyte morphology.

Table 8.2. Factors with a significant effect on the differential WBC counts of stroke patients.

Factor	Groups	Leukocytes	Statistical test employed	P value
Hypertension	Hypertensive vs. normal blood pressure	Monocytes	Two-Sample T-test	0.036
Aspirin	Using aspirin vs. not using aspirin	Lymphocytes	Mann-Whitney U	0.047

8.4.1.3 Other factors

The other factors investigated included the effect of age, gender, hypertension in conjunction with elevated cholesterol, the daily use of aspirin, a previous stroke as well as smoking. All of these factors did not have a significant effect on the percentage of the differential white blood cells counts.

Table 8.3 is a summary of the factors that had no significant effect on the differential white blood cell count.

Table 8.3. Factors with no significant effect on the differential WBC count of stroke patients.

Factor	Groups	Leukocytes	Statistical test employed	P value
Age	Under 65 years of age vs. over 65 years of age	Monocytes	Two-Sample T-test	0.367
		Lymphocyte	Two-Sample T-test	0.248
		Eosinophil	Mann-Whitney U	0.084
		Neutrophil	Two-Sample T-test	0.209
		Basophil	Mann-Whitney U	0.112
Hypertension and cholesterol	Hypertension and cholesterol vs. hypertension alone	Monocytes	Two-Sample T-test	0.927
		Lymphocyte	Two-Sample T-test	0.435
		Eosinophil	Two-Sample T-test	0.828
		Neutrophil	Two-Sample T-test	0.748
		Basophil	Two-Sample T-test	0.808
Gender	Male vs. Female	Monocytes	Two-Sample T-test	0.782
		Lymphocyte	Mann-Whitney U	0.861
		Eosinophil [1]	Two-Sample T-test	0.795
		Neutrophil	Two-Sample T-test	0.876
		Basophil	Mann-Whitney U	0.680
Previous stroke	Previous stroke vs. first time stroke	Monocytes	Mann-Whitney U	0.711
		Lymphocyte	Mann-Whitney U	0.368
		Eosinophil	Mann-Whitney U	0.573
		Neutrophil	Two-Sample T-test	0.906
		Basophil	Mann-Whitney U	0.119
Smoking	Smoking vs. previous smoking vs. non-smoking	Monocytes	One-way ANOVA	0.736
		Lymphocyte	Kruskal-Wallis One-way ANOVA	0.807
		Eosinophil	Kruskal-Wallis One-way ANOVA	0.576
		Neutrophil	One-way ANOVA	0.749
		Basophil	Kruskal-Wallis One-way ANOVA	0.304

[1] Gender: In the case of the eosinophils, due to the lack of equal variance, five randomly selected females where compared with the five male stroke patients

8.5 CONCLUSION

Inflammation is a trait of the pathophysiology of ischaemic stroke. (Emsley and Tyrrell, 2002)

The stroke patients presented with elevated monocyte count and decreased neutrophil count when compared to standard ranges. Additionally, it appears as if hypertension and the daily use of aspirin before the stroke may have an effect on the differential white blood cell, specifically the monocytes and lymphocytes respectively. Other factors including age, gender, elevated cholesterol associated with hypertension, a previous stroke and also smoking appear to have no effect on the differential white blood cell count.

CHAPTER 9: CONCLUSION

The morphology of the external and internal structures of different blood components were investigated to establish the differences found in stroke patients when compared to healthy control individuals.

9.1 PLATELETS AND FIBRIN NETWORKS

TEM as well as SEM analysis revealed alterations in the platelets of stroke patients when compared to healthy controls. Platelets appeared activated since less alpha granules were observed. The OCS pores, associated with the release of alpha granules, were also observed. Platelets release various constituents upon activation. These substances include secreted alpha granule constituents like fibrinogen, factor V and TSP and also dense body secretions like ADP and serotonin. These constituents promote fibrin strand formation and also promote the attraction of additional platelets to form an aggregate.

The layered thin fiber ultrastructure of the fibrin network observed by SEM is indicative of elevated fibrinogen and/or thrombin concentrations in the blood of stroke patients. The uncharacteristic circular morphology seen in some of the micrographs could be as a result of increased thrombin, thrombospondin (TSP) or platelet factor 4 (PF-4) concentrations.

Fibrinogen, TSP and PF-4 are factors released by activated platelets. In light of these observations, it can be assumed that platelets are activated and actively involved in the thrombotic event in the blood of stroke patients. The vasoactive substances released by platelets upon activation also advance the neuronal damage associated with stroke.

It can therefore be assumed that the altered morphology of the fibrin network is present long before the occurrence of the actual thrombotic event.

9.2 BLOOD CELLS

9.2.1 Erythrocytes

Alterations in the morphology of erythrocytes will influence the flow of these cells in the microcirculation. This could augment the ischaemic event. Large amounts of abnormal erythrocytes were observed in the blood of stroke patients. Codocytes, knizocytes,

stomatocytes and echinocytes were identified by SEM and TEM analysis. Hypertensive patients and females independently showed the greatest increase in abnormal erythrocyte forms.

9.2.2 White blood cells

Ischaemic stroke is associated with inflammation. Elevated monocyte count and decreased neutrophil count appeared to be trait of the stroke patients involved in the study. Hypertension and daily aspirin consumption appear to have the greatest influence on the observed differential white blood cell counts.

9.3 FUTURE WORK

The ultrastructural changes seen in the platelets, fibrin networks, erythrocytes as well as differential white blood cell counts could possibly be used to identify possible future thrombotic events or the progress of patients after a stroke. A small, affordable desktop SEM (e.g. a desk-top portable SEM manufactured by ZEOL) could possibly be utilized in a clinical setting as part of the analysis regime. In this way altered fibrin networks could be identified before the thrombotic event and the patient could possibly be treated to prevent a stroke.

Several limitations in this study should however be acknowledged. The study population was limited and analysis of the blood specimens were only analyzed at a single time point. A larger amount of stroke patients as well as a more refined patient enrolment protocol could refine the study. Analysis of follow-up blood samples of each patient could provide additional information about the effectiveness of the treatment regimes on clot ultrastructure and the other blood components.

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APPENDIX A

Etheresia Pretorius, Albe C. Swanepoel (de Lange), Hester M. Oberholzer, Wendy J. van der Spuy, Wiebren Duim, Pieter F. Wessels.

A descriptive investigation of the ultrastructure of fibrin networks in thrombo-embolic ischemic stroke

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