COMPARING PLATELET FUNCTION AND ULTRASTRUCTURE IN SMOKING AND THROMBO-EMBOLIC ISCHEMIC STROKE

by

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Abstract

Stroke is serious neurological disease and is a major cause of death as well as disability throughout the globe. Stroke has a complex pathophysiology that involves inflammatory pathways, excitotoxicity mechanisms, oxidative damage, apoptosis, ionic imbalances, angiogenesis and neuroprotection.

85% of strokes are ischemic and occurs when a cerebral vessel, or any vessel supplying the brain, narrows or loses pressure resulting in subsequent brain ischemia and infarction downstream to the site of obstruction depriving tissues of vital oxygen and nutrients. This may be caused by either atherosclerotic thrombi or distant emboli defined as a mass of clotted blood or other material.

It is estimated that over a billion people currently smoke cigarettes or use other tobacco products, seeing as smoking is a major risk factor for stroke this is of major concern. Platelets are hematopoietic cells produced by bone marrow megakaryocytes. Platelets play a role in the development of ischemic stroke primarily by means of their participation in the formation of thromboemboli, the presence of abnormal platelet function may predispose patients to a pro-thrombotic, pro-inflammatory state. The reorganization of the cytoskeleton in platelets is an important factor in the complex mechanisms found in thrombosis and haemostasis. The platelet membrane contains a large number of receptors which specifically bind agonists that stimulate the physiological platelet response.

Oxidative stress is one of the mechanisms involved in the neuronal damage of stroke. Oxidative stress is a state of imbalance between free radical production, in particular, reactive oxygen species (ROS), and the ability of the organism to neutralize them, leading to progressive oxidative damage. Smoking is known to result in the generation of various free radicals.

Flow cytometric analysis of the platelets of thrombo-embolic ischemic stroke patients and smokers revealed that the membranes of the two groups were altered in some form as well as an increased activation in both groups when compared to healthy individuals. Superoxide levels in the platelets were higher in smokers when compared to stroke patients, while hydrogen peroxide levels were elevated in the platelets of both groups. Superoxide was elevated in the whole blood samples of both groups. The production and subsequent reactions of reactive oxygen species appear to be influential in stroke and smoking and may likely be a crucial factor in the development of a pro-thrombotic, pro-inflammatory state which may prove to be a hallmark in the pathophysiology of stroke and smoking.

Confocal microscopy and Scanning electron microscopy showed that platelets of stroke patients and smokers appear to be more activated and more prone to form tight clots. Furthermore an increased amount of superoxide is present in the platelets of stroke patients and smokers, specifically in the centre of clots. This may be an indication that once platelets have aggregated and started to fuse together, the mitochondria are expelled from the platelets and “trapped” within the clot.

Atomic force microscopy also indicated both the stroke patients and smoker’s platelets appear to be in a more activated state than the control group. Here it is apparent that some form of cytoskeletal rearrangement takes place to a more severe extent in the stroke group than in the smokers. Necrosis may be present in the platelets of stroke patients while neither apoptosis nor necrosis can be identified in the platelets of smokers however some form of membrane alteration is likely present.

All the techniques used showed an increase in platelet activation in stroke patients and smokers, necrotic platelets may be present in the stroke patients while the platelet membrane of smokers seems to be altered. ROS is present and alters the platelet function of smokers and stroke patients in some way. It appears as if thrombo-embolic ischemic stroke patients and smokers’ platelets have similar trends in activation but the processes involved to achieve this differ as there are structural differences present.

These differences may prove a useful tool to further understand the pathophysiology behind thrombo-embolic ischemic stroke as well as to discover new therapeutic pathways.

Declaration

I, Jeanette Noel Du Plooy, hereby declare that this research dissertation is my own work and has not been presented for any degree at another University.

Signed: ……..

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Abbreviations, Symbols and Chemical Formulae

DCF	2,7-dichlorofluorescein
DCFDA	2,7-dichlorofluorescein diacetate
AIDS	Acquired immune deficiency syndrome
APC	Activated protein C
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
α-granules	Alpha granules
AFM	Atomic force microscopy
AF	Atrial fibrillation
BP	Blood pressure
BBB	Blood–brain barrier
BM	Bone marrow
Ca^{2+}	Calcium ion
CO_2	Carbon dioxide
GPIIb	CD41
GPIX	CD42a
GPV	CD42d
GPIIIa	CD61
CDC	Centers for Disease Control and Prevention
Cl^-	Chloride ion
CT	Computed Tomography scan
CI	Confidence interval
Cu^+	Copper ion(I)
Cu^{2+}	Copper ion(II)
CHD	Coronary heart disease
CRP	C-reactive protein
PSGL-1/PSGL-Ig	Cross-linked recombinant soluble P-selectin glycoprotein ligand-1
cGMP	Cyclic guanosine monophosphate
COX	Cyclooxygenase
δ-granules	Delta granules
DTS	Dense tubular system
DNA	Deoxyribonucleic acid
DBP	Diastolic blood pressure
O_2	Dioxygen
DALYs	Disability Adjusted Life Years
et al.	Et alia (and other)
e.g.	Exempli gratia (for example)
$\alpha_{IIb}\beta_3$, GPIIb-IIIa,	Fibrinogen receptor ,CD41/CD61
GSH	Glutathione
GEMs	Glycolipid-enriched membranes
GP	Glycoprotein
GPIIb/IIIa	Glycoprotein IIb/IIIa or integrin $\alpha_{IIb}\beta_3$
g	Grams
HSCs	Haematopoietic stem cells
HE	Hydroethidine

H^+	Hydrogen ion
H_2O_2	Hydrogen peroxide
$HO^{\bullet}_2$	Hydroperoxyl radical
QH_2	Hydroquinone
OH•	Hydroxyl radical
HOCL	Hypochlorous acid
IL-1β	Interleukin-1 beta
Fe(II)/Fe^{2+}	Iron ion (II)
Fe^{3+}	Iron ion (III)
kDa	Kilo Daltons
kHz	Kilohertz
kPa	Kilopascal
IL-6	Knterleukin-6
LIBS	Ligand-induced binding site
Lp-PLA_2	Lipoprotein-associated phospholipase A_2
LAMP3	Lysosomal membrane associated glycoprotein 3
λ-granules	Lysosomes
MRI	Magnetic resonance imaging
MMPs	Matrix metalloproteinases
MMP-1	Matrix metalloproteinases-1
MMP-9	Matrix metalloproteinases-9
MCFI	Mean channel fluorescence intensity
MKs	Megakaryocytes
MPa	Mega Pascal
µl	Microliter
µm	Micro meter
METC	Mitochondrial electron transport chain
mAB	Monoclonal antibody
NOX	NADPH oxidase
nm	Nano metre
NIHSS	National Institutes of Health Stroke Scale
nNOS	Neuronal nitric oxide synthase
N/m	Newton metre
NO•	Nitric oxide
NOS	Nitric oxide synthases
NO_2	Nitrogen dioxide
OCS	Open canalicular system
OC's	Oral contraceptives
oxLDL	Oxidised low density lipoproteins
O	Oxygen
OFR	Oxygen-free radicals
%	Percentage
ONOO-	Peroxynitrite anion
PBS	Phosphate buffered saline
GP Ibα	Platelet glycoprotein Ib alpha, CD42b
GPIbβ	Platelet glycoprotein Ib beta also/ CD42c
PRP	Platelet rich plasma
PAF	Platelet-activating factor
TGFβ	Platelet-derived growth factor

PAH	Polycyclic aromatic hydrocarbons
PKA	Protein Kinase A
CD62P/ GMP-140/PADGEM	P-selectin
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
GSH	Reduced glutathione
NADPH	Reduced nicotinamide dinucleotide phosphate oxidase
RIND	Reversible ischemic neurologic defect
RNA	Ribonucleic acid
SEM	Scanning electron microscopy
$Q^{-}\bullet$	Semiquinone
SCR	Short consensus repeats
Na^{+}/K^{+} ATPase	Sodium-potassium adenosine triphosphatase
sP-selectin	Soluble P-selectin
SD	Standard Deviation
SAH	Subarachnoid haemorrhage
SOD	Superoxide dismutase
$O_2\bullet^{-}$	Superoxide radical
SCCS	Surface-connected canalicular system
RS•	Thiyl radicals
TXA_2	Thromboxane
TF	Tissue factor
TFPI	Tissue factor protein inhibitor
tPA	Tissue plasminogen activator
t-PA	Tissue plasminogen activator
TIAs	Transient ischemic attacks
TM4	Transmembrane 4 superfamily
$CCl_3\bullet$	Trichloromethyl
TNF-α	Tumor necrosis factor-alpha
VWD	von Willebrand disease
Vwf	von Willebrand factor
vWfR,(GPIbαβ/IX)2V or GPIb–IX–V or CD42a-d	von Willebrand factor receptor
E	Young's modulus

CHAPTER 1: INTRODUCTION

The world health organization defines stroke as a clinical syndrome characterized by rapidly developing clinical symptoms and/or signs of focal, and at times global, loss of cerebral function, with symptoms lasting more than 24 hours or leading to death, with no apparent cause other than that of vascular origin. This definition includes stroke due to both cerebral infarction and intracerebral and subarachnoid haemorrhage (Sudlow and Warlow, 1996).

According to a 2012 update report from the American Heart Association; Heart disease and Stroke statistics, on average a person has a stroke every 40 seconds, and dies of one every 4 minutes in the United States (Roger et al., 2012). Stroke is the third most common cause of death worldwide of which more than two-thirds of those deaths occur in developing regions such as sub-Saharan Africa (Connor et al., 2005).

Stroke is serious neurological disease comprising a major cause of not only death but also disability throughout the globe. Stroke has a complex pathophysiology that involves inflammatory pathways, excitotoxicity mechanisms, oxidative damage, apoptosis, ionic imbalances, angiogenesis and neuroprotection (Deb et al., 2010).

There are a large number of risk factors for stroke which reflects the heterogeneity of the disease. They are classified as modifiable, potentially modifiable and non-modifiable.

One of the most profound and preventable risk factors is cigarette smoking, it doubles the risk of stroke especially when combined with hypertension and/or diabetes which results in an immense increase in risk. This risk varies with the amount of cigarettes smoked; there is a 50% reduction of risk by quitting smoking (Cubrilo-Turek, 2004). Evidence suggests that second-hand smoking might contribute to the progression of carotid atherosclerosis in addition to endothelial dysfunction therefore might also increase the risk of stroke (Hawkins et al., 2002). Smoking is known to be a factor in the increase of thrombosis, oxidative stress, inflammation and atherosclerosis (Insull, 2009; Pretorius et al., 2010).

An important factor in the pathogenesis of stroke is the formation of a thrombus. This process is the result of a complicated interplay between blood components such as platelets, coagulation factors, and fibrinolytic factors which individually play a part but also interact with each other (Wang et al., 2005). Platelets play a major role in arterial thrombosis. Atherothrombotic stroke is a platelet-dependant disease since stroke is attributable to platelet-rich thrombi formed on atheromatous plaques in extracranial or intracranial major arteries (Uchiyama et al., 2003). Smoking is also known to increase the risk for a thrombotic event.

Platelets are hematopoietic cells produced by bone marrow megakaryocytes. Under normal conditions in the intact blood vessel most platelets don't ever undergo firm adhesion; however, when vessel wall injury occurs they rapidly adhere to the exposed extracellular matrix (ECM), become activated and form a platelet plug, preventing blood loss. In atherosclerotic arteries upon plaque rupture the same process can lead to acute vessel occlusion, resulting in life threatening myocardial infarction or ischemic stroke (Varga-Szabo et al., 2009). The reorganization of the cytoskeleton in platelets is an important factor in the complex mechanisms found in thrombosis and haemostasis. The cytoskeleton is primarily responsible for regulating platelet shape (Fox, 2001).

The platelet membrane contains a large number of receptors which specifically bind agonists that stimulate the physiological platelet response. The interaction between a platelet-activating agonist and its receptor causes rapid mobilization of signalling molecules within the platelet which are sufficient to initiate and complete shape change and aggregation responses (Willoughby et al., 2002).

Na^+/K^+ ATPase is an integral membrane protein that plays a key role in cellular osmotic regulation through the maintenance of the trans-membrane gradients of Na^+ and K^+, therefore acting as a marker of membrane function whose activity greatly depends on its interaction with the chemical-physical properties of the microenvironment where it is embedded.

A study conducted by Nanetti et al in 2008 showed a decreased platelet Na^+/K^+ ATPase activity in patients affected by acute stroke compared to that in controls as well as significantly lowered membrane fluidity (Nanetti et al., 2008).

Platelet membrane fluidity with regards to microviscosity is a critical determinant of platelet aggregation and secretion, therefore its decrease in stroke patients may play a role in the pathogenesis of ischemic damage. Nanetti et al found a decrease in the platelets Na^+/K^+ ATPase activity in patients, because the enzyme is located in a less fluid cellular microenvironment induced by alterations related to acute stroke (Nanetti et al., 2008). This enzymatic inhibition leads to altered internal and external membrane ionic concentrations (Mazzanti et al., 1997).

This ionic imbalance causes a derangement in cellular enzymatic systems, leading to membrane depolarization which in turn could compromise platelets survival (Rabini et al., 2003).

Na^+/K^+ ATPase activity and membrane fluidity are correlated to stroke severity as measured by the National Institutes of Health Stroke Scale (NIHSS). Alterations in platelet membrane chemical–physical (decreased fluidity) and functional properties (reduced Na^+/K^+ ATPase activity) rise proportionally with NIHSS increase (Nanetti et al., 2008).

It has also been found that smoking induces alterations in platelet membrane fluidity and NA^+/K^+ ATPase activity as well as a change in the membrane cholesterol/phospholipid ratio (Padmavathi et al., 2010). Pretorius and co-workers in 2012 suggest that this changed membrane fluidity is visible ultrastructurally and translates into a more globular and bulbous appearance of the membrane surface in smokers (Pretorius, 2012).

According to Alexandrova & Bochev oxidative stress is one of the mechanisms involved in the neuronal damage of stroke. Oxidative stress is a state of imbalance between free radical production, in particular, reactive oxygen species (ROS), and the ability of the organism to neutralize them, leading to progressive oxidative damage (Alexandrova and Bochev, 2005). ROS are a group of highly reactive molecules that are produced through sequential reductions of Oxygen (O_2) and include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-) (Orrenius, S, Gogvadze, V, Zhivotovsky, 2007).

ROS are short- living compounds initiating complex chain reactions that produce a range of molecular structures, many of which are yet unknown. An elevation in lipid peroxidation products in the circulation and the weakened cellular antioxidant defence system are considered an indirect proof of oxidative stress in stroke.

Risk factors bring about a chronic change in the walls of blood vessels that include additional activation of inflammatory and free radical mechanisms (Alexandrova and Bochev, 2005).

Inflammation takes part in the mechanisms of cerebral injury and recovery after stroke (Lindsberg and Grau, 2003). Inflammation in stroke is mediated by both molecular and cellular components, many of which having anti- or pro-inflammatory properties with beneficial or destructive effects (Emsley, 2003). Inflammation is accompanied by mobilization and activation of leukocytes through the activation of platelets and endothelium, resulting in an excessive production of ROS and oxidative endothelial and tissue damage (Schaller and Graf, 2003).

The brain is highly susceptible to free radical damage as it is rich in polyunsaturated fatty acids, which are particularly vulnerable to free radical induced peroxidation, but also contains a low content of antioxidant enzymes, such as catalase and glutathione peroxidase (Cherubini et al., 2005).

Both smoking and thrombo-embolic stroke are therefore typically associated with inflammation. It is also well-known that smoking is a profound risk factor for stroke. The question arises whether platelet function and ultrastructure is similar in smokers (before a thrombotic event) compared to patients directly after a thrombotic event.

Studies have been done relating to platelet function in normal and diseased conditions using various techniques including flow cytometry and the ultrastructural analysis of platelets, however no research has been done comparing the physiology of platelets in stroke patients and smokers, especially inflammation and ROS, using various fluorescent probes in flow cytometry as well as confocal microscopy using the same probes. Therefore, the current study aims to investigate the changes in platelets in thrombo-embolic ischemic stroke patients and smokers without any apparent health problems.

CHAPTER 2: LITERATURE REVIEW

2.1 CHAPTER OBJECTIVES

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

2.2 INTRODUCTION

Stroke is an on-going reality and challenge facing many people today. The pathophysiology behind stroke is intricate and complicated and many facets of it remain unknown. Platelets play a major role in haemostasis and thrombosis as well as in other processes like inflammation. The negative health consequences of smoking are countless, notably smoking is a major risk factor for stroke. Oxidative stress and reactive oxygen species may well be role players in both stroke and smoking pathophysiology. Therefore stroke, smoking, platelets and reactive oxygen species will now be further discussed in this chapter.

2.3 STROKE AT A GLANCE

The world health organization defines stroke as a clinical syndrome characterized by rapidly developing clinical symptoms and/or signs of focal, and at times global, loss of cerebral function, with symptoms lasting more than 24 hours or leading to death, with no apparent cause other than that of vascular origin. This definition includes stroke due to both cerebral infarction and intracerebral and subarachnoid haemorrhage (Truelsen et al., 2001).

Stroke can be seen as a syndrome, representing a collection of disease processes which all result in cerebral ischemia. The different processes have different clinical phenotypes, different aetiological mechanisms, and different risk factor profiles (Markus, 2003).

Four neurologic phenomena have been defined for stroke based on their duration: transient ischemic attacks (TIAs), reversible ischemic neurologic defect (RIND), stroke in evolution, and completed stroke.

A TIA is an abrupt, short-lasting, focal neurologic deficit or ‘‘mini’’ stroke resulting from temporary and localized brain ischemia. These neurologic deficits are usually reversible within 24 hours but can be a sign of an impending stroke. RIND describes a neurologic affliction that is reversible but the recovery time will exceed 24 hours. A stroke in evolution is defined as stroke-associated symptoms that progressively worsen over time, and a completed stroke is defined as neurologic signs and symptoms that have been stable for more than 24 hours (Kelley et al., 2003).

Strokes are sub classified into ischemic and haemorrhagic types, based on the underlying pathogenesis. 85% of strokes are ischemic and occurs when a cerebral vessel, or any vessel supplying the brain, narrows or loses pressure resulting in subsequent brain ischemia and infarction downstream to the site of obstruction depriving tissues of vital oxygen and nutrients (Collins, 2007). This may be caused by either atherosclerotic thrombi or distant emboli defined as a mass of clotted blood or other material.

Embolic stroke which is the most common results from cerebral ischemia secondary to blockage of the vessel by an embolus, furthermore embolic strokes are classified into arterial, cardio-embolic, and cryptogenic subtypes, depending on the origin of the emboli (Fatahzadeh and Glick, 2006).

Cerebral emboli often arise from various sources including atherothrombi in the carotid bifurcation or aortic arch, cardiac disease, and spontaneous thrombosis in hyper coagulation conditions. Cryptogenic strokes are cerebrovascular events where the source of occlusive emboli remains unknown.

Generally speaking, the neurologic symptoms seen in thrombotic stroke develop slowly, contrasting the sudden, multifocal, and maximal neurologic deficits which if found at the onset is indicative of an embolic stroke. Early seizures and haemorrhagic transformation are more frequent with embolic events (Kelley et al., 2003).

15% of all strokes are haemorrhagic in nature. Intracerebral bleeding causes two-thirds of haemorrhagic strokes while the other third may be due to aneurysmal rupture and subarachnoid haemorrhage (Felberg and Naidech, 2003).

The management of these subtypes differ greatly; therefore the clinical distinction between the subtypes is one of the most important factors in stroke management. This distinction has been revolutionised by the introduction of CT (Computed Tomography scan) and MRI (Magnetic resonance imaging). CT has been the main tool in stroke diagnosis during the past 20 years, more recently MRI is as useful as, if not more so than, CT (Donnan et al., 2008; Fiebach, 2002).

2.4 RISK FACTORS

There are a large number of risk factors involved in the pathophysiology of stroke which is a reflection of the heterogeneity of the disease. Variation in stroke mortality due to differences in risk factors has been established by several population-based studies (Cubrilo-Turek, 2004; Fatahzadeh and Glick, 2006; Kirshner, 2009; Stroebele et al., 2011). Epidemiologic studies have identified many predisposing factors associated with stroke, primary prevention

focuses on the modification of those risk factors in the general population therefore it is a noteworthy topic to examine. Risk factors are classified as modifiable, potentially modifiable and non-modifiable;

2.4.1 NONMODIFIABLE RISK FACTORS

Nonmodifiable risk factors for stroke cannot be changed and include (Sacco et al., 1997);

2.4.1.1 Age

Age is one of the most important and most powerful risk factors for stroke. Each 10 years after the age of 55, the stroke rate is more than double for men and women, possibly due to increased exposure to environmental risk factors and higher prevalence of risk factors including atrial fibrillation (AF), hypertension, diabetes, and coronary heart disease (Cubrilo-Turek, 2004; Feigin et al., 2010).

2.4.1.2 Gender

There is uncertainty pertaining to the effect of gender on the outcome and mortality of stroke as various studies have made different findings. Feigin *et al*. in 2010 stated that men have higher age-specific stroke rates than women and men are more likely to have their first-ever stroke at a younger age. Women however experience more strokes due to an increased stroke incidence for women >84 years (Feigin et al., 2010).

Women tend to have poorer outcomes post-stroke, this may be attributed to women being older at stroke onset; having poorer pre-stroke functioning; having more co-morbidities; being less likely to have social support etc. According to Reeves *et al*. stroke has a greater effect on women than men because women have more events and are less likely to recover (Reeves et al., 2008).

2.4.1.3 Race/Ethnicity

Numerous studies indicate ethnic disparities in prevalence and incidence of ischemic stroke as well as disability and mortality (Feigin et al., 2010). According to a study done by Jiang *et al*. there is no race difference in post stroke all-cause mortality among patients with any type of stroke, however they did find that the adjusted risk of death caused by cerebrovascular disease was relatively lower among non-white stroke patients.

Furthermore they found that the majority of studies on race differences in ischemic or nonspecific stroke mortality found higher mortality in non-white or black patients than in white patients (Jiang et al., 2006).

In South Africa specifically Hoffman analysed 320 young stroke patients between the years of 15 and 49, he stated that race and endemic disease were important determinants of the underlying cause and risk factor profile. Caucasians seemed to have more traditional risk factors like hypertension while blacks usually had presented with an infection in the 2 weeks prior to the stroke (Conner, Myles Bryer, 2006; Hoffmann, 2000).

However, some studies did not find a significant 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).

2.4.1.4 Positive Family History

Ischemic stroke tends to aggregate in families, with a positive family history. Familial history of stroke has often been hypothesized as a risk factor for stroke by many studies (Brown et al., 1996; Howard et al., 1989; Wolf et al., 1992). When taking the disease process into consideration, it is possible that a positive parental history of stroke could lead to an increased risk of stroke events through:

- Genetic heritability of stroke risk factors, such as elevated blood pressure, elevated serum cholesterol and diabetes
- The inheritance of susceptibility to the effects of such risk factors
- Familial sharing of cultural/environmental and lifestyle factors, such as higher-sodium and higher-fat diet, lower physical activity, and lower socioeconomic status
- The interaction between genetic and environmental factors

As with sex as a risk factor there are also conflicting findings regarding family history, Marshall, Welin *et al.*, Carrieri *et al.*, and Kiely *et al.* have found some degree of familial and/or parental aggregation of stroke (Carrieri et al., 1994; Kiely et al., 1993; Marshall, 1973; Welin et al., 1987).

However, Boysen *et al*, Brass and Shaker and Herman *et al* found no statistical association (Boysen et al., 1988; Brass and Shaker, 1991; Frenken et al., 1983).

Roger *et al* states in the 'Heart disease and stroke statistics 2012 update: a report from the American Heart Association' that a family history of cardiovascular disease (CVD) increases

risk of CVD, with the most increase in risk occurring if the family member's CVD was premature (Roger et al., 2012).

According to Lisabeth *et al* the most focus has been on identifying genetic determinants of stroke risk, many aspects of the genetic epidemiology of ischemic stroke remain relatively unstudied. Apart from the relationship between a positive family history of stroke and stroke risk, aspects of ischemic stroke for which genetic factors are plausible, such as stroke subtype, stroke outcomes and stroke severity have not yet been thoroughly investigated.(Lisabeth et al., 2005) There may be certain differences in genetic susceptibility to stroke based on ischemic stroke subtype (Lisabeth et al., 2005).

A meta-analysis of cohort studies showed that a positive family history of stroke increases risk of stroke by approximately 30% (Flossmann et al., 2004; Goldstein et al., 2011). Genetic influences on stroke risk can be considered on the basis of individual risk factors, genetics of common stroke types, and uncommon or rare familial stroke types.

2.4.1.5 Other Nonmodifiable Risk Factors

Other non-modifiable risk factors include; diseases e.g. cancer, chronic kidney disease, types of arthritis, abnormal arteries and veins, pregnancy, and low birth weight.

2.4.2 MODIFIABLE RISK FACTORS

Modifiable risk factors can be managed through various lifestyle adjustments and pharmacological and surgical interventions to effectively reduce the risk of recurrent stroke (Kirshner, 2009). Continuing research suggests differential association of risk factors throughout stroke subtypes, (Di Carlo et al., 2006; Ohira et al., 2006; Paganini-Hill, 2003; Schulz and Rothwell, 2003) despite this treatment guidelines currently make distinctions only between cardioembolic and non-cardioembolic stroke, and this only with regards to antiplatelet therapy versus anticoagulation therapy (Kirshner, 2009).

2.4.2.1 Hypertension

Hypertension is one of the most important risk factors which contribute to 70% of all strokes. Hypertension is the strongest modifiable and independent risk factor for both haemorrhagic and ischemic stroke in both middle and late age. The relationship between blood pressure (BP) and stroke risk is consistent, graded, strong, independent and etiologically significant (Goldstein et al., 2011).

The risk of stroke rises in proportion to BP, for females as well as for males, and nearly doubles for each 7.5 mm Hg increment in diastolic blood pressure (DBP). Among the elderly isolated systolic hypertension notably increases the risk of stroke as systolic BP rises with increasing age.

The general recommendation and target goal for general population is a systolic/diastolic BP of <140/90 mm Hg. Diabetic patients with hypertension have a lower target: < 130/85 (Cubrilo-Turek, 2004). The prevalence of hypertension is increasing and high, this going hand in hand with the increase of overweight and obese people in the world.

More than two thirds of people that are 65 years of age and above are hypertensive (Chobanian et al., 2003). Even though antihypertensive therapy is so efficient and the diagnosing and monitoring of it so easy, a large section of the population remains undiagnosed and inadequately treated. This is seen especially in the elderly and minority populations (Hyman and Pavlik, 2001).

2.4.2.2 Cigarette Smoking

Practically every multivariable assessment of stroke risk factors identifies cigarette smoking as a strong risk factor for ischemic stroke

Cigarette smoking approximately doubles the risk of stroke and increases both ischemic and haemorrhagic stroke risk. This is significant when smoking is combined with e.g. diabetes and/or hypertension: doubling a larger number. Cigarette smoking is an independent risk factor for ischemic stroke in women and men (Cubrilo-Turek, 2004).

Results from studies mainly conducted in older age groups provide evidence of a dose-response relation between the number of cigarettes smoked per day, and the risk of stroke, and this has further been extended to young women from an ethnically diverse cohort (Bhat et al., 2008; Cubrilo-Turek, 2004). Smoking is also associated with a 2- to 4-fold increased risk for subarachnoid haemorrhage (SAH) (Feigin et al., 2005; Kurth et al., 2003a, 2003b).

A meta-analysis of 32 studies done by Shinton and Beevers found a relationship between smoking and both ischemic and haemorrhagic stroke, particularly at young ages. The risk of stroke associated with cigarette smoking seems to be present in all age groups but appear to be far greater in younger population groups (Shinton and Beevers, 1989; United States Department of Health and Human Services, 1989).

The yearly number of stroke deaths due to smoking in the United States is estimated to be between 21 400 (without adjustment for potential confounding factors) and 17 800 (after

adjustment), which indicates that smoking may contribute to 12% to 14% of all stroke deaths (Thun et al., 2000).

A study done by the Centers for Disease Control and Prevention (CDC); National Health Interview Survey and death certificate data for 2000 to 2004, revealed that smoking resulted in an average of 97 681 stroke deaths among women and 61 616 stroke deaths among men in the United States (Centers for Disease Control and Prevention, 2008).

Cigarette smoking may also enhance the effects of various other stroke risk factors, including systolic BP,(Nakamura et al., 2008) vital exhaustion (unusual irritability, fatigue, and feelings of demoralization),(Schwartz et al., 2004) and oral contraceptives (OC's) (Poulter et al., 1996).

The relationship between cardiovascular disease and smoking is well documented in the literature, as is the association of smoking with elevated levels of inflammatory markers and accelerated atherosclerosis.

Smoking is able to trigger an immunologic response to vascular injury, which is associated with increased levels of inflammatory markers, such as white blood cell count and C-reactive protein (Bakhru and Erlinger, 2005).

According to the results of an international, multicentre, case-control study on ischemic stroke and oral contraceptives; there is a clear synergistic effect between the use of OC's and cigarette smoking on the risk of cerebral infarction (Poulter et al., 1996).

Unfortunately not only active smokers are in danger as exposure to environmental tobacco smoke, other wisely known as "second-hand" or passive cigarette smoke is known and established to be a risk factor for heart disease (Barnoya and Glantz, 2005).

Numerous studies provide evidence that exposure to environmental cigarette smoke is a substantial risk factor for stroke, with risk almost approaching double that found for active smoking,(Bonita et al., 1999; Qureshi et al., 2005; Zhang et al., 2005) although 1 study found no notable association regarding stroke but did find an association with coronary heart disease (CHD) (Whincup et al., 2004).

Smoking most likely contributes to the increased stroke risk through both acute effects on the risk of thrombus formation in atherosclerotic arteries as well as chronic effects related to increased atherosclerosis (Burns, 2003). The smoking of merely 1 cigarette increases mean BP, heart rate, and cardiac index and decreases the arterial dispensability (Kool et al., 1993; Silvestrini et al., 1996).

Beyond the known immediate effects of cigarette smoking, both active and passive exposure to smoke is associated with the pathophysiological development of atherosclerosis (Howard et al., 1998).

As well as placing persons at an increased risk for both embolic and thrombotic stroke, cigarette smoking nearly triples the risk of cryptogenic stroke in people with a low atherosclerotic burden and no clear evidence of a cardiac source of emboli (Karttunen et al., 2002).

The most effective preventive measures remains never to smoke and to minimize exposure to environmental cigarette smoke, risk is how ever reduced with smoking cessation. Quitting smoking is associated with a rapid decrease in the risk of stroke (50%) and other cardiovascular events to a level that approaches but does not reach that of those who never smoked (Burns, 2003; Cahill et al., 2012; Cubrilo-Turek, 2004; Preventive Service Task Force, 2009; Song and Cho, 2008; Stead et al., 2008). Even though sustained smoking cessation is very difficult to achieve, effective pharmacological and behavioural treatments for nicotine dependence are widely available (Cahill et al., 2012; Preventive Service Task Force, 2009).

2.4.2.3 Cholesterol

Various epidemiological studies have shown a correlation between increased total blood cholesterol levels and the risk of myocardial infarction (Piechowski-Jóźwiak and Bogousslavsky, 2004).

However the correlation between cholesterol levels and stroke prevalence is debated in the literature. Most epidemiological studies with few exceptions found a correlation between elevated cholesterol levels and an increased risk of ischemic stroke. Contrasting this many but not all studies found a correlation between lower cholesterol levels and an increased risk of haemorrhagic stroke (Goldstein et al., 2011).

There may be many reasons for not being able to find a clear correlation between cholesterol level and stroke occurrence including; analysis of the occurrence of stroke subtypes and differentiation between cholesterol components. The prophylactic treatment used in high cholesterol patients may also influence the occurrence of stroke (Piechowski-Jóźwiak and Bogousslavsky, 2004).

Corvol *et al* found in 2003 strong evidence for the role of cholesterol in stroke as they found a documented correlation between stroke incidence and the degree of cholesterol reduction, baseline cholesterol level, and final cholesterol level (Corvol et al., 2003).

According to a study done by Engstrom on 22444 men, cholesterol was found to be associated with ischemic stroke among patients who had elevated levels of inflammation-sensitive plasma proteins, and not among people with lower levels of these markers (Engstrom, 2002).

Therefore, cholesterol may possibly not be a major risk factor in isolation, but may play a role when interacting with other factors to assist the progression of atherosclerosis (Thrift, 2004).

Cholesterol is most certainly associated with stroke but its defined role as a risk factor has yet to be determined.

2.4.2.4 Diabetes

Patients with diabetes have an increased susceptibility to atherosclerosis as well as a higher prevalence of pro-atherogenic risk factors, including hypertension and abnormal blood lipids (Goldstein et al., 2011).

Case-control studies and prospective epidemiological studies of stroke patients have confirmed that diabetes independently increases the risk of ischemic stroke, ranging from a 1.8-fold to nearly 6-fold increase (Thomas, 1996).

Age-specific incidence rates and rate ratios showed that diabetes increased incidence of ischemic stroke for all ages, but that the risk was most prominent before age 55 in blacks and before age 65 in whites (Ooth, 2006). The stroke risk associated with diabetes is found mostly in people with type 2 diabetes as in the age group most strokes take place, type 2 is more common than type 1.

Diabetes causes atherosclerotic changes in the cerebropetal arteries as well as the heart and is associated with different subtypes of ischemic stroke. The risk of arterial fibrillation, which is a major cause of thrombo-embolic stroke, increases by 40% in individuals with diabetes (Luitse et al., 2012).

Diabetes is a well-known strong risk factor for ischemic stroke; this may be because of accompanying risk factors, such as obesity, hypertension and lipid abnormalities (Benson and Sacco, 2000; Cubrilo-Turek, 2004).

2.4.2.5 Infection and Inflammation

Inflammation influences the initiation, growth, and destabilization of atherosclerotic lesions,(Libby and Ridker, 2006) however the application of this information to risk assessment and treatment in the prevention of stroke is controversial (Goldstein et al., 2011).

Inflammation is a vital feature of the host defence response to infection and is needed for the containment and removal of the pathogen and eventually promoting tissue repair and normalizing function. However, many studies suggests that the known beneficial effects of infection-induced inflammation are counter acted by the potentially damaging aggravation of inflammatory pathways that leads to a predisposition and/or exacerbation of acute ischemic brain injury and accelerates the progression of chronic neurodegeneration (Emsley and Hopkins, 2008; Tuttolomondo et al., 2010).

The relationship between infections and acute ischemic stroke is complex and bidirectional. Many studies found that infections can result in ischemic stroke, and the rate of infection is increased after ischemic stroke (McColl et al., 2009, 2007).

A growing amount of evidence elucidates the important role of inflammation, which forms an integral component of the complex interactions between the immune and nervous systems, in stroke pathology and etiology.

Extensive data supports the contribution of inflammation in various phases of stroke, including; post-ischemic mediator of brain damage, as possible biomarker and modifier of susceptibility, putative predictor of prognosis and recurring cerebrovascular events and finally proximate trigger (Barone and Kilgore, 2006; Delbrutto, 2005; McColl et al., 2009).

Emerging evidence shows that inflammatory events occurring outside the brain prior to, during, and after stroke notably influence stroke outcome and susceptibility. According to numerous studies systemic concentrations of various inflammatory markers have been found in conjunction with stroke incidence (Rallidis et al., 2006; Tuttolomondo et al., 2010).

C-reactive protein (CRP) is a sensitive and dynamic marker of inflammation which is synthesized by the liver and found in plasma (Kaptoge et al., 2010). Slightly elevated CRP levels may indicate chronic “low-grade” inflammation and many studies have suggested that patients with elevated CRP concentrations are at an increased risk of stroke, CRP is the most commonly used marker of inflammation (Chei et al., 2011; Kuo et al., 2005). However, recent studies have put into question the utility of CRP as an independent predictor of risk

itself (Bos et al., 2006; Sattar et al., 2009) this may suggest that CRP is a surrogate marker for other present cerebrovascular risk factors.

Other proposed serum markers of inflammation include fibrinogen, serum amyloid A, Lp-PLA2, and interleukin-6 (IL-6). Several studies suggest a relationship between Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) and stroke risk (Goldstein et al., 2011). IL-6 is the most prominent inflammatory cytokine that has been found to be a predictive marker of stroke risk (McColl et al., 2009).

Mechanisms for infection and inflammation suggest that both cellular and molecular inflammatory mediators which are shared by many inflammatory diseases are possible candidates as effectors that regulate stroke vulnerability through various processes including; modifying thrombotic potential and endothelial dysfunction.

During inflammation the mobilization of leukocytes is a common feature, increased total leukocyte and neutrophil counts acts as a predictive factor for first-time and recurrent stroke risk (Grau et al., 2004).

The exact nature of the inflammatory and immune profiles of various disease conditions differ depending on the type of pathology. Some overlap exists but generally speaking, heightened activity of innate immune cells, specifically neutrophils, is known to be stimulated by bacterial infection when stroke risk is greatest, during the acute phase. Whereas most chronic non-infectious diseases like rheumatoid arthritis and atherosclerosis are dictated by macrophage and T cell-driven mechanisms (Libby, 2002).

Despite the potential diversity in immune profile, acute and chronic infections, bacterial and viral, non-infectious and infectious diseases are all known risk factors for stroke. It is important to consider that the characteristic immune profile associated with each predisposing disease condition is likely to be most apparent at the local site of pathology, e.g. at the inflamed synovial membrane, atherosclerotic plaque or site of infection.

However, a convergence may take place at the systemic level where molecular and cellular mediators of inflammation, which are commonly found in multiple diseases, come together.

These mediators mentioned previously which act as markers of stroke susceptibility, may well act in a uniform manner to increase predisposition to an ischemic event, possibly through modification of endothelial function. Further investigation is needed; however numerous studies have reported an increase in severe neurological deficits amongst stroke patients who present with a preceding infection (Smith et al., 2006; Zeller et al., 2005).

According to Zeller *et al* in parallel with increased platelet activation, increased platelet-leukocyte aggregation is present in stroke patients with infection compared with those without 24 hours after a stroke (Zeller et al., 2005).

A study done by Emsley showed that plasma CRP concentration and total leukocyte counts were significantly elevated up until 3 months after the stroke occurred compared with control subjects and this increase was less severe in patients with no preceding infection (Emsley, 2003). Other studies found elevated CRP levels and leukocyte numbers in stroke patients presenting with infection in the week prior to stroke occurrence (Zeller et al., 2005).

Stroke itself initiates a profound inflammatory reaction involving local cellular activation in the brain as well as the production of a range of inflammatory mediators, including proteases, chemokines, cytokines, vascular adhesion molecules and reactive oxygen species that will be discussed later (Wang et al., 2007).

Systemic responses are also initiated that include a hepatic-driven acute phase response and the mobilization of bone marrow-derived leukocytes which are transported to the ischemic brain (Price et al., 2003).

The blood–brain barrier (BBB) is an important interface where peripherally-derived molecular and cellular inflammatory mediators can affect the brain. McColl *et al*. found that the exacerbated peripheral inflammatory response to stroke induced by preceding systemic inflammation has destructive actions on components of the neurovascular unit, specifically the inter-endothelial tight junctions that are vital for BBB integrity (McColl *et al*., 2008).

Systemic inflammation activates both neural and humoral signalling pathways that may cause activation of brain cells (Konsman et al., 2002). This periphery-brain signalling is an important part of the host defence response to infection as the activation of specific brain regions triggers physiological and behavioural alterations that assist in fighting the infection.

According to Perry in 2004 pre-activation of certain cells, in particular microglia, inside the brain due to an infection or other causes of systemic inflammation, could sensitize cells to ischemic brain injury via signalling pathways and eventually enhance the post-ischemic inflammatory reaction found in the brain (Perry, 2004).

Platelets are well known to be essential for primary haemostasis, however they also play an important pro-inflammatory role. Platelets normally circulate in an inactive state. When activated, platelets can secrete and present a variety of molecules, change their shape and the expression pattern of some adhesion molecules.

These changes relate to the adhesion of platelets to leukocytes as well as the vessel wall. The interaction of platelets with neutrophils promotes the recruitment of neutrophils into inflammatory tissue therefore participating in host defense.

The above mentioned interaction of neutrophils with platelets is thought to be mainly mediated through P-selectin (which will be discussed later) and ß2 and ß3 integrins (CD11b/CD18, CD41/CD61). Platelets are also able to interact with endothelial cells and monocytes. Adherent platelets promote the so called “secondary capture” of neutrophils and other leukocytes.

Platelets also secrete endothelial and neutrophil activators inducing production of inflammatory cytokines. Therefore, platelets are important amplifiers of acute inflammation and will be discussed further in following section (Zarbock et al., 2007).

2.4.2.6 Other Risk Factors

Other risk factors include; Alcohol consumption, drugs e.g. cocaine, obesity/body fat distribution, dyslipidemia, arterial fibrillation, asymptomatic carotid stenosis, Sickle cell disease, postmenopausal hormone therapy, oral contraceptives, diet and nutrition, physical inactivity. Novel risk factors: increased Vwf (von Willebrand factor), high white blood cell count, migraine, metabolic syndrome, sleep disordered breathing, Elevated Lipoprotein(a) and Hypercoagulability.

2.5 PREVALENCE OF STROKE

According to a 2012 update report from the American Heart Association; Heart disease and Stroke statistics, on average a person has a stroke every 40 seconds, and dies of one every 4 minutes in the United States. Each year about 795 000 people experience a new or recurrent stroke, of these approximately 610 000 are first attacks and 185 000 are recurrent attacks (Roger et al., 2012).
In 2002, the stroke-related disability was determined to be the sixth most common cause of reduced disability-adjusted life-years (DALYs-the sum of life-years lost as a result of premature death and years lived with disability adjusted for severity). It is estimated that by 2030 stroke-related disability in western societies will be ranked as the fourth most import cause of DALYs (Donnan et al., 2008).

Of all deaths in industrialized countries 10–12% is due to stroke, and about 88 per cent of the deaths attributed to stroke are amongst people over 65 years of age (Markus, 2003).

Stroke is the third most common cause of death worldwide of which more than two-thirds of those deaths occur in developing regions such as sub-Saharan Africa (Connor et al., 2005).

2.6 PATHOPHYSIOLOGY OF STROKE

According to Brey and Coull the coagulation system is responsible for maintaining blood flow in the blood vessels while at the same time ensuring vessel integrity which encompasses repairing fissures in order to prevent seepage from the vessel that may occur under normal circumstances.

When the above mentioned balance is disturbed it can result in the induction of thrombosis. In most cases of ischemic stroke, the initiation of blood coagulation leading to thrombosis is needed to cause ischemia. The rapid initiation of haemostasis usually leads to thrombus formation. The pathological decline of blood flow occurs when there is injury to the endothelium within the heart or within an atherosclerotic precerebral artery.

Within the ischemic cerebrovascular bed, there are two major zones of injury: the core ischemic zone and the ischemic penumbra (the term generally used to define ischemic but still viable cerebral tissue).

While haemorrhagic stroke is attributed to several distinctive defects in haemostasis, most of the coagulation disorders that lead to ischaemic stroke are not well described. A predisposition to thrombosis known as a prothrombotic or hypercoagulable state, may be present after blood coagulation is activated, when fibrinolysis is impaired or when platelet reactivity is increased (Coull and Brey, 2004).

As mentioned above a disturbance in the coagulation system may result in stroke, therefore STROKE AFFECTS HAEMOSTASIS.

The following paragraphs will discuss haemostasis in order to understand the importance of stroke and the haemostatic process.

In the human body the vitally important flow of blood is kept in working order by the presence of a sensitive balance between haemostasis and fibrinolysis. The maintenance of this balance involves a mutually dependent network of physiological processes. To summarize, haemostasis involves the interplay of three factors: vasoconstriction, thrombocyte aggregation and blood clotting (Rau et al., 2007).

When damage occurs at the blood vessel wall, repair must take place as quickly as possible to minimize and prevent blood loss into the surrounding environment (Silverthorn, 2004).

The pressure that is exerted within the blood vessel complicates this process; this is where haemostasis plays a crucial role. Haemostasis entails the cessation of bleeding and involves both blood coagulation and contraction of the damaged blood vessel (Norris, 2003). Haemostasis primarily involves the cleavage of inactive fibrinogen into fibrin. This conversion process is thrombin-mediated (Wolberg and Campbell, 2008).

The procoagulant phase of the haemostatic process is divided into three steps: The first step involves the vasoconstriction of the damaged vessel resulting in a temporary decrease in the pressure and blood flow within the blood vessel which greatly contributes to the formation of a platelet plug (Silverthorn, 2004).

The second step involves the formation of a platelet plug (mechanical seal) that closes off the area which has been damaged. The formation of the platelet plug initiated by the adhesion of thrombocytes to the exposed collagen in the wall of the (Silverthorn, 2004).

The third step takes place simultaneously with the second step and is characterized by the coagulation cascade. Thrombin plays an important role in maintaining the balance between procoagulant and anticoagulant pathways (haemostasis) (Bates and Weitz, 2006; Di Cera, 2011), it converts fibrinogen into fibrin which acts as a reinforcement to the platelet plug now known as a blood clot (Silverthorn, 2004).

The coagulation cascade consists of two pathways: the intrinsic and extrinsic pathways. The Intrinsic Pathway is activated by the exposure of collagen and various other activating factors found in the damaged area (Silverthorn, 2004).

The Extrinsic Pathway is activated when tissue thromboplastin or tissue factor (a mixture of protein phospholipids) is exposed in the area where damage occurred. At one stage the Intrinsic and Extrinsic pathways converge to follow a Common Pathway. In the course of the Common Pathway thrombin activates fibrinogen to form fibrin. Fibrin is insoluble and forms the fibre mesh that reinforces and stabilizes the platelet plug (Silverthorn, 2004).

The platelets and fibrin mesh together trapping erythrocytes, they are used for further reinforcement. Plasmin molecules are also incorporated by the fibrin network that are essential for the dissolving of the clot after repair has been completed (Silverthorn, 2004).

Thrombin is activated by a cascade of enzymatic reactions that is activated in turn by injury or damage to a blood vessel (Sugo et al., 2006). Throughout the process of fibrinolysis, thrombin is controlled by thrombomodulin (Di Cera, 2008).

Fibrinolysis is initiated by the activation of plasminogen by thrombomodulin and the thrombin complex that results in the degradation of the blood clot (Norris, 2003). The fibrinolytic pathway also involves Protein C which is activated by the coagulation cascade. The main objective of fibrinolysis is to prevent potentially damaging excessive clot formation which could result in thrombosis and to removed clots after the damaged vessel has been restored (Rau et al., 2007).

The process of fibrinolysis involves the action of the proteolytic enzyme plasmin (inactive: plasminogen or fibrinolysin) that dissolves the clot (Norris, 2003).

Plasminogen can be activated by a variety of factors including both extrinsic and intrinsic compounds. The intrinsic factors consist of kallikrein, factor XII and XI. Extrinsic factors consist of the tissue plasminogen activator (t-PA) which is released throughout the fibrinolytic process (Ajjan and Grant, 2006).

Blood coagulation is regulated by an array of anticoagulant factors. Under normal physiological conditions the coagulation formed thrombus (Esmon, 2003; Rau et al., 2007). Thrombomodulin functions as a cofactor to thrombin which in turn amplifies the rate in activation of protein C (Dahlbäck and Villoutreix, 2005a). promoters are outweighed by anticoagulant factors (Dahlbäck and Villoutreix, 2005b; Esmon, 2003). A paramount factor in the regulation of this pathway is the vitamin K-dependent Protein C (Dahlbäck and Villoutreix, 2005a; Esmon, 2003).

There is an undoubted relationship between arterial occlusive disease, including stroke, and coagulation mechanisms. Thrombosis is central to the main pathophysiological mechanisms of ischaemic stroke.

In the majority of cases the damaging thrombotic process becomes activated secondarily to an underlying vascular pathology, however some cases of ischaemic strokes results primarily from an alteration of the haemostatic mechanisms which likely results in unchecked and unregulated thrombus formation within the cerebral circulation (Greaves, 1993).

Many of the haemostatic disorders found in stroke are associated with dysfunction of the vascular endothelium as well as abnormalities of or interference with the natural anticoagulant proteins such as protein C, protein S, and antithrombin III (Coull and Clark, 1993).

Furthermore **abnormal platelet activation**, platelet count and volume have also been implicated as risk factors of ischaemic stroke (Greaves, 1993).

Basic research studies have implicated smoking in various abnormalities of coagulation. In smokers an inflammatory state exists, this is of interest as the possible influence of inflammation on coagulation offers further potential to contribute to thrombogenesis.

Smokers have been found to have significant elevations in numerous plasma factors and increased platelet aggregation and fibrinolysis. Additionally plasma fibrinogen levels are elevated in smokers as well as Factor XIII, which is involved in stabilizing fibrin clots and thrombin generation seems to be induced (Tapson, 2005).

Lastly cigarette smoking is a major risk factor for the development of atherosclerosis. Plausible mechanisms for this include platelet and leucocytes activation, and damage to the endothelium. Any of these mechanisms may contribute to changes in thrombosis and haemostasis. A variety of studies have demonstrated that smoking may result in an altered endothelial function by a direct toxic effect which in turn may trigger haemostatic activation and thrombosis (Cacciola et al., 2007).

In conclusion acquired, possibly short-lived, abnormalities of platelets, coagulation, and fibrinolysis may contribute greatly to stroke in synergy with other mechanisms e.g. a pro-inflammatory state in a person who smokes. Stroke and smoking therefore likely share common factors regarding abnormalities found in haemostasis and the coagulation cascade.

A reason must exist for these haemostatic changes. One of the main culprits may be oxidative stress as a result of reactive oxygen species (ROS), in the following chapter ROS will be discussed as well as the affect it has on stroke and the affect smoking has on it in order to gain a better understanding of how all these factors are inter linked in the pathophysiology of stoke and smoking.

2.7 REACTIVE OXYGEN SPECIES, STROKE AND SMOKING

The generation of free radicals and other reactive oxygen and nitrogen species occur in vivo via various processes whether it is by an 'accident' of chemistry or useful metabolic purposes. The balance of these radicals is regulated by a network of antioxidant defence systems that are assisted by repair systems. Tissue injury in the pathology of human disease is often accompanied by an imbalance of the oxidant/ antioxidant status, resulting in oxidative stress. Oxidative damage to biomolecules is thought to play an important role in the pathophysiology of numerous human diseases and can be treated by therapeutic intervention with appropriate antioxidants (Halliwell, 2001).

2.7.1 What are free radicals

Electrons in atoms occupy areas of space known as orbitals. Each orbital can hold only two electrons, which are spinning in opposite directions. According to Halliwell a free radical can most simply be defined as any species able to exist independently containing one or more unpaired electrons.

Containing only paired electrons most biological molecules are non-radicals. When an electron occupies an orbital by itself it has two possible directions of spin. Seeing as electrons are more stable when paired in orbitals, radicals tend to be more reactive than non-radical species in general, although there is considerable variation present in their reactivity. Radicals can react with other molecules in various ways (Halliwell, 1989).

The simplest free radical is an atom of hydrogen with one proton and a single electron. Superoxide ($O_2^{\bullet -}$) and hydroxyl ($OH^{\bullet}$) are examples of oxygen-centred radicals and trichloromethyl ($CCl_3^{\bullet}$) is an example of a carbon-centred radical. Sulphur centred radicals are Thiyl radicals ($RS^{\bullet}$), and nitric oxide ($NO^{\bullet}$) is a free radical where the unpaired electron is delocalized between two different atoms. A dot is always used to denote free radicals (Halliwell, 2001).

When a free radical reacts with a non-radical, it results in the creation of a new free radical. (Halliwell, 2001) Oxygen-free radicals (OFR), or more broadly speaking, reactive oxygen species (ROS), and reactive nitrogen species (RNS) are the products of normal cellular metabolism (Valko et al., 2006).

The term reactive oxygen species, is used often in the biomedical free radical literature, is a collective term that includes oxygen-centred radicals such as $O_2^{\bullet -}$ and $OH^{\bullet}$, but also some non-radical derivatives of oxygen, such as hydrogen peroxide (H_2O_2) and hypochlorous acid

(HOCl). A major source of ROS is generated by the mitochondria as the toxic by-products during oxidative phosphorylation, the energy generating pathway (Ridnour et al., 2005).

2.7.2 Oxidative Stress

The harmful effect of free radicals that has the ability to cause biological damage is known as oxidative stress and nitrosative stress. The term oxidative stress refers to the state where there is a serious imbalance between generation of reactive species and antioxidant defence (Valko et al., 2001).

Antioxidant defence systems function by scavenging, and minimizing the formation of ROS but they are not 100% effective. For this reason repair systems exist to manage molecules that have been oxidatively damaged. This defence system is multifactorial and includes both endogenous and diet-derived molecules.

ROS and RNS are well known for being either harmful or beneficial to living systems. It is estimated that the average person has about 10 000 – 20 000 free radicals attacking their body per day (Valko et al., 2006).

In spite of the cell's antioxidant defence system in place to counteract oxidative damage from ROS, radical-related damage of proteins and DNA have been suggested to play a key role in the development of several degenerative processes including Alzheimer disease, Parkinson disease, ischemic heart disease, arthritis, cancer and aging (Pala and Gurkan, 2008).

Oxidative stress has also been implicated as a major role player in pathological conditions involving cardiovascular disease and ischemia/reperfusion amongst others (Dhalla et al., 2000).

2.7.3 Formation of reactive species

Many reactive species and free radicals are continuously generated in the human body. See figure 2.1 for examples of how radicals are generated in the body. Some are generated by mishaps of chemistry e.g. a steady stream of $O_2^{\bullet -}$ is generated when electrons leak directly on O_2 from the intermediate electron carriers of the mitochondrial electron transport chain.

OH• and H• radicals are formed in living organisms when they are exposed to ionizing radiation, this results in the splitting of the O – H bonds in water. OH• is a highly reactive molecule and when it is synthesized in vivo it damages anything it is generated next to (Halliwell, 1989).

$NO^{\bullet}$

Contrasting to the deleterious effects of OH•, other less reactive radicals may be useful in vivo. One of these radicals is NO• which is synthesized from the amino acid L-arginine by three types of nitric oxide synthases (NOS). NOS type I and III are Ca^{2+} -dependent and constitutively expressed, primarily in vascular endothelial cells and nerve tissue.

NO• is a water- and lipid-soluble free radical with an extremely short half-life of only a few seconds. Up regulation of NOS type II (inducible enzyme) is mediated transcriptionally by a range of cytokines.

Nitric oxide assists in regulating blood pressure and is thought to be involved in the killing of parasites by macrophages, and has a variety of other functions (Brouns and De Deyn, 2009; Halliwell, 2001).

Superoxide

Superoxide radical is produced by phagocytic cells and assists in killing bacteria. Research also suggests that other cell types like lymphocytes, fibroblasts and endothelial cells may produce smaller amounts of superoxide as intercellular signalling molecules (Halliwell et al., 2000). In phagocytes superoxide is generated by a membrane bound enzyme complex called reduced nicotinamide dinucleotide phosphate (NADPH) oxidase (Perner et al., 2003).

A big portion of $O_2\bullet^-$ synthesized in vivo likely undergoes a dismutation reaction shown by the overall equation below in which hydrogen peroxide (H_2O_2) is formed (Halliwell, 2001).

$$2O_2\bullet^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Other sources of superoxide include NOS, xanthine oxidase, cyclooxygenase (COX), and mitochondrial electron transport chain enzymes, which play a minor role in the production (Perner et al., 2003). Superoxide is able to cross the cell membrane via the anion channel (Brouns and De Deyn, 2009).

Superoxide is very short lived and largely cell impermeable. It is the precursor of several biologically relevant and important ROS. Superoxide reacts with $NO^{\bullet}$ to inactivate $NO^{\bullet}$ in a diffusion-limited fashion, leading to $NO^{\bullet}$ deficiency and endothelial dysfunction, a defining characteristic of many vascular diseases, including atherosclerosis, hypertension and diabetic vascular complications (Cai et al., 2007).

Superoxide can react with ferric iron in the Haber-Weiss reaction to produce Fe(II) thereby effecting redox cycling of the iron, see Figure 2.2 (Kell, 2010):

$O_2^{\bullet -} + Fe^{3+} \rightarrow O_2 + Fe^{2+}$

Hydrogen Peroxide

H_2O_2 has a similar molecular structure as water and is highly diffusible within and between cells. H_2O_2 can also be produced by the action of various oxidase enzymes found in cells including amino acid oxidases and xanthine oxidase. Hydrogen peroxide is lipid soluble and readily crosses cell membranes (Brouns and De Deyn, 2009).

Hydrogen peroxide is produced in vivo by the dismutation of superoxide radical non-enzymatically, as well as enzymatically catalysed by superoxide dismutase. A range of oxidase enzymes produce hydrogen peroxide including monoamine and glycollate oxidases. Furthermore hydrogen peroxide is produced by the peroxisomal pathway for β-oxidation of fatty acids (Halliwell et al., 2000).

H_2O_2 also amplifies its own production and regulates endothelial function through complex mechanisms (Cai et al., 2007).

H_2O_2 is poorly reactive in chemical terms; it can act as mild oxidizing or reducing agent, unless molecules have hyper-reactive thiol groups or methionine residues H_2O_2 will not oxidize most biological molecules readily.

The deleterious effects of H_2O_2 mainly comes from its conversion to the indiscriminately reactive hydroxyl radical ($OH^{\bullet}$) via exposure to ultraviolet light shown below (a) or by the interaction with a variety of transitional metal ions via the Fenton reaction, most significantly likely being iron in vivo (b).

(a) $H_2O_2 \xrightarrow{uv} 2OH^{\bullet}$

(b) $H_2O_2 + Fe^{2+}$ (or Cu^{+}) $\rightarrow$ intermediate products $\rightarrow OH\bullet + OH^{-} + Fe^{3+}$ (Cu^{2+})

When iron is not properly liganded and in the ferrous form, it can react with hydrogen peroxide or (per)oxidases via the Fenton reaction leading to the very damaging hydroxyl radical as shown above (Kell, 2010).

Figure 2.1 Examples of the production of free radicals by different biochemical processes within the body (Betteridge, 2000).

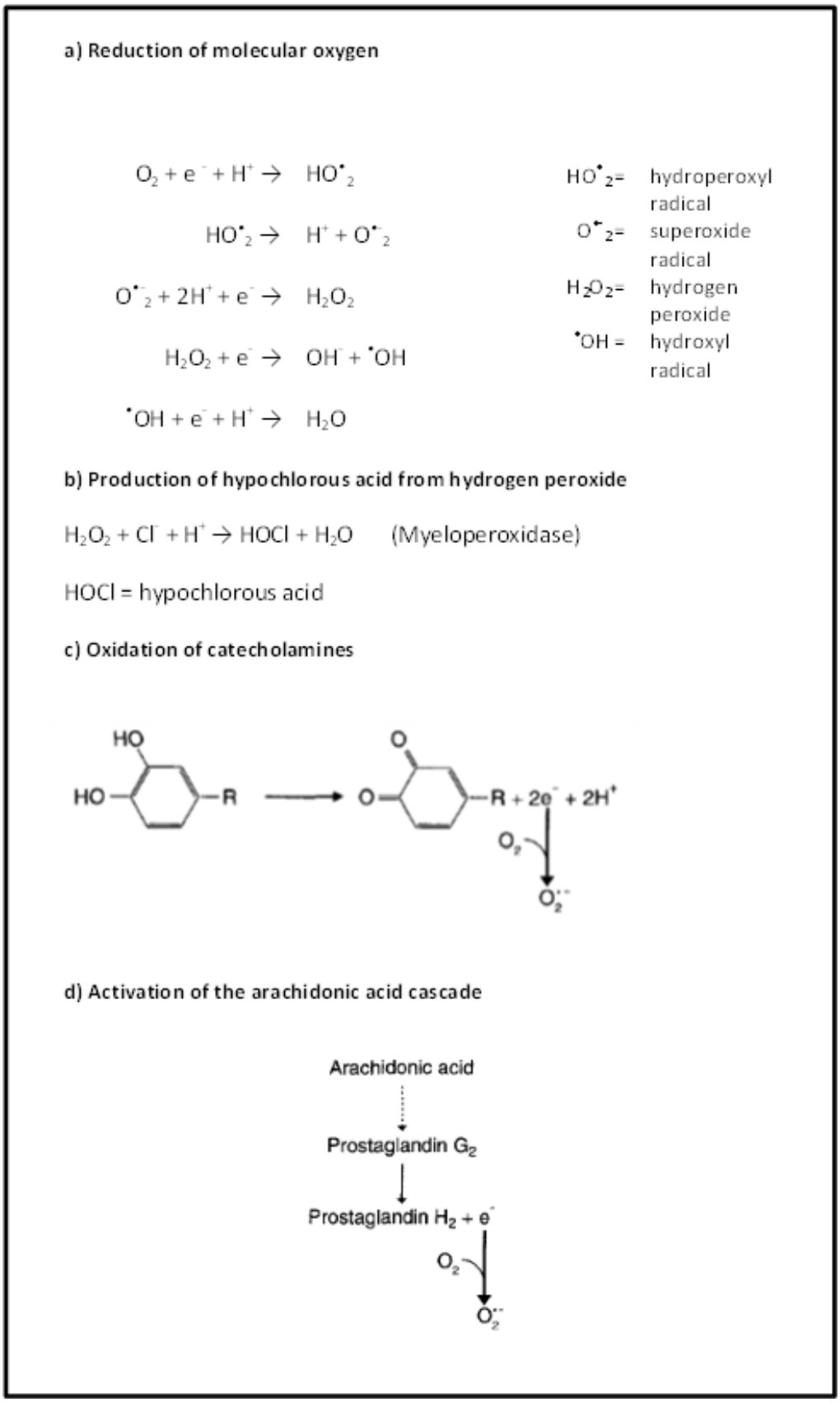
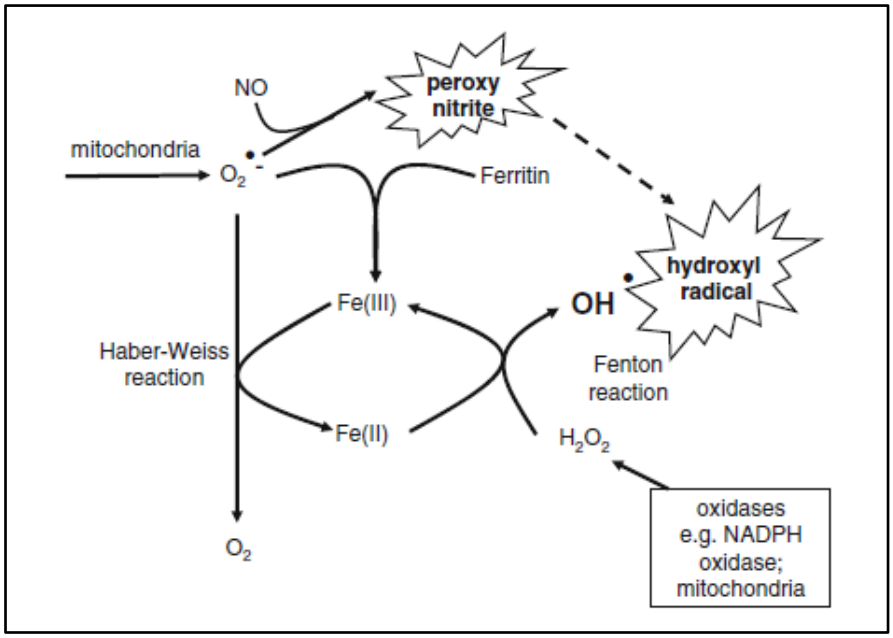


Figure 2.2. The Haber-Weiss and Fenton reactions combine using poorly liganded iron in a catalytic cycle to produce the very damaging hydroxyl radical. Poorly liganded iron can also be liberated via the destruction of haem and other iron-containing substances. Peroxynitrite anion (ONOO-) is produced by the reaction of superoxide and nitric oxide (NO•) which when protonated decomposes to OH• and NO_2 (Kell, 2010).



Living organisms have adapted in such a way that mechanisms exist to sequester transition metal ions into protein-bound forms that are unable to catalyse $OH^{\bullet}$ formation and also other free radical reactions in vivo. The above mentioned mechanisms are specifically important in extracellular fluids like the blood plasma.

Regarding inflammation H_2O_2 generated at the site of inflammation by activated phagocytes seem to modulate the inflammatory process (Halliwell et al., 2000).

2.7.4 Consequences of oxidative stress

Free radicals exhibit a wide spectrum of cellular effects including damage to the cytoskeleton and DNA, inactivation of enzymes, protein denaturation, release of calcium ions from intracellular stores, lipid peroxidation and chemotaxis (Brouns and De Deyn, 2009).

Oxidative stress can result in:

1. Adaptation: for example the up regulation of antioxidant defence systems; when adult rats are slowly acclimatized to increased O2 levels they are able to tolerate pure O2 for significantly longer periods than control rats, apparently due to increased production of antioxidant defence enzymes and reduced glutathione (GSH) in the lung. Ischaemic

preconditioning is another example where ROS produced by ischemia are initially harmful, but may also lead to a response that is protective against subsequent insult.

2. Tissue injury: oxidative stress can cause damage to a variety of molecular targets as mentioned above. The primary cellular target of oxidative stress differs depending on the type of tissue. For example, when H_2O_2 is added to several mammalian cells, increased DNA strand breakage occurs before detectable oxidative protein damage or lipid peroxidation.

3. Cell death: Both apoptosis and necrosis can result from oxidative stress. During necrotic cell death, the cell swells and ruptures, releasing its contents is into the surrounding area affecting adjacent cells. The released contents can include antioxidants such as catalase and pro-oxidants such as iron and copper ions. Therefore if a cell dies because of other mechanisms than oxidative stress, necrotic cell death may lead to oxidative stress in the immediate surrounding environment. However in apoptosis, the cell's intrinsic 'suicide mechanism' is activated; cells undergoing apoptosis do not release their contents therefore generally speaking no damage is caused by apoptosis to surrounding cells (Halliwell, 2001). Also severe oxidative stress may cause cell death through necrosis where moderate oxidation on the other hand can trigger apoptosis (Brouns and De Deyn, 2009).

2.7.5 Reactive species and Human Disease

Free radicals have been implicated in the pathology of over 100 human diseases, ranging from haemorrhagic shock and ulcerative colitis to AIDS (acquired immune deficiency syndrome) and cystic fibrosis. Few human diseases are caused primarily by oxidative stress however increased formation of reactive species more often occur as a consequence of the disease pathology. The resulting oxidative stress can play a significant role in further tissue injury (Halliwell, 2001).

2.7.6 Oxidative stress in stroke

It is noteworthy to mention that many of the risk factors of stroke discussed earlier contribute to disease progression at least partly via oxidative stress. E.g. Cigarette smoke contains significant amounts of free radicals and also regulates their production. Also oxidative stress and DNA damage are induced by oxidised low density lipoproteins (oxLDL) and by diet-induced hypercholesterolaemia. Regarding diabetes many studies have linked type I and II with atherosclerosis via oxidative stress. The role of aging in cardiovascular disease may involve age-related mitochondrial damage and dysfunction (Fearon and Faux, 2009).

Regardless of the mechanism whereby ROS is produced, when their amount is significantly elevated there is a high risk of neuronal damage because of the high susceptibility of the brain to oxidative stress for the following reasons:

(1) The brain represents a mere 2% of body weight, but it consumes an enormous amount of about 20% of the body's oxygen;

(2) The brain is relatively poorly supplies with protective antioxidants enzymes or substances;

(3) The brain has relatively high concentrations of easily peroxidizable lipids, such as polyunsaturated fatty acids, which represent one-third of brain fatty acids, and particularly those with many double bonds such as arachidonic acid and docosoexaenoic acid

(4) The brain has many regions that are highly enriched in iron (Cherubini et al., 2005).

2.7.6.1 Free radicals in ischemia and reperfusion

A large amount of evidence supports the finding of an increased production of free radicals during ischemia and reperfusion (Morimoto et al., 1996). Various studies show that ROS and RNS are important mediators of tissue injury in acute ischemic stroke as shown in detail in Figure 3 (Kontos, 2001).

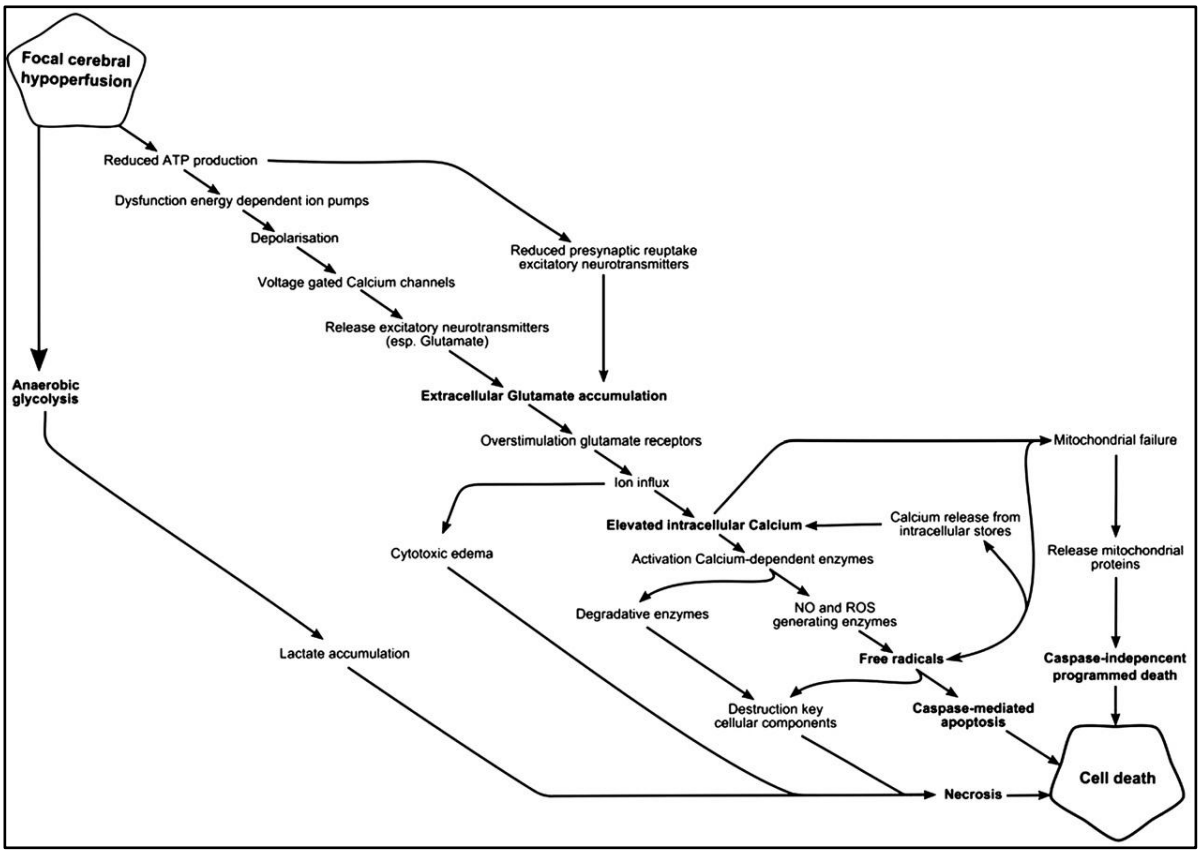
2.7.6.2 Inflammation and ROS following stroke

During stroke the cessation of cerebral blood flow leads to energy depletion as well as necrotic neuronal death. This in turn can trigger immune responses that ultimately lead to inflammatory cell activation and infiltration. Next reperfusion of the occluded vessel, due to compensation by the accompanying circulation, or spontaneous or therapeutic canalization leads to the generation of ROS either via reperfusion with oxygenated blood or by production within brain and immune cells.

ROS are now able to stimulate ischemic cells causing them to secrete inflammatory cytokines and chemokines that cause peripheral leukocyte recruitment and adhesion molecule upregulation in the cerebral vasculature respectively. Once the inflammatory cells are activated, they can release a variety of cytotoxic agents including even more cytokines, matrix metalloproteinases (MMPs), NO and more ROS (Figure 2.4).

These substances are able to induce more cell damage and disruption of the BBB and extracellular matrix.

Figure 2.3. Diagrammatic representation of processes leading to bioenergetic failure, excitotoxicity, oxidative stress and eventually cell death after acute focal cerebral hypoperfusion (ATP (adenosine triphosphate); NO, ROS,) (Kontos, 2001).



Lastly secondary damage develops as a result of brain oedema, vasomotor/hemodynamic deficits and post ischemic microvascular stasis leading to hypoperfusion and post-ischemic inflammation, thereby involving activation of microglia and potentially damaging brain infiltration of peripheral inflammatory cells (Wang et al., 2007).

From the above mentioned paragraphs it is clear that two significant pathophysiological mechanisms are involved in ischemic stroke; oxidative stress and inflammation. As mentioned previously brain tissue is not well equipped with antioxidant defenses, hence why reactive oxygen species and other free radicals or oxidants, released by several inflammatory cells, threaten tissue viability in the perimeter of the ischemic core (Lakhan et al., 2009).

Within seconds to minutes after loss of blood flow to a region of the brain, rapid initiation of the ischemic cascade occurs, which entails a series of subsequent biochemical events that lead to the disintegration of cell membranes and neuronal death at the core of the infarction.

Focal hypoperfusion found at the onset of ischemic stroke leads to excitotoxicity and oxidative damage which in turn leads to microvascular injury and blood brain barrier dysfunction additionally initiating post-ischemic inflammation. These events all exacerbate the initial injury and may lead to permanent cerebral damage (see Figure 2.5).

Figure 2.4. Inflammation following stroke. Brain ischemia triggers inflammatory responses due to the presence of necrotic cells, generation of ROS and production of inflammatory cytokines even within neurons.

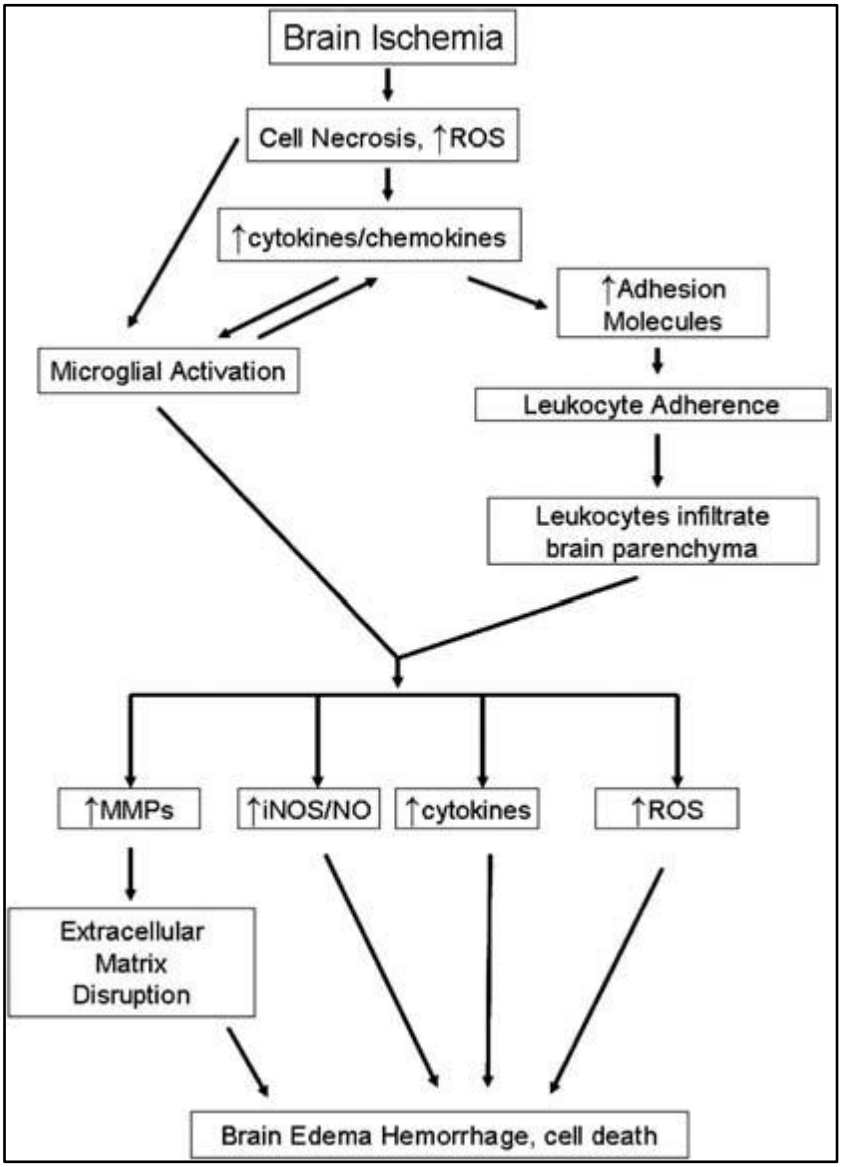
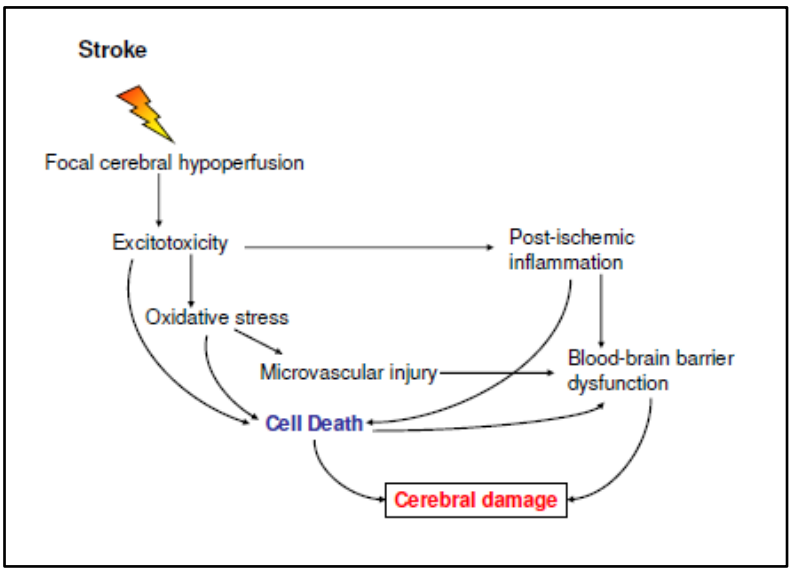


Figure 2.5. Ischemic cascade leading to cerebral damage. Ischemic stroke leads to hypoperfusion of a brain area that initiates a complex series of events. Excitotoxicity, oxidative stress, microvascular injury, blood-brain barrier dysfunction and post ischemic inflammation lead ultimately to cell death of neurons, glia and endothelial cells. The degree and duration of ischemia determines the extent of cerebral damage (Lakhan et al., 2009).



Oxidative stress leading to ischemic cell death involves the formation of ROS and RNS through multiple injury mechanisms, such as Ca^{2+} overload, mitochondrial inhibition, reperfusion injury, and inflammation (Lakhan et al., 2009). Specific ROS formed during stroke will now be discussed further.

2.7.6.3 Superoxide and Hydrogen Peroxide

During brain ischemia followed by reperfusion superoxide is generated, through the leakage from the mitochondrial electron transport chain and via xanthine oxidase.

Under these conditions superoxide is generated during reperfusion. The concentration of superoxide is at its maximum in the early phases of reperfusion and decreases over the next 2 hours. Consistent with this Peters *et al*., directly showed an increase in superoxide radical anions in the penumbral region not only during the ischemic phase but also after reperfusion (Peters et al., 1998). A doubling in superoxide radical anion formation has been shown in the peri-infarct penumbra during and after focal ischemia (Fabian et al., 1995). As mentioned previously superoxide is converted to hydrogen peroxide via superoxide dismutase therefore one would expect an increase in hydrogen peroxide in stroke patients.

A variety of other radicals are produced as a result of the interaction of oxygen radicals with other molecules found in tissue. Of great importance is the formation of the highly toxic

peroxynitrite, which is formed from superoxide and nitric oxide, most of the toxicity as a result of superoxide has been ascribed to the formation of peroxynitrite (Beckman and Koppenol, 1996; Beckman et al., 1996; Dugan and Choi, 1994; Fridovich, 1978; Iadecola, 1997; Wei and Kontos, 1990; Wei et al., 1996).

Peroxynitrite can be protonated spontaneously to produce the hydroxyl radical which is the most reactive oxygen radical, and probably causes the most tissue injury. Peroxynitrous acid can also directly increase brain damage by reacting with a variety of important biological molecules or it can be converted into other ROS and RNS (Beckman, 1994; Kontos, 2001).

Free radicals are known to influence cerebral blood flow; they can act as strong cerebral vasodilators (Wei et al., 1985) and the interaction between nitric oxide and superoxide leads to the alteration of vascular reactivity to CO_2 consequently inducing vasoconstriction instead of vasodilation (Kontos, 2001). Oxygen radicals also increase platelet aggregability (Brouns and De Deyn, 2009; Kontos, 2001; Love, 1999)

2.7.6.4 NO

Ischemia causes an increase in NOS type I and III activity in neurons and vascular endothelium. In terms of brain ischemia, NOS type I and II are deleterious, but production of NO in blood vessels by NOS type III readily improves blood flow to the ischemic penumbra via vasodilation and inhibition of platelet adhesion. It also scavenges oxygen radicals and has anti-inflammatory effects through the inhibition of leukocyte adhesion to the endothelial cells.

NO can have protective and damaging roles, mostly depending on the cell type producing it and the phase of ischemia as shown by various studies. Inhibition of platelet aggregation and the prevention of leukocyte endothelial adhesion is amongst some of the protective effects of NO (Castillo et al., 2000).

2.7.6.5 Ischemia itself and free radical production

Independent of the intricate mechanisms responsible for ischemic stroke, ischemia itself sets a cascade of events in motion that can lead to the increase free radical production through several different pathways.

The major change affecting neurons during ischemia is the depletion of ATP, due to the lack of the substrates needed for its production (oxygen and glucose). This energy failure causes

membrane depolarization, because of the reduced activity of ATP-dependent ion pumps, like Na^+/K^+-ATPase.

Transmembranous ionic gradients are compromised because of this impairment resulting in an influx of extracellular Ca^{2+} through voltage-sensitive Ca^{2+} channels and the uncontrolled release of several excitatory amino acids, such as glutamate into the extracellular space (Lipton, 1999).

The end result of ischemia is the accumulation of intracellular Ca^{2+} from multiple sources. This induces the activation of a range of Ca^{2+}- stimulated enzymes, such as lipases, proteases, protein kinases, nucleases, and NOS.

Some Ca^{2+}-dependent enzymes like phospholipase A2 and COX, generate oxygen free radicals and neuronal nitric oxide synthase (nNOS) produces nitric oxide during the ischemic phase and early reperfusion (Lee et al., 1999; Lipton, 1999).

The modified function of the mitochondrial electron transport chain is most likely a source of oxidative stress during ischemia and reperfusion (Sims and Anderson, 2002).

Now that the course and formation of ROS during stroke has been discussed the following paragraphs will focus on ROS and the vascular system including haemostasis, coagulation and platelets.

2.7.6.6 Vascular function and ROS

Two effector systems that are often implicated in the vascular changes associated with inflammation involve the production of ROS and RNS. Research in this field mostly supports the view that the enhanced production of ROS and decreased bioavailability of NO, found in an inflammatory response, plays a crucial role in mediating the microvascular dysfunction.

Furthermore it is believed that restoration of the normal physiological balance between ROS and NO will normalize vascular function (Granger and Kubes, 1996; Suematsu et al., 2002; Tailor et al., 2005; Tyml, 2011; Wolin, 2009; Wolin et al., 2002).

Oxidative stress pathways generating ROS are prominent in endothelial injury, both ischemic and non-ischemic, with prominent roles of enzyme- and non-enzyme mediated pathways; mitochondria once again have a critical role, particularly in the non-enzymatic pathways generating ROS. Inflammation also contributes to vascular endothelial injury, and endothelial cells have the capacity to rapidly increase expression of inflammatory mediators following ischemic challenge; this leads to enhanced leukocyte-endothelial interactions mediated by selectins and adhesion molecules (Fisher, 2008).

2.7.6.7 ROS, Coagulation and thrombosis

It is well recognized that coagulation and inflammation are closely linked processes that can evoke a vicious cycle because the activation of the one process can lead to activation of the other (Esmon, 2005; Levi and van der Poll, 2005; van der Poll, 2001).

This interdependence of coagulation and inflammation is evident in a variety of acute and chronic inflammatory diseases where changes in haemostatic biomarkers may suggest subclinical activation of the coagulation system, also an increased prevalence of thromboembolic events is present (Broeders, 2002; Danese et al., 2007; Egbrink et al., 2005; Granger et al., 2010; Leopold and Loscalzo, 2009; Levi et al., 1997; Remková and Remko, 2010; Tailor et al., 2005; Yoshida and Granger, 2009).

ROS can disrupt the highly controlled coagulation system found in healthy tissue by, altering the expression and activity of major components of the coagulation cascade (Leopold and Loscalzo, 2009).

Redox-sensitive regulatory mechanisms have been indicated as a role player in the production/activation of tissue factor (TF). The gene encoding TF is redox sensitive (Herkert and Görlach, 2002). Exposure of endothelial cells and monocytes to ROS e.g. H_2O_2 results in an increased TF mRNA and protein expression and enhanced TF procoagulant activity (Cadroy et al., 2000; Golino et al., 1996).

Many studies have linked ROS-dependent induction or activation of TF to NADPH oxidase in neutrophils, platelets, and endothelial cells (Djordjevic et al., 2004; Görlach et al., 2000).

Other components in the coagulation pathway are similarly influenced by ROS to favour a pro-coagulant environment.

The protein C pathway is influenced by ROS in several ways. ROS can induce the shedding of endothelial protein C receptor (EPCR) through endothelial cells. Antioxidants have been shown to attenuate the EPCR shedding caused by cytokines. (Menschikowski et al., 2009) Activated protein C (APC) can be directly inactivated by oxidants inducing changes in the structure of the APC active site (Nalian and Iakhiaev, 2008).

Thrombomodulin, which plays a role in the activation of protein C, is inactivated via oxidation of specific methionine residues by neutrophil-derived oxidants (Glaser et al., 1992).

The oxidized thrombin exhibits a reduced capacity to interact with the antithrombin III–heparin complex, thrombomodulin, protein C, as well as a decrease in its ability to activate platelets (Gray and Barrowcliffe, 1985).

The prothrombotic and procoagulant effects of ROS are also as a result of effects on fundamental components of the fibrinolytic system. Fibrinogen modified by oxidation forms fibrin at an accelerated rate and also has a reduced ability to stimulate tissue plasminogen activator in order to convert plasminogen to plasmin, when compared to non-oxidized fibrinogen (Upchurch Jr et al., 1998).

ROS enhances fibrinolysis by inhibiting plasminogen activator (Lawrence and Loskutoff, 1986) and by increasing the transcription of PAI-1 in vascular smooth muscle cells, endothelial and other cells (Dimova et al., 2004; Swiatkowska et al., 2002).

2.7.6.8 Thrombus formation and ROS

Based on the knowledge gathered on the known actions of ROS and NO on the coagulation cascade as well as platelet function, it seems likely that ROS promote thrombus development while NO is antithrombotic.

Superoxide dismutase (SOD), less specific free radical scavengers and catalase, has been shown to decrease thrombus development in numerous in vivo models (Peire and Puig-Parellada, 1998; Yao et al., 1993). The protection against thrombosis attributed to SOD has been linked to preservation of NO bioavailability (Meng et al., 1995).

According to Hashimoto *et al.* iron chelation with desferrioxamine has proven effective in disabling thrombosis, (Hashimoto et al., 1999) whereas according to Day *et al* chronic iron administration seems to accelerate arterial thrombosis (Day et al., 2003). This suggests that iron-catalyzed radicals originating from superoxide and hydrogen peroxide likely contribute to thrombogenesis.

Some studies implicate both NADPH oxidase (Begonja et al., 2005) and xanthine oxidase (Kuwano et al., 1996) as sources of the ROS that are involved in thrombus development.

The relative contributions of endothelial cells, platelets, and other cells (e.g., leukocytes, macrophages) to the ROS and NO that modulate thrombus formation remain unknown (Kvietys and Granger, 2012).

2.7.6.9 Platelets and ROS

As mentioned before the activation, adhesion, and aggregation of platelets, a critical component of thrombus formation, represents the primary response to vascular injury (Andrews and Berndt, 2004; Furie and Furie, 2008; Mackman et al., 2007).

A further consequence of platelet activation and aggregation is the enhanced production of ROS, which is associated with an increase in oxygen consumption as well as elevated levels of glutathione disulfide (Bressler et al., 1979; Burch and Burch, 1990).

Stimulated platelets have been shown to produce hydrogen peroxide, superoxide, and hydroxyl radicals (Caccese et al., 2000; Finazzi-Agrò et al., 1982; Krötz et al., 2004; Wachowicz et al., 2002). Superoxide anions released into the bloodstream by platelets act as autocrine messengers that are involved in further platelet activation and the recruitment of platelets to the sites of thrombus formation (Sill et al., 2007).

Furthermore several studies have suggested that platelet-derived ROS likely play a more important role in paracrine or autocrine signalling, than a phagocytic function (Essex, 2009; Krötz et al., 2004).

Even though platelets have the ability to produce ROS from several enzymatic sources, most studies have attributed platelet-derived ROS to NADPH oxidase (Essex, 2009; Freedman, 2008; Krötz et al., 2004).

Platelets are known to express Nox2-containing NADPH oxidase (Nox are a family of catalytic enzymes found in the catalytic subunit of NADPH oxidase) Activated Nox produces superoxide (See Figure 2.6) It has been shown that collagen independently increased platelet production of superoxide mediated by Nox (Arthur et al., 2008).

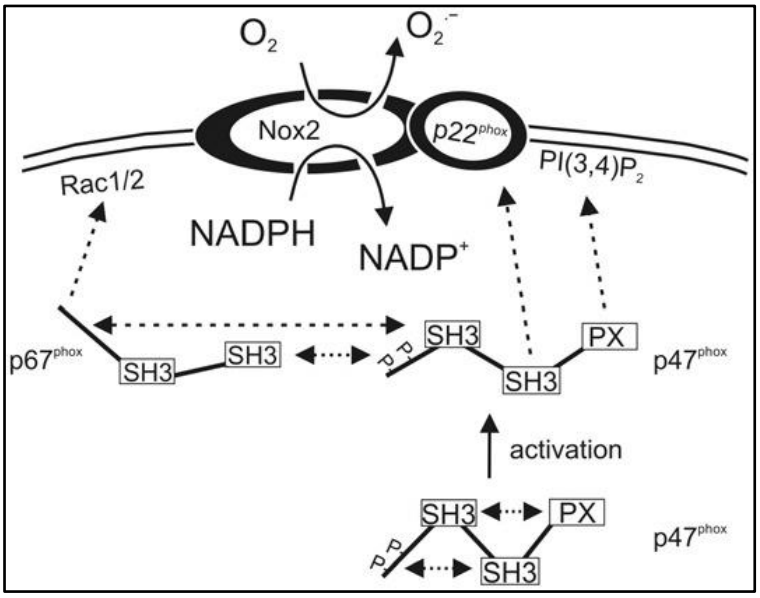
Platelets stimulated with thrombin, produce cytosolic superoxide anions via NADPH oxidase and these oxidant anions are thought to participate, in some manner in signalling leading to GP IIb/IIIa (glycoprotein IIb/IIIa) receptor activation (Sill et al., 2007).

Even though collagen-induced GPIIb/IIIa- mediated platelet aggregation is known to be associated with enhanced superoxide generation via NADPH oxidase (Görlach, 2005), the time course of superoxide production lags behind the aggregation response, indicating that superoxide is not critical a critical factor for the initiation of aggregation (Essex, 2009).

Regardless, both superoxide scavengers and NADPH oxidase inhibitors have been found to blunt platelet aggregation and thrombus formation on collagen. Megakaryocytes and platelets also express the same isoform (NOSIII) of nitric oxide synthase that is present in endothelial cells (Gkaliagkousi et al., 2007).

Under normal conditions platelet-associated NOS generates NO, but when certain cofactor levels are limited, the enzyme is also able generate superoxide (Wolin et al., 2002).

Figure 2.6. Platelet NAD(P)H oxidase complex (Nox2). Activation of p47phox by phosphorylation leads to recruitment of the p47phox/p67phox complex (cytosolic components) to the Nox2/ p22phox subunits (membrane associated) to form an active complex that converts NADPH to NADPþ and generates O_2^-. The p47phox subunit interacts with PI(3,4)P2, p22phox via N-terminal PX and SH3 domains, and p67phox which, in turn, interacts with Rac1/2 (Arthur et al., 2008).



According to Freedman *et al.* “Uncoupled” NOS may be a role player in platelet superoxide production as platelets derived from eNOS- deficient mice showed a marked attenuation of superoxide flux (Freedman et al., 2000).

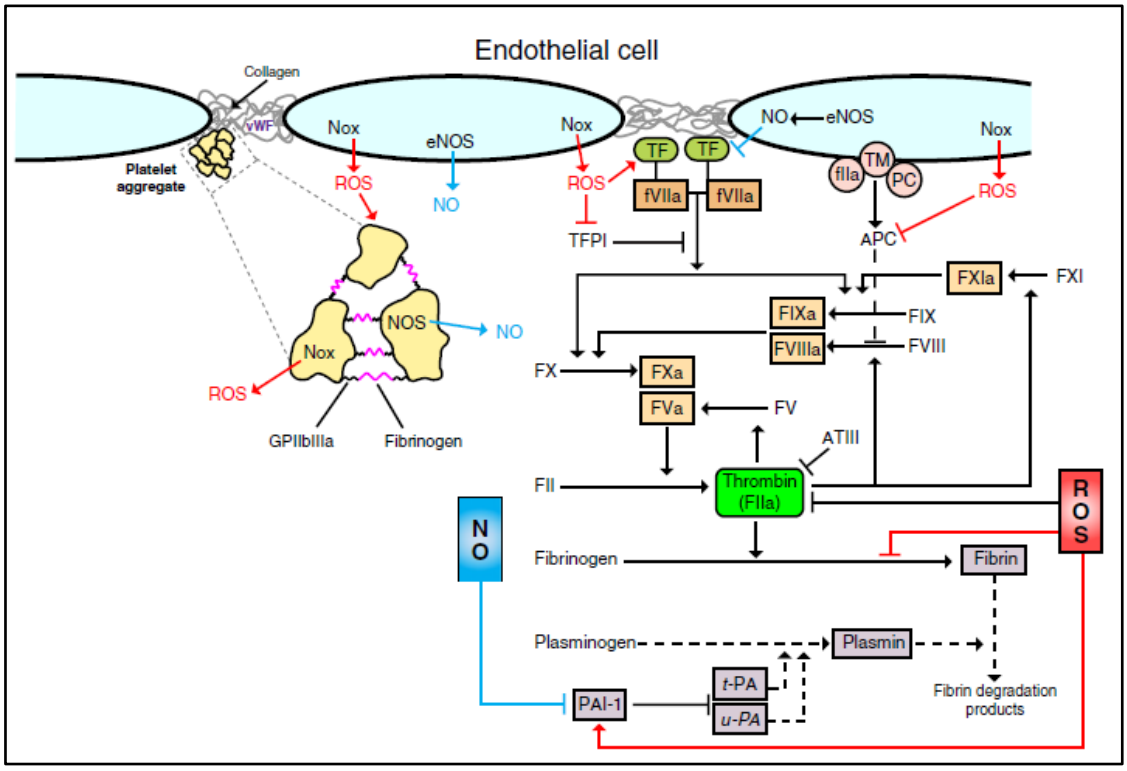
A number of mechanisms have been implicated in the pro-aggregation actions of ROS, including the inhibition of redox-sensitive ecto-ADPases, inactivation of NO, and enhanced reactivity of platelets to agonists such as collagen, ADP (Adenosine diphosphate), and thrombin. See figure 2.7 below.

Both nitric oxide and superoxide have been suggested to be involved in the activation of αIIbβ3 integrin and glycoprotein IIb/IIIa (will be discussed later), which mediate platelet aggregation and adhesion. It appears that low levels of ROS generated by platelets are sufficient to modulate platelet function.

Both intra- and extracellular superoxide appear to modulate CD40L expression and release, however studies performed by Bakdash and Williams with extracellular antioxidants suggest

that P-selectin expression is regulated by extracellular superoxide, and αIIbβ3 integrin activation by intracellular superoxide (Bakdash and Williams, 2008).

Figure. 2.7. Role of ROS and NO in coagulation and platelet aggregation. ROS (red pathways) can promote the initiation of coagulation by targeting the TF–fVII complex as well as tissue factor protein inhibitor (TFPI). ROS also promote coagulation and thrombus formation by inhibiting the production of activated protein C (APC), enhancing the conversion of fibrinogen to thrombin and enhancing PAI-1 activity. NO (blue path- ways) tends to exert an opposite effect on the coagulation cascade. In addition, NO targets the platelets to inhibit aggregation, whereas ROS promote this process. NADPH oxidase (Nox) seems to be a major endothelial cell source of the ROS that modulate platelet aggregation (Kvietys and Granger, 2012).



It is known that superoxide enhances platelet aggregation (Handin et al., 1977), but not in the presence of plasma (Ambrosio et al., 1997, 1994a; Salvemini et al., 1991). The effect of superoxide is generally attributed to inactivation of NO; however, it has also been proposed that superoxide may indirectly promote platelet aggregation by inactivating PAF-acetylhydrolase, the enzyme that degrades PAF (Platelet-activating factor), a potent platelet agonist (Ambrosio et al., 1994b).

H_2O_2 is able to either stimulate or inhibit platelet aggregation (Ambrosio et al., 1997), this occurs in in the presence of plasma (Canoso et al., 1974; Levine et al., 1976; Stuart and Holmsen, 1977). The inhibitory effect of H_2O_2 appears to stem from stimulation of guanylate cyclase (Ambrosio et al., 1994a), whereas the stimulatory effect has been attributed to the Ca^{2+} release from the sarcoplasmic reticulum resulting from the oxidation of sulfhydryl groups in the sarcoplasmic/endoplasmic reticulum, Ca^{2+} ATPase and enhanced Ca^{2+} release from mitochondria (Redondo et al., 2004).

It is widely accepted that NO is a significant regulator of platelet function. NO derived from either platelets or endothelial cells has an inhibitory effect on platelet adhesion and aggregation. Also NO is able to break up preformed platelet aggregates and then to inhibit the recruitment of platelets into aggregates.

The inhibitory effects of NO on platelet function are counteracted by superoxide, which inactivates NO, and is stabilized by superoxide dismutase (Kvietys and Granger, 2012).

L-arginine and NO donors inhibit platelet adhesion and aggregation in vivo (Cerwinka et al., 2002; Chen and Mehta, 1996).

Several signalling pathways have been indicated in NO-mediated, cGMP-dependent modulation of platelet function, including cGMP (Cyclic guanosine monophosphate)-dependent protein receptors (Feil et al., 2003).

The net effect of cGMP-mediated activation of these various pathways is a reduction in intracellular calcium, which inhibits the conformation change of GPIIb/IIIa into its active form (Rex and Freedman, 2007). Some of the actions of NO on platelets have been attributed to a cGMP-independent mechanism (Rex and Freedman, 2007).

2.7.7 Cigarette Smoking & ROS

Tobacco smoke is a highly complex aerosol containing approximately 1010 particles/mL, or 1014-1016 free radicals per puff consisting of highly porous carbonaceous polymeric material with adsorbed heavy metals, N-nitrosamines, aza-arenes, polycyclic aromatic hydrocarbons (PAH), reactive aldehydes, quinones and other organic chemicals. The particulate phase of tobacco smoke contains at least 3,500 chemical compounds and a high proportion of them are toxic, carcinogens or mutagens (Hecht, 1999).

Many of these compounds are relatively long lived, such as tar-semiquinone, which is able to generate hydroxyl radicals and H_2O_2 by Fenton reaction in presence of free iron. These agents may induce an oxidative stress by disturbing the oxidant: antioxidant balance which could lead to cellular damage found particularly in the lungs. The oxidative stress caused by

cigarette smoking can lead to destruction of the alveolar wall, resulting in airway enlargement. Important to note the increased oxidative stress is able to trigger pro-inflammatory cytokines (Kode et al., 2006).

2.7.7.1 Cigarette Smoke and Oxidative stress

ROS and RNS are generated when mainstream cigarette smoke (the exhaled smoke smokers release after taking a puff on a lit cigarette) interacts with physiological fluids or aqueous media. Some constituents of smoke become involved in oxidative stress after they are modified chemically by metabolic processes in vivo (Wooten et al., 2006).

2.7.7.2 Free Radicals in the Particulate-Phase (Solid phases/tar)

Exogenous H_2O_2 present in cigarette smoke as well as H_2O_2 that forms by the physiological response to smoke constituents are thought to be a likely source of oxidative stress in smokers.

Quinones are freely formed from cigarette smoke constituents that are vulnerable to auto-oxidation. Generally speaking the toxicity of quinones is believed to via two mechanisms, the formation of covalent bonds with essential biological molecules and the redox cycling mechanism, resulting in the generation of excess ROS as by-products (Wooten et al., 2006).

Trace amounts of various metals are also found in cigarette smoke. The most abundant redox- inactive metals found in cigarette smoke are mercury, lead, cadmium, and arsenic. The most abundant redox-active metals present in cigarette smoke are iron and copper, with copper being more abundant than iron (Stohs et al., 1997).

Numerous studies have suggested that metal-induced oxidative stress may be partially responsible for the toxicity of the above mentioned metals (Ercal et al., 2001). Redox-active metals can undergo redox cycling within oxygenated aqueous solutions, with the accompanying formation of ROS. Redox-inactive metals are able to deplete cells of thiol-containing antioxidants which lead to a reduction in the activity of antioxidant enzymes.

Heavy metals can cause other molecular effects such as the inhibition of DNA repair and activation of cellular signalling (Barchowsky and O'Hara, 2003). Therefore both redox-inactive and redox-active metals can potentially result in an increase in ROS in smokers.

Transition metals present in the tar of cigarette smoke are of importance because of their ability to promote formation of hydroxyl radicals through the Fenton reaction, both in living

tissues and in aqueous extracts of cigarette smoke. Specifically Fe^{2+} and Cu^{1+} are well known to be active in the formation of hydroxyl radicals.

2.7.7.3 Free Radicals in the Gas-Phase

Cigarette smoke contains many oxidizing agents that are present in the gas/vapour phase (Pryor, 1992). NO· which is not itself a radical, combines with molecular oxygen in air, over a period of seconds, to form the nitrating agent and toxic oxidant, NO_2·.

The carbon-centred radicals present in the gas-phase react instantaneously with molecular oxygen to form peroxyl radicals that in turn react with NO· from the smoke gas-phase to form alkoxyl radicals and NO_2·, resulting in a continuous cycle.

The simultaneous generation of superoxide and NO· favours production of the peroxynitrite anion (Beckman et al., 1990). According to Huie and Padmaja this reaction is diffusion controlled, therefore it is likely that competition exists between NO· and SOD for superoxide (Huie and Padmaja, 1993).

Various cigarette smoke constituents may lead to the generation of ROS. Among these constituents, dihydroxybenzenes are potential good candidates.

The oxidation of hydroquinone (QH_2) by means of molecular oxygen in aqueous solution generates semiquinone (Q^{-}•) and superoxide radicals as seen in the reaction below (Wooten et al., 2006) :

$$QH_2 + O_2 \rightarrow Q^{-} \bullet + O_2^{-} \bullet + 2H^{+}$$

Studies have shown that superoxide and hydroxyl radicals form in aqueous extracts of cigarette smoke (Pryor et al., 1998). The hydroxyl radicals are produced via the Fenton reaction from the metal mediated decomposition of H_2O_2 as discussed earlier (Pryor, 1992). Exposure to a variety of oxidant chemicals in cigarette smoke is associated with diminished endogenous levels of antioxidants. Smokers have been shown to have reduced plasma levels of vitamin C and α-carotene (Yanbaeva et al., 2007).

Glutathione (GSH) is an important antioxidant used to maintain vitamins E and C in their reduced and functional forms and to reduce peroxides to non-toxic hydroxyl fatty acids and/or water. Smoking generates ROS that can oxidize GSH to disulfide form which results in diminished plasma GSH levels.

Smoking may also lead to mitochondrial damage, resulting in decreased energy production in the heart muscle and mitochondrial DNA damage (Erhardt, 2009).

In conclusion cigarette smoke contains high levels of superoxide and other ROS (such as hydroxyl radical, hydrogen peroxide, and peroxynitrite) generated from cigarette combustion.

Now that the presence of ROS has been established as a result of smoking, the following paragraphs will describe the effect of smoking and ROS on endothelial dysfunction, coagulation, and haemostasis.

2.7.7.4 Cigarette Smoke Induces Oxidative Injury to the Cerebrovascular System

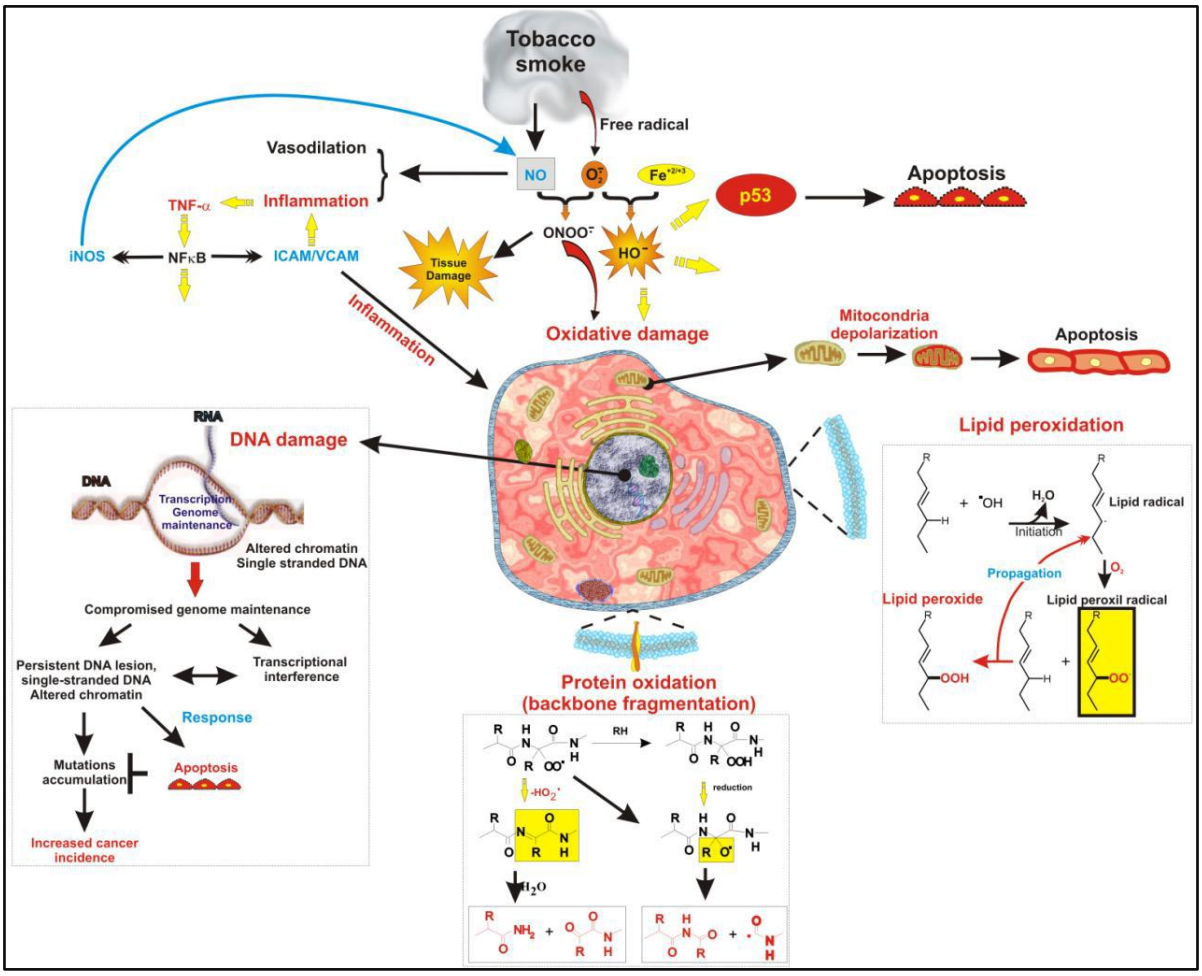
The vascular adverse effects of cigarette smoking may be the result of endothelial exposure to ROS, Figure 2.8 shows multiple pathways by which the exposure to ROS originating from tobacco combustion can induce cellular damage and inflammation (Mazzone et al., 2010).

Elevated CRP levels induced by cigarette smoking, may promote endothelial dysfunction by decreasing the production of NO and reducing its bioactivity (Mazzone et al., 2010).

ROS-induced alterations of macromolecules lead to cell damage. These alterations include peroxidation of polyunsaturated fatty acids found in membrane lipids, DNA strand breakage, protein oxidation, mitochondrial depolarization, RNA oxidation and apoptosis (Mazzone et al., 2010; Yanbaeva et al., 2007).

Several studies have found that antioxidant supplementation is able to prevent, at least to some degree, the inflammation and oxidative damage induced by cigarette smoke exposure. This strongly supports the hypothesis of a ROS-mediated toxicity of cigarette smoke exposure (Mazzone et al., 2010).

Figure 2.8. ROS-induced cellular inflammatory response and oxidative damage. Schematic representation of the multiple pathways by which the exposure to reactive oxygen species originating from tobacco combustion can induce cellular damage and inflammation (Mazzone et al., 2010).



2.7.7.8 Cigarette Smoke Induces Inflammatory and Thrombotic Injury

Some components of cigarette smoke contribute to a pro-atherosclerotic environment by triggering a complex pro-inflammatory response as a result of the recruitment of leukocytes to the site of inflammation by cytokine signalling (such as TNF-α(tumor necrosis factor-alpha) and IL-1β), matrix metalloproteinase up-regulation (e.g., MMP-9 and MMP-1), and by promoting the binding and adhesion of monocytes to the endothelial wall of blood vessels.

Studies show that long-term smoking results in airway inflammation characterized by macrophage, neutrophil and activated T lymphocyte infiltration as well as increased cytokine concentrations such as IL-6 and TNF-α. Additionally almost all smokers show evidence to some extent of lung and systemic cellular and/or humoral inflammation (Tanni et al., 2010).

Figure 2.9 shows Potential pathways and mechanisms for cigarette smoking-mediated cardiovascular dysfunction.

Studies have reported significantly higher levels of nitrated and oxidized fibrinogen, ceruloplasmin (the major copper-carrying protein in the blood which also is involved in iron metabolism), plasminogen and transferrin (iron-binding blood plasma glycoproteins controlling the level of free iron in biological fluids) in smokers than in non-smokers (Yanbaeva et al., 2007).

As a result of increased levels of Von Willebrand factor, platelet activating factor, catecholamines and thromboxane, increased platelet activation aggregation and coagulation activity is often observed in smokers (Mazzone et al., 2010). This increased activation of platelets, increases the risk of thrombus formation which leads to damage being inflicted on the lining of the arteries, aiding the development of atherosclerosis (Erhardt, 2009).

Smoking is also believed to be associated with the upregulation of metalloproteinases (protease enzyme whose catalytic mechanism involves a metal) that are thought to weaken the arterial wall and contribute to the destabilization and rupture of existing atherosclerotic plaques (Erhardt, 2009).

Therefore a pro-thrombotic, pro-inflammatory state exists in individuals who smoke.

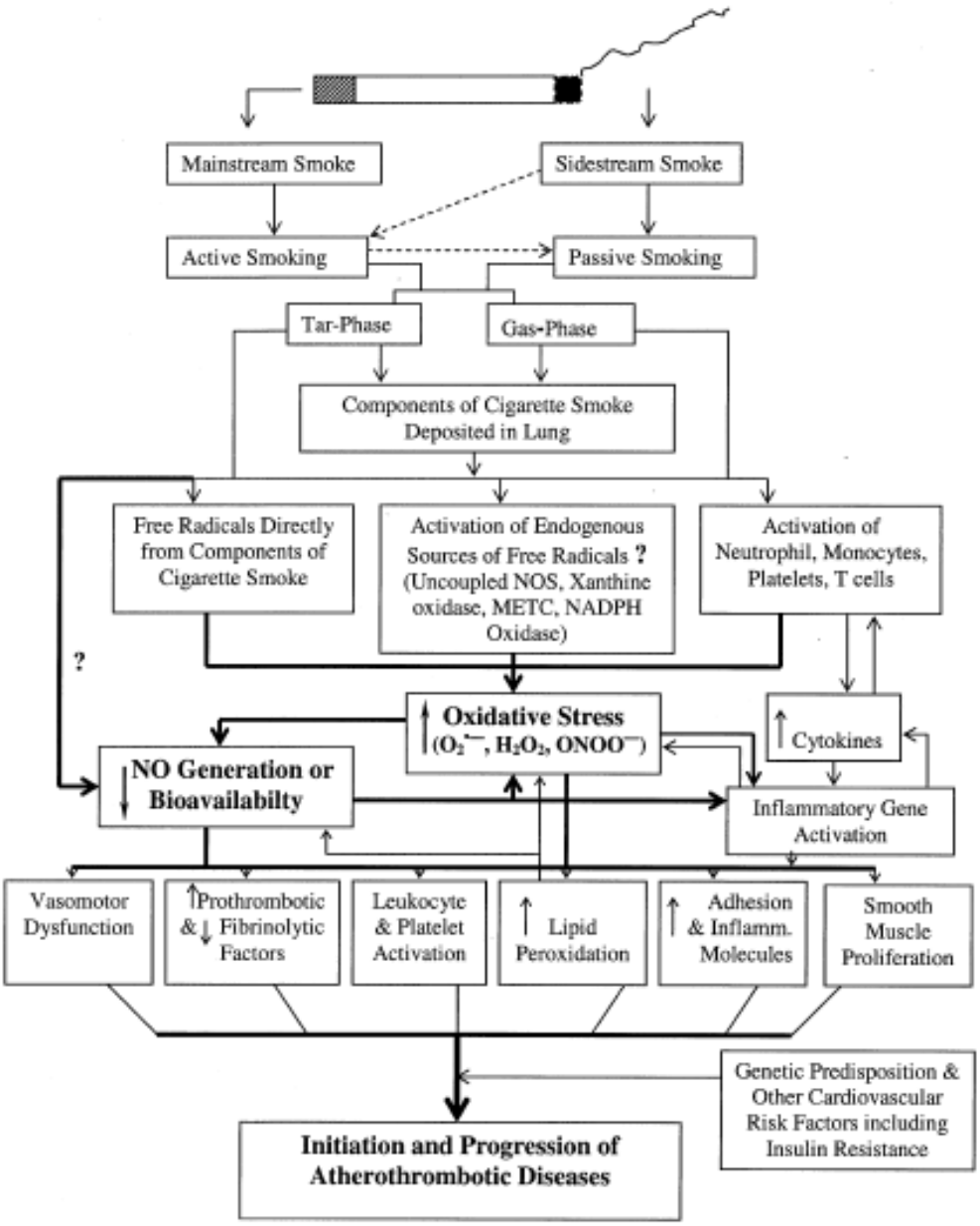
Besides inflammation, proposed potential mechanisms by which smoking increases the risk of atherosclerosis and cardiovascular pathology include several other pathways: systemic haemostatic and coagulation disturbances, vascular endothelial dysfunction, and lipid abnormalities.(Yanbaeva et al., 2007)

In conclusion ROS are considered likely candidates as chemical mediators that can link inflammation to coagulation and thrombosis because of their proven ability to alter platelet function as well as influencing the balance between the procoagulant, anticoagulant, and fibrinolytic systems (Görlach, 2005).

As ROS seem to be present in stroke patients and individuals who smoke, this link is noteworthy to explore further.

In this thesis platelets in particular will be evaluated in stroke patients and smokers and subsequently compared to healthy individuals. For that reason platelet physiology and morphology will now be discussed.

Figure 2.9. Potential pathways and mechanisms for cigarette smoking-mediated cardiovascular dysfunction. The **bold boxes** and **arrows** in the flow diagram represent the probable central mechanisms in the complex pathophysiology of cigarette-smoking-mediated athero-thrombotic disease. H_2O_2 -hydrogen peroxide; METC - mitochondrial electron transport chain; NADPH; NOS; $ONOO^-$; O_2^- (Ambrose and Barua, 2004).



2.8 PLATELET PHYSIOLOGY AND MORPHOLOGY

Blood platelets (thrombocytes) have held a great fascination since the beginning of the 19th century. At the commencement of research, platelets were called “strange little pellets” (Hermann Nasse, 1807–1892). They were not considered as independent blood components then but were thought to be expelled cell nuclei. Giulio Bizzozero (1816–1901) first described the independent nature of platelets and their association with blood coagulation in his essay: “On a new morphological element in the mammalian blood and its role in thrombosis and blood coagulation”. James H. Wright (1869–1928) however was the first who clarified the origin of platelets. He found platelets as “pinch-offs” of the plasma membrane of megakaryocytes in the bone marrow.

Since these early descriptions of platelets, our knowledge in physiology and pathophysiology expanded immensely. Platelets were and still are of major interest in scientific research to which we can attribute in the present day an abundance of information, especially in the area of vascular diseases (Sysmex Xtra Online, 2007).

Platelets are unique cells, they are the smallest blood corpuscles with a diameter of 1-4 µm, young platelets being larger than older more mature ones. 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.

Resting platelets circulate as non-adhesive discoid clear cells and have a life span of approximately 8-10 days (Willoughby et al., 2002). Upon stimulation, granules are liberated from the platelets into the external medium. The granular contents mediate host defense, recruitment and activation of adjacent platelets and leukocytes as well as regulation of tissue repair (Picker, 2011).

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 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 (Andrews and Berndt, 2004).

2.8.1 Platelet Production

Megakaryocytes (MKs) give rise to circulating platelets (thrombocytes) through commitment of the multipotent stem cell to the MK lineage, proliferation of the progenitors and terminal

differentiation of MKs. This process is characterised by DNA endo-reduplication, cytoplasmic maturation and expansion, and release of cytoplasmic fragments as circulating platelets. Within the bone marrow (BM), MKs are derived from haematopoietic stem cells (HSCs), which evolve from the multipotential haemangioblast. The haemangioblast gives rise to all blood and blood vessel precursor cells. During MK maturation, internal membrane systems, organelles and granules are assembled in bulk (Deutsch and Tomer, 2006).

Platelet release occurs when the MK cytoplasm is transformed into proplatelets (precursor of cytoplasmic extensions), followed by the release of 2000–5000 new platelets/cell. (Long et al., 1982) The production of platelets from the MK is an extensive process of compartmentalised apoptosis. The cell's nuclear region and cytoplasmic environment undergoes caspase-dependant programmed cell death.

A large section of the organelle and storage containing cytoplasm is unaffected by the execution of this programme. The enduring plasma membrane system, organelles and cytosol which include the platelet's alpha and dense granules, fragments into the subcellular particles known as platelets (Clarke et al., 2003; Deutsch and Tomer, 2006). The sites of platelet production are typically the bone marrow but it is thought that platelets are also formed in the lungs and blood.

There are two models of thrombopoiesis, which are not mutually exclusive. The first model called the proplatelets model suggests platelet assembly and budding off the tips of proplatelets, which act as assembly lines for platelet production at the end of each proplatelet. In other words this model proposes that megakaryocytes extend plump pseudopodia that give rise to long (>100-µm) branched proplatelet processes that appear "beaded" by virtue of intermediate swellings(Patel et al., 2005). The proplatelets extend into sinusoidal spaces, here they detach and fragment into individual platelets, producing about 2000–5000 new platelets (Choi et al., 1995; Zucker-Franklin, 1970).

The second model of platelet biogenesis is the platelet territory model which suggests that, within the MK cytoplasm, there are defined preformed territories with internal membranes differentiating pre-packaged platelets that are released upon fragmentation of the cytoplasm. Electron microscopy analysis of the internal membranes of MKs stemmed this theory (Mori et al., 1993; Zucker-Franklin and Petursson, 1984). When mature MKs are cultured in contact with subendothelial extracellular matrix, they are stimulated to produce platelets by explosive fragmentation of the entire cytoplasm (Caine et al., 1986; Eldor et al., 1986).

Ex vivo as well as in vivo observations of platelet release from MKs using phase-contrast microscopy greatly support the explosive-fragmentation theory (Deutsch et al., 1995; Kosaki, 2005). However, recently platelet production was observed *in vivo*, confirming the concept of proplatelet formation *in vivo* (Junt et al., 2007).

Junt *et al* observed that megakaryocytes extended dynamic proplatelet-like protrusions into microvessels which seemed to be sheared from their respective trans-endothelial stems by blood flowing past, resulting in the appearance of proplatelets in peripheral blood (Junt et al., 2007). However, the exact mechanism of platelet formation is still unclear.

2.8.2 Platelet morphology

Within a platelet, organelles are randomly distributed throughout the cytoplasm. Various skeletal components, including actin filaments and microtubules, a membranous system and an affluence of glycogen used to generate energy are also present. The microtubules and filaments function as support structures for the platelet. They assist in the maintenance of the platelets shape and also function to secrete granules, as the filaments and microtubules play a role in the retraction of a clot in the process of fibrinolysis (Silverthorn, 2004).

2.8.2.1 Platelet Membrane

The membranous system is firstly comprised of the open canalicular system (OCS) which is responsible for connecting the cytoplasm to the surface and enlarges the platelet surface since the invaginations are continuations of the plasma membrane. Secondly the membranous system contains the dense tubular system (DTS) responsible for the storage of metabolic enzymes (analogous to the endoplasmic reticulum of nucleated cells) (Willoughby et al., 2002).

Open canalicular system (OCS)

Demarcated openings to a complex arrangement of internal membrane channels form the open canalicular system (OCS), also known as the surface-connected canalicular system (SCCS). The cytoplasmic space is permeated by dilated channels. The plasma and membrane receptors are present in the OCS. Upon platelet activation, the receptors are translocated to the surface. Additionally the OCS can also accumulate specific membrane receptors from the plasma following cell activation, via the process of down regulation. For

example the vWf receptor is transported into the OCS by down regulation upon platelet activation (Joseph and Italiano, 2008).

The platelet is surrounded by a plasma membrane that extends through the multiple channels of the surface-connected canalicular system, increasing the surface area of the platelet.

The plasma membrane contains phospholipids; the negatively charged phosphatidylserine and phosphatidylinositol residues are primarily confined to the cytoplasmic side, where they may serve as substrates for phospholipases. The plasma membrane also contains a large number of receptors which specifically bind agonists that stimulate the physiological platelet response. The interaction between a platelet-activating agonist and its receptor causes rapid mobilization of signalling molecules within the platelet which are sufficient to initiate and complete shape change and aggregation responses.

Through this phospholipid bilayer, intrinsic glycoproteins extrude, serving as platelet receptors for activating and inhibiting agents. The dense tubular system is the site where the majority of calcium is sequestered and where enzymes involved in prostaglandin synthesis are localized. This membrane system lies in close contact with the channels of the open canalicular system, forming a membrane complex (Willoughby et al., 2002).

Na^+/K^+ ATPase is an integral membrane protein that plays a key role in cellular osmotic regulation through the maintenance of the trans-membrane gradients of Na^+ and K^+, therefore acting as a marker of membrane function whose activity greatly depends on its interaction with the chemical-physical properties of the microenvironment where it is embedded.

According to Zakim *et al*, Na^+/K^+ ATPase represents an index of membrane functionality involved in the modulation of phospholipid and protein interactions regarding membrane fluidity. A study conducted by Nanetti *et al.* in 2008 showed a decreased platelet Na^+/K^+ ATPase activity in patients affected by acute stroke compared to that in controls as well as significantly lowered membrane fluidity.

Platelet membrane fluidity with regards to microviscosity is a critical determinant of platelet aggregation and secretion, therefore its decrease in stroke patients may play a role in the pathogenesis of ischemic damage. Nanetti *et al.* found a decrease in the platelets Na^+/K^+ ATPase activity in patients, because the enzyme is located in a less fluid cellular microenvironment induced by alterations related to acute stroke. This enzymatic inhibition leads to altered internal and external membrane ionic concentrations (Mazzanti et al., 1997).

This ionic imbalance causes a derangement in cellular enzymatic systems, leading to membrane depolarization which in turn could compromise platelets survival. (Rabini et al., 2003)

Na^+/K^+ ATPase activity and membrane fluidity are correlated to stroke severity as measured by the National Institutes of Health Stroke Scale (NIHSS). Alterations in platelet membrane chemical–physical (decreased fluidity) and functional properties (reduced Na^+/K^+ ATPase activity) rise proportionally with NIHSS increase. (Nanetti et al., 2008)

It has also been found that smoking induces alterations in platelet membrane fluidity and NA^+/K^+ ATPase activity as well as a change in the membrane cholesterol/phospholipid ratio (Padmavathi et al., 2010). Pretorius and co-workers in 2012 suggest that this changed membrane fluidity is visible ultrastructurally and translates into a more globular and bulbous appearance of the membrane surface in smokers (Pretorius, 2012).

2.8.3 Platelet Secretion

Platelets contain three major types of granules: Alpha-granules, dense granules and lysosomes. Platelet secretion or exocytosis releases molecules at the site of injury to activate other cells or to facilitate cellular adhesion. Platelets secrete molecules from intracellular granules. These molecules play central roles in haemostasis, thrombosis, and vascular remodelling (Rendu and Brohard-Bohn, 2002; Roberts et al., 2012)

Alpha-granules (α-granules)

α-granules are the largest and most numerous granules found in human platelets. They are single membrane spherical organelles. α-granules are involved in haemostasis, cell-matrix interactions and wound healing as they store a collection of plasma proteins that are effectors in these processes.

α-granules contain adhesive proteins such as vWf, fibrinogen, thrombospondin, and glycoprotein receptors such as the vWfR or the fibrinogen receptor $\alpha IIb\beta_3$ embedded in their membranes. α-granules also contain several growth factors, such as insulin like growth factor 1, platelet factor 4 (a heparin-binding chemokine), platelet-derived growth factor (TGFβ) and the adhesion molecule P-selectin (Roberts et al., 2012). The α-granules can then release these stored proteins at the location of vessel injury when required (Rendu and Brohard-Bohn, 2002).

Delta-granules (Dense bodies)

Delta granules (δ-granules) are also known as dense core granules or dense bodies. Delta granules are the smallest granules found in the cytoplasm. Dense bodies act as pools for intracellular calcium and contain small non-protein molecules, like ADP, ATP, histamine, granulophysin, calcium and serotonin. Evidence shows that delta granules contain proteins like P-selectin, which was thought to be exclusively in α-granules (Israels et al., 1992). 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).

Lysosomes

Lysosomes (λ-granules) are granules found within platelets that are involved in the release reaction of platelets. Lysosomes contain acid hydrolases such as acid proteases, glycosidases and cationic proteins with bacterial activity. Only powerful stimuli will result In these enzymes being secreted from the lysosomes. The free hydrolytic enzymes will, by means of the action of hydrolytic degradation, assimilate substances in platelet aggregates and subsequently break these substrates down to their most basic units (Rendu and Brohard-Bohn, 2002; Zarbock et al., 2007).

Mitochondria

Platelets contain actively metabolizing mitochondria. They play a crucial role in the cell specifically relating to its energy metabolism. Mitochondria can independently support the energy requirements of the platelet seeing as anaerobic glycolysis doesn't influence the level of ATP in the platelet or normal platelet functioning. Direct evidence of the role of mitochondria in coagulation stems from findings that changes in the permeability of mitochondrial membranes were linked to changes in the coagulation activity (Barile et al., 2012). In 2012 Barile *et al.* found a unique class of anti-coagulants that inhibit platelet function, presumably by inhibiting mitochondrial respiration, this implies that platelet activation and platelet-activated blood clotting are dependent on mitochondrial function (Barile et al., 2012). Mitochondria contain calcium which may possibly be just as important as the DTS and extracellular calcium in the activation of platelets (White, 2007).

Glycogen

Glucose is the primary energy supply for platelets and is absorbed from the plasma relatively quickly. Around half of the assimilated glucose is involved in supplying energy for synthetic

purposes or alternatively converted into glycogen for storage, during basal settings (Calverley and Thiehelt, 2009). Glycogen is present in isolation but may also be found in large clusters of particles within the platelet (Rendu and Brohard-Bohn, 2002).

2.8.4 Platelets and haemostasis

Haemostasis is an intricate process which involves cellular components, the blood vessel wall and soluble factors in circulation. Pertaining to primary haemostasis platelets are the most important cellular components in this process, while coagulation factors are vital for secondary haemostasis (Marcucci et al., 2008).

Primary haemostasis is activated upon damage to a blood vessel. Exposed collagen will interact with platelets at the location of injury to the vascular endothelium through the following processes: platelets will adhere to the collagen (adhesion), resulting in the platelets becoming activated (activation), contents of their granules will be liberated (secretion) and lastly the platelets will collect around the area (aggregation). Platelet aggregation is also supported by factors like thromboxane, vWf and fibrinogen (Ramasamy, 2004).

Following primary haemostasis, the coagulation cascade is initiated by coagulation factors. This cascade results fibrin fibers forming. This fibrin formation is known as secondary haemostasis, which is responsible for the formation of a stable clot (Marcucci et al., 2008).

The metabolic processes of the platelet are controlled by mitochondria and the DTS while the platelet release reaction is controlled by the secretions of granules (alpha granules, delta granules and lysosomes). It is the synergistic actions of the DTS and mitochondria that supply the metabolic energy and manage the cytosolic calcium essential for the secretion of the various granule components (Rendu and Brohard-Bohn, 2002). A big quantity of vasoactive substances is kept within the secretory products of platelets. Serotonin, calcium, thromboxane (TXA_2), ADP and ATP are examples(Holmsen, 1987).

2.8.5 Platelets and stroke

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

Joseph *et al.* studied platelet morphology, specifically the secretory organelles, in acute ischemic stroke patients (Joseph et al., 1989b).

There found that there are alterations present in the number of mitochondria, alpha granules and dense bodies in the platelets of the stroke patients when compared to healthy control individuals (Joseph et al., 1989a). Additionally fewer mitochondria might indicate that the activated platelets of stroke patients release or consume mitochondria more readily than healthy individuals‟ platelets (Joseph et al., 1989b).

Various animal studies confirm the hypothesis that stroke may be exacerbated by vasoactive substances that are released from various platelet organelles. This is plausible as these substances can pass through the disrupted blood brain barrier thereby coming into direct contact with cerebral tissue (Fieschi et al., 1975; Fujimoto et al., 1985; Furlow and Bass, 1975). Consequently, neuronal and vascular injury can arise from these released substances (De Clerck et al., 1985, 1984).

2.8.6 Previous Research Done On Ultrastructure Of Platelets In Disease Conditions

2.8.6.1 Stroke

Nucleated cells undergo cell death during thromboembolic events, platelets are also affected by parameters causing these incidents. In a study done by Pretorius *et al.* in 2012 on thromboembolic ischemic stroke patients where they studies smears of platelet-rich plasma (PRP) using scanning electron microscopy. Their results indicated that thromboembolic ischemic stroke causes membrane tears and swollen platelets, which is indicative of necrosis. They concluded that this morphology might be due to the pro-coagulant activity characteristic of the disease. Particularly, initiation of necrotic cell death at sites of vascular injury may play an important role in inducing inflammatory and repair processes (Pretorius et al., 2012).

2.8.6.2 Tobacco Smoke

Pretorius and co-workers in 2010 showed that fibrin network ultrastructure is changed due to smoking, and the authors coined the term sticky fibrin phenomenon. It was found that a typical netted fibrin fiber morphology is present in smokers and major, thick fibers tend to stick together to form thickened matted masses of fibrin (Pretorius et al., 2010).

Research shows that smoking causes changes in platelet membrane fluidity and Na^+/K^+ATPase activity (Miura et al., 2011). In 2012 Pretorius *et al.* conducted a study to determine if the changes in the membrane fluidity of smokers are ultrastructurally visible. Using a scanning electron microscope they found a difference in the globular nature of the platelet membrane of smokers was visible as well as more pronounced surface pseudopodia than in healthy individuals. No apoptotic or necrotic platelet morphology were noted. They concluded that, changes in membrane fluidity is structurally visible and translates into a more globular and bulbous appearance of the membrane surface (Pretorius, 2012).

One of the techniques used in this study makes use of certain platelet receptors, therefore these receptors will now be discussed in detail.

2.9 PLATELET RECEPTORS AND ACTIVATION

Platelet activation, adhesion and aggregation are mediated by specific surface receptors that include integrins, such as the fibrinogen receptor $\alpha IIb\beta_3$; the leucine-rich von Willebrand factor receptor, and the immunoglobulin-family receptors such as GPVI and P-selectin. The major glycoproteins in circulating platelets are the von Willebrand factor receptor (vWfR) and the fibrinogen receptor $\alpha_{IIb}\beta_3$(GPIIb-IIIa or CD41/CD61) which interact with both soluble and tethered ligands to activate platelets (Shattil and Newman, 2004).

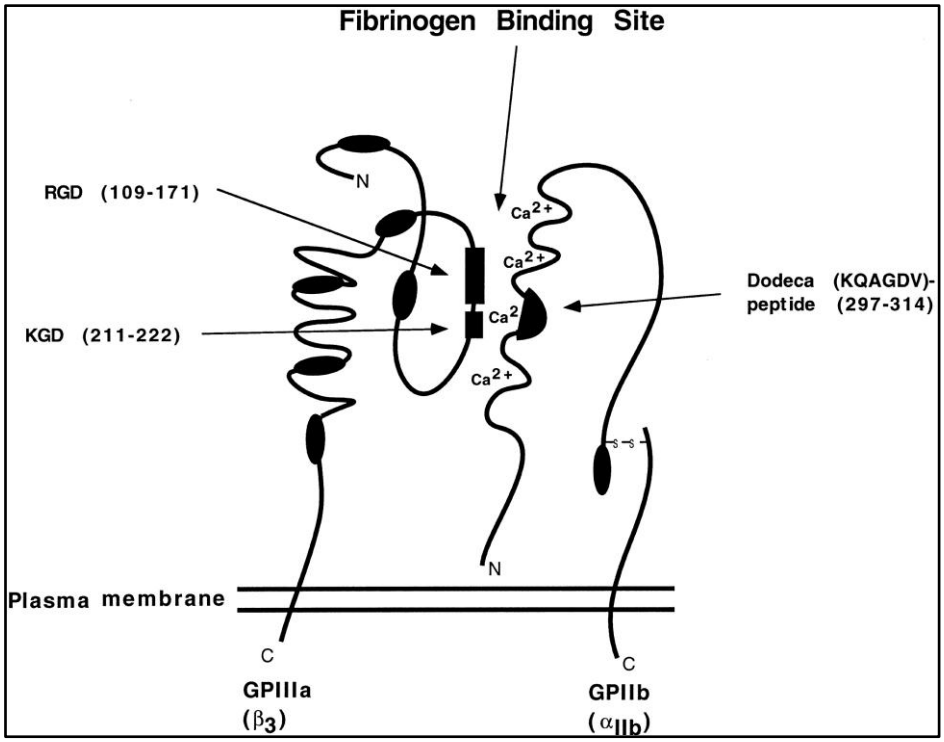
2.9.1 CD41/CD61 or GPIIb-IIIa（$\alpha_{IIb}\beta_3$ integrin)

Integrins are non-covalent heterodimers composed of α- and β-subunits that mediate cell to cell and cell to matrix interactions. Integrins are a large family of cell surface receptors. At least 24 integrins have been found in various cell surfaces. On platelets, β3 (αIIbβ3, αVβ3) and β1(α2β1, α5β1, α6β1, α8β1) integrins have been found. Their expression ranges from 2–4000 copies (α2β1, α5β1, αVβ3) to 50– 120,000 copies (αIIbβ3) per cell.

CD41 (GPIIb) is a glycoprotein composed of 2 chains i.e. GPIIbα of 120 kDa and GPIIbβ of 23 kDa linked by disulfide bond. The integrin β3 chain, also called CD61 (GPIIIa), is a 110 kDa glycoprotein that belongs to integrin family of proteins.

CD41 (GPIIb) is non-covalently associated with CD61 (GPIIIa). Both these receptors are present on platelets and megakaryocytes. Together they form the CD41/CD61 complex (GPIIb/IIIa or $\alpha_{IIb}\beta_3$ complex/integrin) as seen in Figure 2.10. The CD41/CD61 complex ($\alpha_{IIb}\beta_3$) is the predominant protein on the platelet surface, accounting for about 17% of the total platelet membrane protein(Ni and Freedman, 2003), therefore the CD41 antibody is a useful tool to identify platelets in research by using techniques such as flow cytometry and confocal microscopy as seen in this thesis .

Figure 2.10. GP IIb/IIIa (CD41/CD61) structure. GP IIb/IIIa is composed of an α-subunit (GP IIb) and a β-subunit (GP IIIa). GP IIb contains 4 putative Ca^{2+} binding sites (Htun et al., 2006).



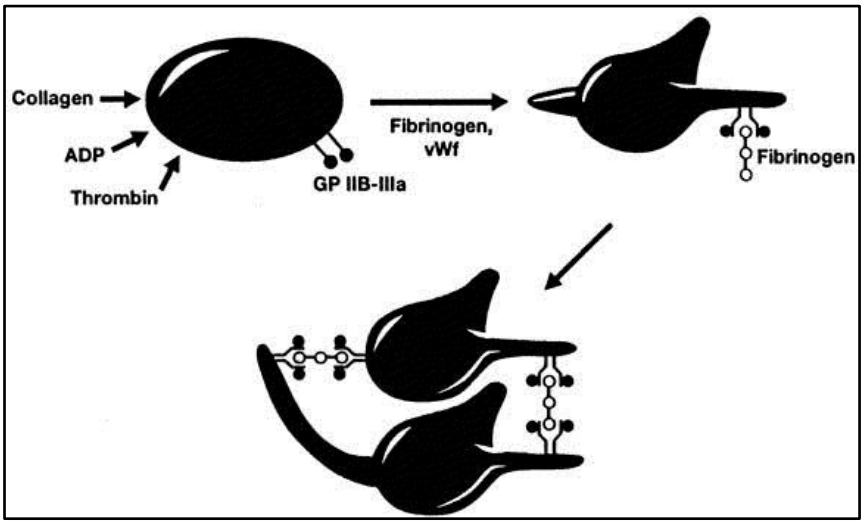
Previous studies demonstrated that GPIIb and GPIIIa form a calcium-dependent complex in the platelet membrane that functions as a receptor for fibrinogen and other adhesive glycoproteins. The CD41 P2 monoclonal antibody (mAb) reacts with GPIIb (CD41) in the intact complex with GPIIIa (CD61) but not with the separated subunits (Saboor et al., 2012).

Platelet aggregation depends on fibrinogen binding to the CD41/CD61 complex. The resting form of the CD41/CD61 complex on non-activated platelets has a very low affinity for fibrinogen. However, after platelet activation the complex receives signals from inside the cell to rapidly change its conformation and binds to immobilized fibrinogen as shown in Figure 2.11 and the complex becomes a receptor for soluble fibrinogen, fibronectin, vWf, vitronectin and thrombospondin.

The integrin CD41 has an important role in platelet aggregation, and antibodies against it are used clinically to reduce the risk of ischemic complications. CD41 is also of interest as a marker for hematopoietic differentiation, because in mature cells it is specifically expressed within the megakaryocytic lineage (Zhang et al., 2007).

The adhesive protein binding function of CD41/CD61 is activation-dependent and is contained entirely within the heterodimer formed by the large extracellular domains. Activation of the CD41/CD61 complex required for ligand binding takes place in response to chemical agonists of platelets as well as to vWf binding to GP Ibα.

Figure 2.11. Role of GP IIb/IIIa in platelet aggregation. On unstimulated platelets, GP IIb/IIIa is in a conformation that has low affinity for soluble fibrinogen. When platelets are activated, they undergo morphologic and physiologic changes, and GP IIb/IIIa molecule alters its conformation, becoming a high-affinity receptor for fibrinogen. Each fibrinogen molecule can bind to 2 GP IIb/IIIa molecules and therefore cross-link receptors on adjacent activated platelets and ultimately lead to formation of platelet-rich thrombi. vWf, von Willebrand factor (Phillips and Scarborough, 1997).



Platelet activation up regulates the number of surface complexes by 50%. The integrin CD41/CD61 is exposed to the extracellular and the intracellular environment and has been found to function as a mediator of bi-directional signalling, therefore signalling from the inside- out and the outside-in as seen in figure 2.12.

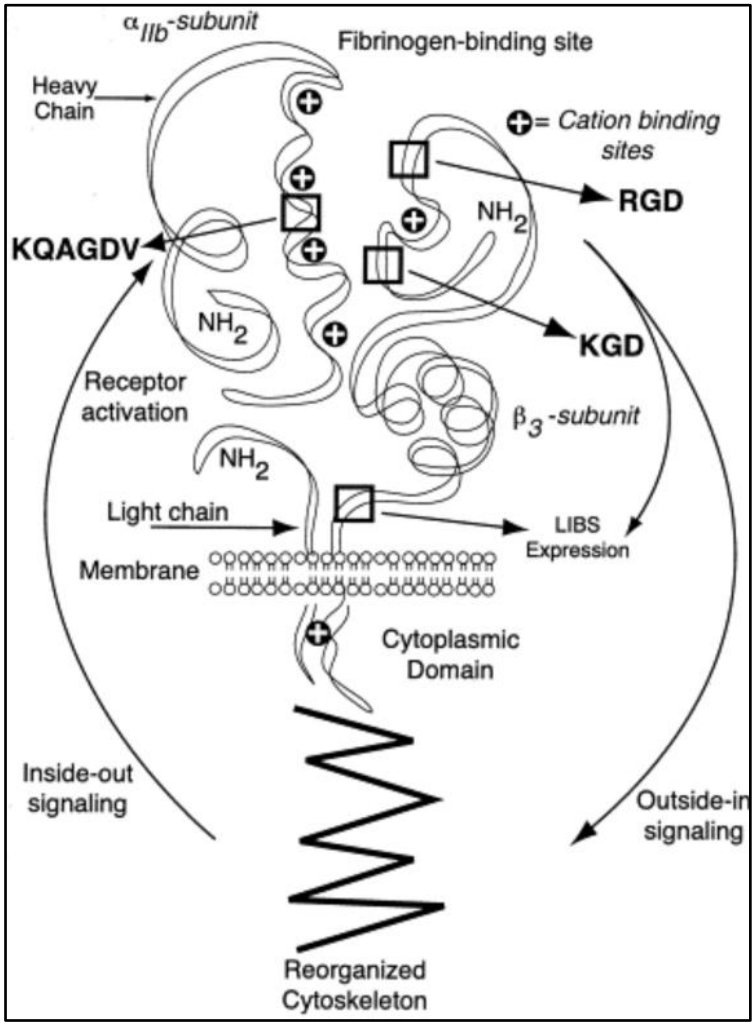
During signalling from within the cell to the outside, primary platelet agonists stimulate signal transduction pathways and changes in cytoskeletal proteins, resulting in the activation of numerous platelet functions.

This signalling is specific to the activation of the receptor function for the soluble adhesive proteins, vWf and fibrinogen. These adhesive proteins are able to crosslink platelets when binding to activated CD41/CD61complexes on stimulated platelets to initiate platelet aggregation because of their multivalent properties.

Signalling from the outside of the cell inwards takes place during platelet aggregation and adhesion. This process is induced by fibrinogen and vWf binding to the extracellular domain of CD61 (GPIIIa). This signalling from the CD41/CD61 complex induces a variety of

functions that are crucial to platelet physiology, platelet aggregation and responses of the vessel wall (Schmugge et al., 2003)

Figure 2.12. Platelet glycoprotein IIb/IIIa structure and function. Heterodimeric structure with cation binding sites, RGD, KGD and KQAGDV binding sites and intracellular tail mediating both inside-to-outside and outside-to-inside signaling. Inside-out signals lead to increased platelet glycoprotein IIb/IIIa receptor activation, while outside-in signals increase ligand-induced binding site (LIBS) expression and cytoskeletal responses (Chew and Moliterno, 2000).



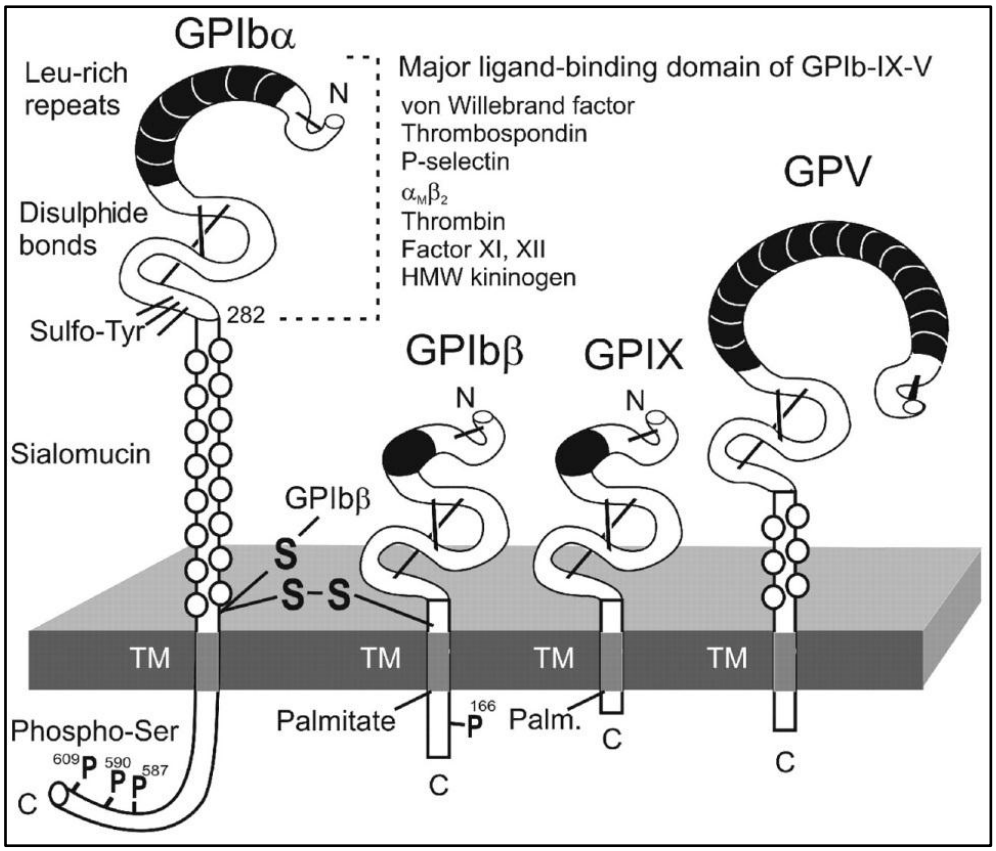
2.9.2 The von Willebrand factor receptor (vWfR), (GPIbαβ/IX)2V or GPIb–IX–V (CD42a-d)

The von Willebrand factor receptor is a complex of 4 polypeptides as shown in figure 2.13:

- GPIbα (Platelet glycoprotein Ib alpha chain also known as CD42b)
- GPIbβ (GPIb also known as CD42c)
- GPIX (CD42a)
- GPV (CD42d)

Therefore the von Willebrand factor receptor is known under the following abbreviations $(GPIb_{\alpha\beta}/IX)_2V$ or GPIb–IX–V (CD42a-d). GPIb–IX–V is a unique platelet adhesion receptor; the four transmembrane subunits of the complex is each a member of the leucine-rich repeat protein superfamily.

Figure 2.13. The GPIb-IX-V complex composed of GPIbα disulphide-linked to two GPIbß subunits, and noncovalently associated with GPIX and GPV. Disulphide bonds within domains either side of leucine-rich repeat domains are depicted as solid black bars. The position of sulphated tyrosine residues (Sulfo-Tyr at 276, 278 and 279 of GPIbα), phosphorylated serine residues (Phospho-Ser) and palmitylated Cys residues of GPIbß and GPIX are indicated. C, C-terminus; N, N-terminus; TM, transmembrane domain (Berndt and Andrews, 2011).



There are ~25,000-30,000 copies of this receptor complex per platelet surface. In resting platelets, this vWfR complex is linked to underlying actin filaments by filamin A molecules. The vWfR complex mediates activation and platelet adhesion to collagen bound vWf under high shear rates, after vessel wall injury, platelet GPIb-IX-V mediates the initial adhesion contact to vWf bound to collagen within the exposed vascular sub-endothelium under arterial shear rates, allowing platelets to roll at much slower velocities (Hartwig and DeSisto, 1991).

The CD42b mAb used in this study specifically binds to CD42b also known as the Platelet glycoprotein Ib alpha chain (GPIbα).

The GPIbα (CD42b) subunit is 610 amino acids in length and with a molecular weight of 135 kDa, contains the binding sites for vWf, P-selectin, thrombin, high molecular weight kininogen and factor XII. The cytoplasmic tails of GPIbα and GPIbβ contain binding sites for signalling molecules, such as 14-3-3ζ, and for proteins of the platelet cytoskeleton, such as actin-binding protein and a protein kinase A-phosphorylation site, that seem to regulate platelet actin polymerization in response to agonist stimulation.

The platelet GP Ib-IX-V receptor complex plays a pivotal role in the development of arterial thrombosis. Following endothelial injury (including atherosclerotic plaque rupture), it is involved in the initiation of thrombus formation at the site of the blood vessel wall lesion through its ability to attach platelets to vWf (Meisel et al., 2004).

The binding interaction between vWf and GPIbα involves three sequences of the GP Ib:

1. The N-terminal 282 amino acids of GP Ibα,
2. The C- terminal flanking region (involved in platelet type von Willebrand disease (VWD) and it appears to regulate an affinity conversion of the receptor from a low affinity to a higher affinity state, potentially equivalent to that taking place in the receptor under shear conditions,
3. The N-terminal flanking sequence and the leucine-rich repeat region. The signalling events induced by vWf binding to GPIb–IX–V include elevation of cytosolic Ca^{2+}, and activation of protein kinase C and tyrosine kinases (Schmugge et al., 2003).

It has been found that a subset of GPIb-IX–V is imperatively associated with the lipid rafts in resting platelets and that additional copies of the receptor are recruited to these micro-domains upon stimulation with vWf.

Lipid rafts (glycolipid-enriched membranes [GEMs]) are dynamic assemblies of cholesterol and sfingolipids with the appearance of a more ordered structure than the rest of the plasma membrane and are thought to act as platforms for signal transduction.

GPIb-IX–V interacts constitutively with a number of intra-cellular proteins, including actin-binding protein, 14-3- 3 ζ, and calmodulin. Calmodulin is a calcium-binding messenger protein that transduces calcium signals by binding calcium ions, modifying its interactions with various target proteins. These interactions possibly regulate different GPIb-IX–V-

dependent cellular events, such as platelet adhesion, rolling, cytoskeleton reorganisation, and transmembrane signalling.

In resting platelets, calmodulin has been found to be constitutively associated with GPIb-V–IX. Upon platelet stimulation with thrombin, calmodulin dissociates from the receptor and relocates to the cytoskeleton (Andrews, 2001). Although the physiological role of calmodulin association with GPIb-V–IX is unknown, this interaction may potentially regulate different aspects of receptor function and signalling (Canobbio et al., 2004).

A binding site for actin-binding protein is found in the cytoplasmic tail of the GPIbα subunit within the residues. Actin-binding protein directly interacts with actin filaments; therefore it anchors the entire receptor to the membrane skeleton. In addition, the interaction of GPIb-IX–V with actin-binding protein has been shown to play a role in cell activation and aggregation.

The 14-3-3 proteins are 30-kDa ubiquitous proteins that regulate the activity of many signalling molecules and control different physiological processes such as cell cycling, mitogenesis, and apoptosis. The 14-3-3 isoform most commonly found in signalling transduction pathway is 14-3-3 ζ (Xiao et al., 1995).

It is usually expressed as a dimer, therefore it may bind simultaneously two cytoplasmic proteins. GPIb-IX–V binds to 14-3-3 ζ through both GPIb and GPV.

The 14-3-3 ζ interaction with GPIbβ requires the phosphorylation of Ser^{166} by Protein Kinase A (PKA). It has recently been demonstrated that the PKA-mediated phosphorylation of GPIbβ at Ser^{166} negatively regulates vWf binding to GPIbIX (Canobbio et al., 2004).

2.9.3 P-Selectin

P-selectin (CD62P/ GMP-140/PADGEM) is a 140 kDa member of the C-type selectin family. Like other selectins (CD62E and CD62L), P-selectin contains an amino terminal lectin-like domain, followed by an EGF domain, nine short consensus repeats (SCR), a transmembrane domain and a short cytoplasmic domain (Saboor et al., 2012).

P-selectin is present in megakaryocytes, in Weibel-Palade bodies of endothelial cells and in alpha-granules of platelets.

Upon platelet activation with any number of agonists P-selectin is phosphorylated and rapidly translocated from α-granules of platelets and Weibel-Palade bodies of endothelial cells to the surface membrane. P-selectin mediates rolling of platelets and neutrophils on

activated endothelial cells (Frenette et al., 1995). It is also involved in the interaction between activated platelets with monocytes and neutrophils (Larsen et al., 1989).

There are approximately 10 000 P-selectin molecules on the surface of an activated platelet, translating to a density of perhaps 350 sites/µm2 , a density that exceeds that on even a thrombin or histamine stimulated endothelial cell in vitro by an approximate factor of ten (McEver, 2001).

It has been shown that the interaction of P-selectin with cross-linked recombinant soluble P-selectin glycoprotein ligand-1 (PSGL-1/PSGL-Ig) or membranous sulfatides in platelets increases calcium spikes in single platelets, and triggers platelet GP IIb/IIIa (CD41/CD61)-activation thereby enhancing platelet aggregation, forming larger more stable platelet aggregates and platelet-leukocyte aggregate formation (Merten and Thiagarajan, 2000; Théorêt et al., 2011).

P-selectin up-regulates tissue factor in monocytes and leads to leukocyte accumulation in areas of vascular injury associated with thrombosis and inflammation (Furie et al., 2001).

Platelet P-selectin has been found to be a thrombo-inflammatory molecule that plays significant roles in platelet function and thrombosis (Théorêt et al., 2011).

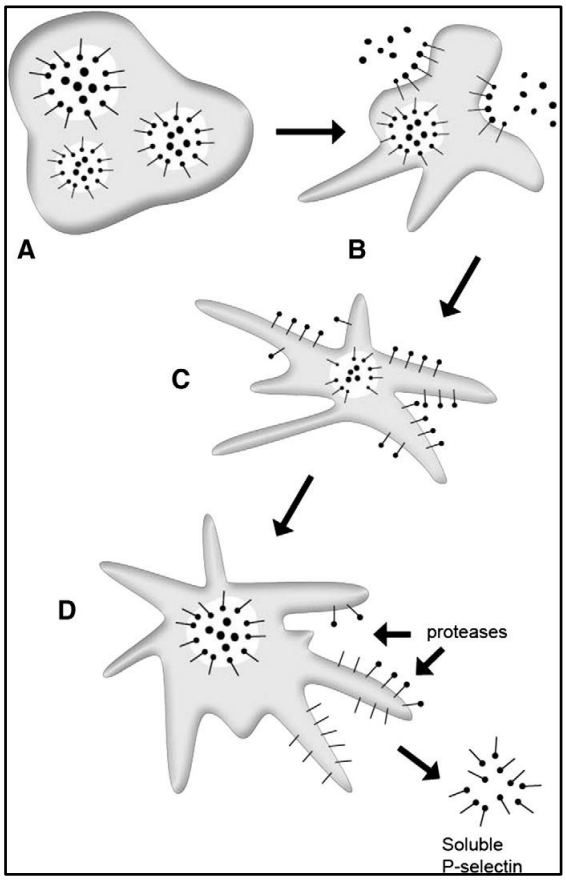
After translocation of P-selectin to the surface of the activated platelet, separation from the membrane, possibly by serum proteases, simple shedding, or active cleavage from cell surface, possibly by a non-specific enzyme or other mediators that may come from leukocytes can occur (Blann and Draper, 2011).

This process generates a soluble fraction that can be detected in the plasma known as soluble P-selectin (sP-selectin) (Fig. 2.14). Thus sP- selectin may be taken to be a surrogate of membrane P-selectin, and thus of the alpha granule mobilisation that is a key aspect of platelet activation (Blann and Draper, 2011).

As P-selectin is present in endothelial cells and platelets, the debate arises whether or not raised plasma levels of P-selectin reflect endothelial dysfunction, platelet activation or both.

Semenov *et al*. reported strong correlations between soluble P-selectin and platelet count (Semenov et al., 1999) and Fijnheer *et al*. suggests that under normal conditions, the majority of the P-selectin is from platelets. Fijnheer *et al*. also concluded that endothelial cell activation is associated with an increased P-selectin concentration per platelet (Blann and Lip, 1997; Blann et al., 1997; Fijnheer et al., 1997).

Figure 2.14. Platelet activation and P selectin. A resting platelet with three intra-cellular alpha granules. B. Upon activation, perhaps by ADP or collagen, there is a shape change with the appearance of pseudopodia. Alpha granules migrate to the exterior and merge with the cell membrane, and the content of the alpha granules is discharged into the plasma. C. Thus an activated platelet is characterised by pseudopodia and an increased expression of membrane bound P-selectin. This latter fact in itself is exploited in defining platelet activation by flow cytometry. D. Serum proteases digest membrane P selectin, resulting in the appearance of soluble P selectin in the plasma (Blann and Lip, 1997).



2.9.4 CD63-PE

CD63 was first described in the granules of resting platelets and on the surface membrane on activated platelets. CD63 was later identified as granulophysin, a 53 kDa platelet dense granule glycoprotein and is also known as lysosomal membrane associated glycoprotein 3 (LAMP3). Its surface expression is associated with lysosomal secretion.

CD63 is a member of the tetraspanin family alternately known as the transmembrane 4 superfamily (TM4). CD63 and has four transmembrane domains with three N-linked glycosylation sites clustered on one of the luminal regions (McNicol and Israels, 1999).

The major extracellular domain is heavily glycosylated and contains poly-N-acetyllactosamines. The intracellular domains of CD63 are small and have no clear motif known to be involved in signal transmission (Skubitz et al., 2000).

Studies have shown that CD63 is a marker for granule release as CD63 is located in the membrane of dense granules and is translocated to the plasma membrane after activation (Nishibori et al., 1993). CD63 modulates platelet spreading and platelet tyrosine phosphorylation on immobilized fibrinogen. CD63 is used as a marker of in vivo platelet activation (Saboor et al., 2012).

The CD63 MoAb used in this study recognizes the activation-specific fusion of the lysosomal granule membrane with the plasma membrane, therefore only binds to the surface of activated platelets.

It has also been suggested that CD63 may protect the plasma membrane from degradation by lysosomal enzymes (Marquardt, 2002).

In conclusion, the haemostatic system consists of 4 areas that may be impaired alone or in combination including: platelets, vessels, plasmatic coagulation, and fibrinolysis. Various methodologies exist to assess damage to the coagulation system. In this thesis platelets will be focussed on as a tool to assess potential damage or abnormalities found in stoke patients and smokers.

3 Methods can be used:

- Flow Cytometry
- Confocal Microscopy
- Atomic Force Microscopy

Therefore in the following chapters the above mentioned techniques will be discussed.
Out of the literature it is clear that stroke is a major burden in society today, similarly tobacco smoke and its harmful properties challenge the health of many smokers in an on-going basis. Both stroke patients and smokers seem to have abnormalities in the coagulation system, they appear to be in a pro-thrombotic, pro-inflammatory state which may be attributed at least in part to the presence of reactive oxygen species.

Therefore the aim of the current study is to investigate changes in platelets in thrombo-embolic ischemic stroke patients and smokers using flow cytometry, atomic force microscopy and confocal microscopy, the objective being to compare the profiles between the groups, with healthy individuals that act as controls.

CHAPTER 3: PATIENTS AND HOSPITAL PROCEDURES

3.1 PATIENTS INFORMATION

3.1.1 Stroke Patients

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 as well as the investigator interacted with the patients and informed consent was obtained from either the patient or a family member before blood was drawn, see the patient information leaflet and informed consent from below. 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 10 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.

3.1.2 Smokers

Individuals who have been smoking for at least 5 years and are not on any chronic medication volunteered to take part in this study. Informed consent was obtained from all participants. A total number of 10 smokers were included in the study. After receiving informed consent, blood was drawn by a nurse or a finger prick was done where needed.

3.1.3 Control Subjects

Healthy control subjects were used to compare the smokers and stroke patients to. These participants were non-smokers, don't use any chronic medication and don't have a history of thrombotic disease.

3.2 HOSPITAL PROCEDURES

On day of admission, 5ml of blood was drawn from each stroke patient in a citrate tube (0,5 ml of Sodium Citrate (3,8%) for 4,5ml of blood). For smokers 5ml of blood was drawn in a citrate tube or a finger prick was done where 20µl of blood was collected and stored in an eppendorf tube containing 1ml sheath fluid and 10µl citrate.

Blood was also collected from 10 healthy, control individuals. 5ml of blood was drawn into a citrate tube and like mentioned above 20µl of blood was collected from finger-pricks.

3.3 PATIENT INFORMATION LEAFLET AND INFORMED CONSENT FORM

TITLE OF STUDY:

COMPARING PLATELET FUNCTION AND ULTRASTRUCTURE IN SMOKING AND THROMBO-EMBOLIC ISCHEMIC STROKE

Principal investigator:

Prof E Pretorius

Department of Anatomy

INTRODUCTION

You are invited to participate in a laboratory-based research study conducted by the Department of Anatomy (School of Medicine) from the University of Pretoria. This information leaflet is to help you to decide if you would like to participate. Before you agree to take part in this study you should fully understand what is involved. If you have any questions, which are not fully explained in this leaflet, do not hesitate to ask the investigator.

PURPOSE OF STUDY

The researcher is investigating and comparing platelet function and ultrastructure in smoking and thrombo-embolic ischemic stroke. Various microscopy techniques will be used as well as flow cytometric analysis.

PROCEDURES

As part of your routine blood investigation, we take an additional 2 ml of blood. This blood will be used in the various techniques mentioned above. You will also be asked to complete a short questionnaire attached at the back of this document (page 5 of 5).

HAS THE TRIAL RECEIVED ETHICAL APPROVAL?

Yes, this protocol has been granted written approval by the Faculty of Health Sciences Research Ethics Committee, University of Pretoria. The study has been structured in accordance with the Declaration of Helsinki (last update: October 2000), which deals with the recommendations guiding doctors in biomedical research involving humans/subjects. A copy of the Declaration may be obtained from the investigator should you wish to review it.

WHAT ARE MY RIGHTS AS A PARTICIPANT IN THIS STUDY?

Your participation in this study is entirely voluntary and you can refuse to participate or stop at any time without stating any reason. Your withdrawal will not affect your access to other medical care. The investigator retains the right to withdraw you from the study if it is considered to be in your best interest.

COULD ANY OF THESE STUDY PROCEDURES RESULT IN DISCOMFORT OR INCONVENIENCE?

Venipunctures (i.e. drawing blood) are normally done as part of routine medical care and present a slight risk of discomfort. Drawing blood may result in a bruise at the puncture site, or less commonly fainting or swelling of the vein, infection and bleeding from the site. Your protection is that the procedures are performed under sterile conditions by experienced personnel. A total of 5 ml of blood (i.e. 1 teaspoon) will be collected during your single donation.

WHAT ARE THE RISKS INVOLVED IN THIS STUDY?

In previous studies some patients have reported experiencing side effects, which included bruising, or swelling of the vein.

ARE THERE ANY WARNINGS OR RESTRICTIONS CONCERNING MY PARTICIPATION IN THIS STUDY?

None

INSURANCE AND FINANCIAL ARRANGEMENTS

Neither you nor your medical aid scheme will be expected to pay for the study. During a study-related injury the Department of Anatomy assumes no obligation to pay for the medical treatment of other injuries or illnesses.

CONFIDENTIALITY

All information obtained during the course of this study is strictly confidential. Data that may be reported in scientific journals will not include any information which identifies you as a patient in this study. In connection with this study, it might be important for domestic and foreign regulatory health authorities and the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, as well as your personal doctor, to be able to review your medical records pertaining to this study.

Any information uncovered regarding your test results or state of health as a result of your participation in this study will be held in strict confidence. You will be informed of any finding of importance to your health. Information will not be disclosed to any third party in addition to the ones mentioned above without your written permission.

INFORMED CONSENT

I hereby confirm that I have been informed by the investigator, Prof E Pretorius about the nature, conduct, benefits and risks of study.

I have also received, read and understood the above written information (Patient Information Leaflet and Informed Consent) regarding the clinical trial.

I am aware that the results of the study, including personal details regarding my sex, age, date of birth, initials and diagnosis will be anonymously processed into a study report.

I may, at any stage, without prejudice, withdraw my consent and participation in the study. I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.

Patient's name (Please print)

……

Patient's signature ..... Date ..... .

Witness's name (Please print)

……

Witnesses's signature .....Date ..... .

I, Prof E Pretorius herewith confirm that the above patient has been informed fully about the nature, conduct and risks of the above trial.

Investigator's name
Prof E Pretorius

Investigator's signature .....Date ..... .

Questionnaire

Patient Lab Number:

DEVELOPING A NOVEL SCREENING TOOL USING ULTRASTRUCTURE TO PREDICT THROMBOTIC EVENTS

How old are you?			
male		female	

If you are a female are you pre- or post- menopausal. Please mark.

Pre-menopausal		**Post-menopausal**	

If you are a female do you use hormone replacement therapy?

YES		NO	

If yes, please specify name of medication.

Do you suffer from any of the following? Mark with a cross

High blood pressure	YES		NO	
High cholesterol	YES		NO	
Do you smoke?	YES		NO	
Have you had a stroke previously?	YES		NO	
If yes, when did you have this stroke?				
Do you use Aspirin?	YES		NO	
Do you use Wafarin?	YES		NO	
Any other medication, please specify?				

CHAPTER 4: FLOW CYTOMETRIC ANALYSIS OF PLATELET FUNCTION IN THROMBO-EMBOLIC ISCHEMIC STROKE AND SMOKERS

4.1 CHAPTER OBJECTIVES

In this chapter the physiological changes in platelets of stroke patients and smokers compared to healthy individuals will be investigated using flow cytometry.

4.2 INTRODUCTION

Flow cytometry rapidly measures the specific characteristics of a large number of individual cells. Before flow cytometric analysis, cells in suspension are fluorescently labelled, typically with a fluorescently conjugated MoAb. In the flow cytometer, the suspended cells pass through a flow chamber and, at a rate of 1000 to 10 000 cells per minute, through the focused beam of a laser. After fluorescent activation of the fluorophore at the excitation wavelength, a detector processes the emitted fluorescence and light scattering properties of each cell. Flow cytometry allows the simultaneous detection of surface antigens in a sensitive and specific manner.

4.3 MATERIALS AND METHODS

Hospital procedures were followed as described in chapter 3.

For each sample taken from patients 6 tubes was prepared; each tube containing 1ml sheath fluid from Beckmann and Coulter and 20µl of blood, or 20µl of the blood, sheath and citrate mixture from the finger pricks.

The various tubes were stained with 20µl of CD41-FITC and 20µl of one of the following probes: CD41-PE, CD42b-PE, CD62P-PE and CD63-PE (from Beckman Coulter). For ROS analysis one tube contained 1 µl Hydroethidine (HE) and 20µl CD41-FITC and the other 1 µl 2,7-dichlorofluorescein diacetate (DCFDA) and 20µl CD41-PE.

The samples stained with different probes, were incubated at room temperature in the dark for 20 minutes before being analyzed by a flow cytometer (FC 500, Beckman Coulter).

Forward scatter and 90º side scatter were displayed on logarithmic scales. Two platelet gates were set. The first gate was set according to the morphological characteristics of platelets while the second gate was set according to CD41-FITC fluorescence, a platelet specific marker. The fluorescence of the different antibodies was plotted on 256-channel log

histograms. The results were expressed in arbitrary units as mean channel fluorescence intensity (MCFI).

4.4 STATISTICAL ANALYSIS

Since, for the experimental group, patients were chosen to be similar on the basis that they do not use any chronic medication, have a history of thrombotic disease, are healthy individuals and are smokers, the MCFI observations for the 10 participants can be regarded as being independent and identically distributed random variables for each of the different blood-preparation techniques. Furthermore, since for each participant the MCFI was calculated as the mean fluorescence of a large sample of platelets (10 000 platelets per individual), the well-known Central Limit Theorem assures us that the Normal distribution is a close approximation for the distribution of the MCFIs for the experimental group. A similar argument follows for the control group with the exception that they were chosen to be non-smokers, and again we can assume a Normal distribution for the MCFIs of the control group. This allows us to make use of the two-sided Student's t-test to detect a significant shift in location of the mean of the MCFI values between the experimental and the control group. No assumption of equivalence of variance between the two groups was made and the test was performed at a 5% level of significance (A P value of ≤ 0.05 was considered significant).

4.5 RESULTS AND DISCUSSION

Table 4.1: Flow cytometric analysis of control subjects, stroke patients and smokers. Results presented as mean ± Standard Deviation (SD) of MCFI and percentage activated platelets. (n=60 000 0 platelets total analyzed for each of the groups)

MoAb	Control Subjects	Stroke Patients	Smoking group
CD41-PE	23 ± 2.6	0.38 ± 0.33	4.3 ± 5.3
CD42b-PE	14.7 ± 2.8	21.74 ± 3.6	20.3 ± 7.6
CD62P-PE X-mean	15.5 ± 3.9	11.1 ± 0.8	12.5 ± 1.6
CD62P-PE % Activated Platelets	54.4% ± 19%	31.92% ± 5%	32.7% ± 8%
CD63-PE X-mean	12.6 ± 3.1	11.4 ± 0.3	12 ± 1.2
CD63-PE % Activated Platelets	15.5% ± 6%	31.4% ± 3%	38.8% ± 11%
HE Platelets	10.2 ± 2.4	6.5 ± 2.1	19.6 ± 17.6
HE whole blood	12.51 ± 1.3	16.28 ± 1.2	18.1 ± 6.7
DCFDA platelets	13.4 ± 6.9	25.7 ± 7.7	36.8 ± 8.3
DCFDA whole blood	46.21 ± 15.8	57.9 ±12.6	55.2 ± 15.6

4.5.1 Stroke versus Control

Results

Figure 4.1. Platelet membrane markers (CD41-PE and CD42b-PE) in stroke patients and control subjects. SD indicated and statistically significant differences with an asterisks*.

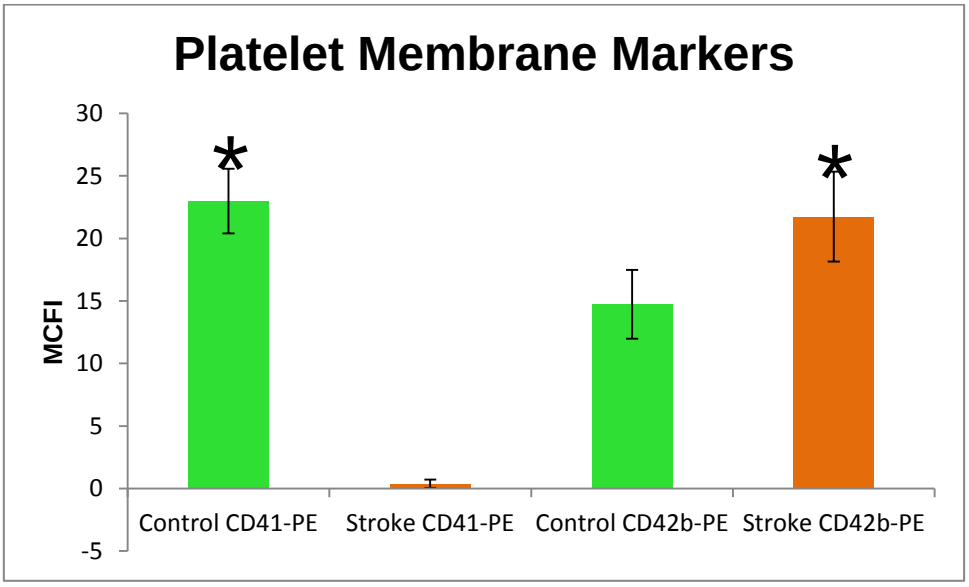


Figure 4.2. Histogram overlay plot of CD41-PE expression measured by MCFI. A: control subject, B: stroke patient and C: smoker .

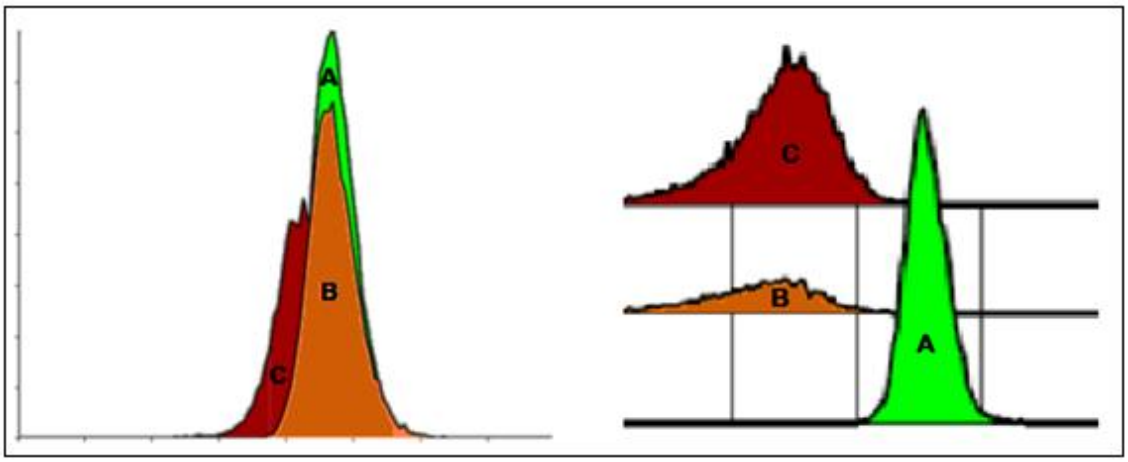
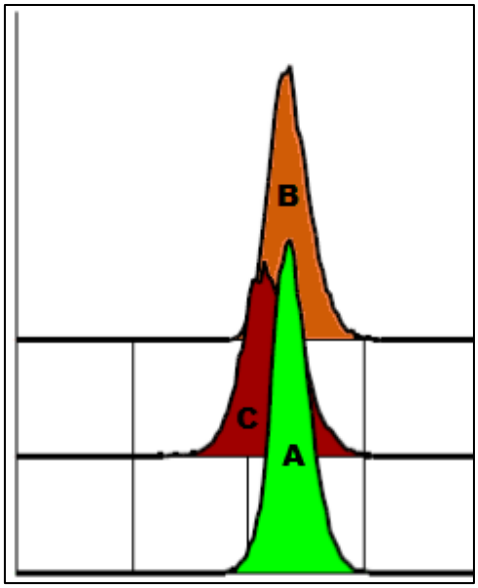


Figure 4.3. Histogram overlay plot of CD42b-PE expression measured by MCFI. A: control subject, B: stroke patient and C: smoker.



Regarding the MoAb CD41-PE, there is a statistically significant difference between the control group and the stroke patients ($p<0.05$), control subject having a higher MCFI as seen in figure 4.1 and 4.2.

Regarding MoAb CD42b-PE, there is a statistically significant difference between the control group and the stroke patients ($p<0.05$), stroke patients having a higher MCFI as seen in figure 4.1 and 4.3.

Regarding MoAb CD62P-PE, there is a statistically significant difference between the control group and the stroke patients FL3 X-mean as well as the % activated platelets ($p<0.05$), control subjects having a higher MCFI and activation. In stroke patients 31.92% of the platelets were activated and 54.36% of the control subjects' platelets were activated as seen in figure 4.4.

Regarding MoAb CD63-PE, there no significant difference between the control group and the stroke patients FL3 X-mean however there is a statistically significant difference in the % activated platelets ($p<0.05$), stroke patients having a higher activation of 31.37% compared to the 15.54% activation in control subjects as seen in figure 4.4

Platelet activation via both probes is also illustrated in figure 4.6.

Figure 4.4. Platelet activation markers (CD62P-PE and CD63-PE) in stroke patients and control subjects. SD indicated and statistically significant differences with an asterisks*.

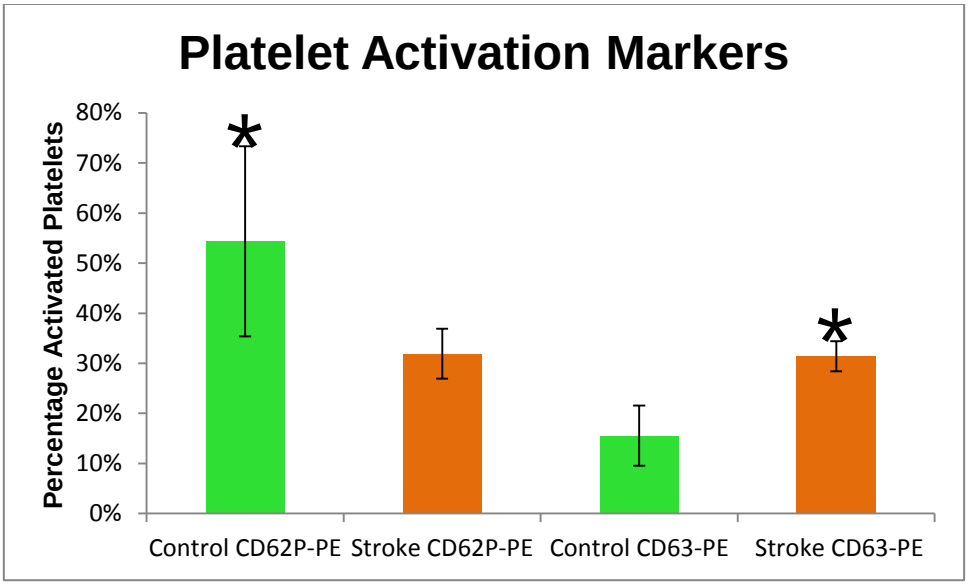


Figure 4.5. ROS levels in platelets (HE representing superoxide and DCFDA representing hydrogen peroxide) in stroke patients and control subjects. SD indicated and statistically significant differences with an asterisks*.

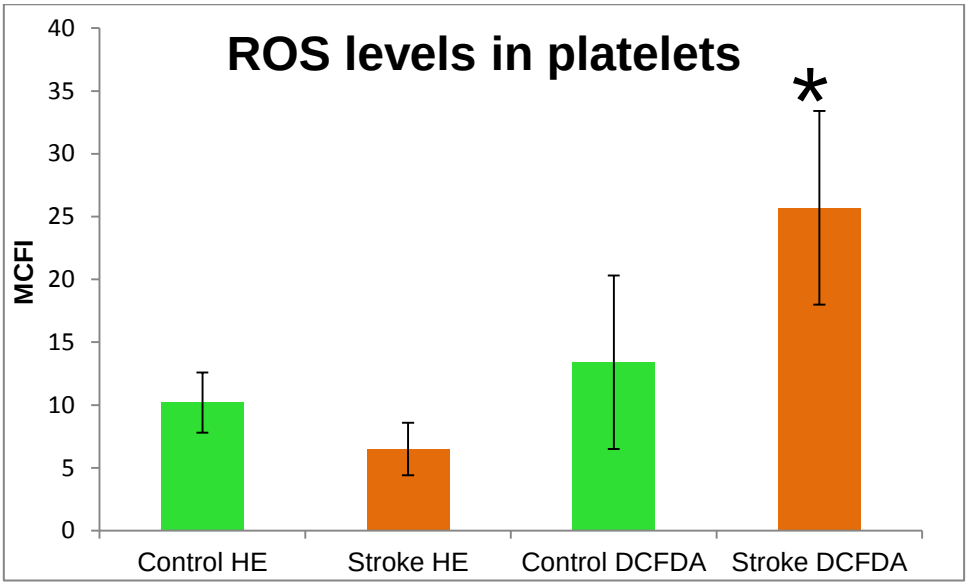
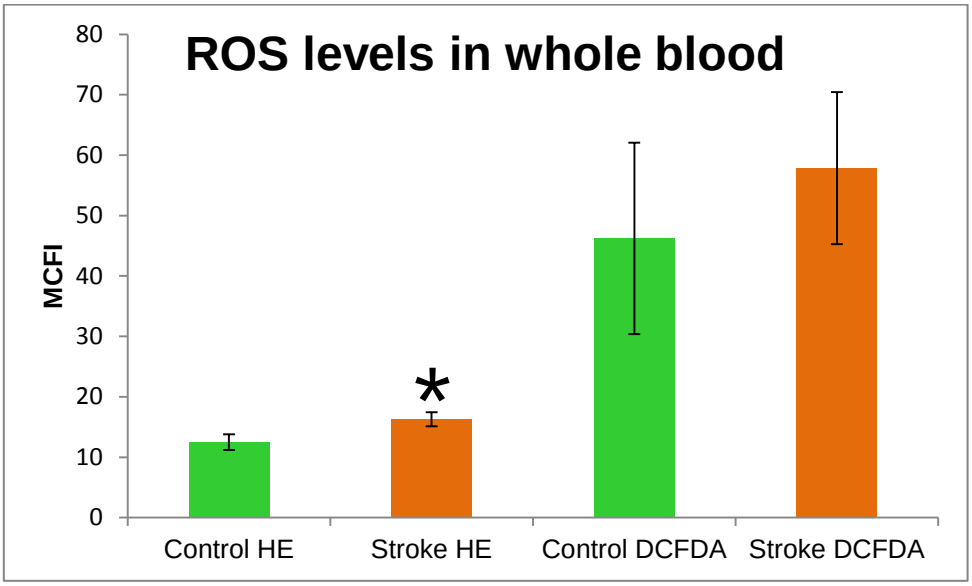


Figure 4.6. ROS levels in whole blood (HE representing superoxide and DCFDA representing hydrogen peroxide) of stroke patients and control subjects. SD indicated and statistically significant differences with an asterisks*.



Regarding HE labelling, there was no significant difference between stroke patients and control subjects platelet levels however the was a significant difference in the superoxide levels in the whole blood of stroke patients compared to control subjects, stroke patients having higher values (Fig 4.6).

Regarding DCFDA in the platelets there is a statistically significant difference between the groups, stroke patients having a higher MCFI than control subjects as seen in figure 4.5 and 4.8. Stroke patients had slightly higher values of hydrogen peroxide in whole blood but not at a statistically significant level.

Figure 4.7. Platelet activation probes of a control subject, stroke patient and a smoker. The upper right quadrant F2 represents the population of platelets that are activated.

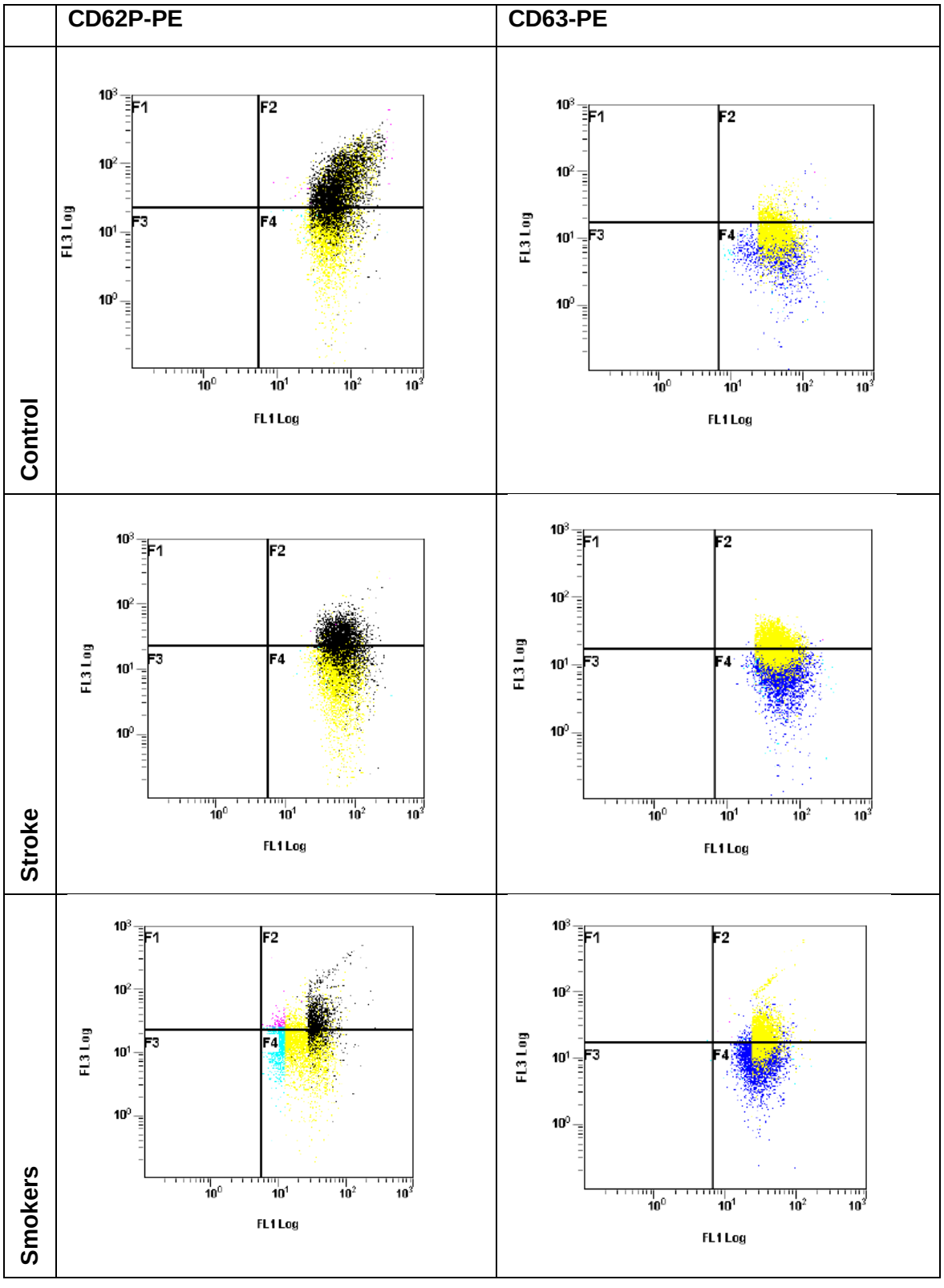
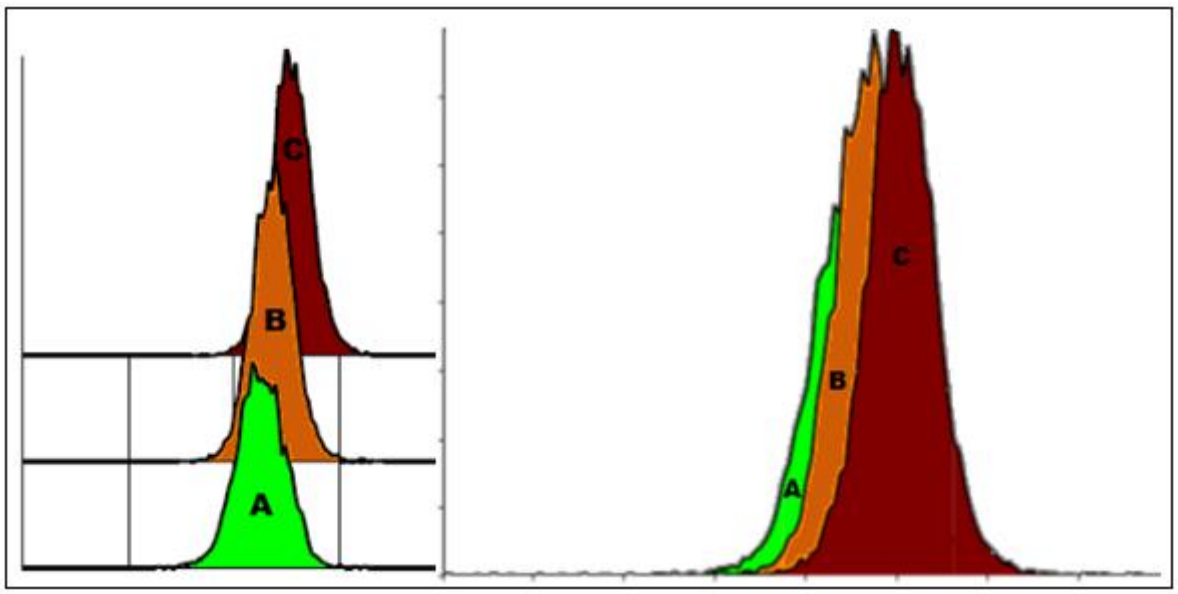


Figure 4.8. Histogram overlay plot of DCFDA (hydrogen peroxide) in platelets measured by MCFI. A: control subject, B: stroke patient and C: smoker.



Discussion and Conclusion

Platelet Probes

As mentioned previously GpIIb–IIIa is the receptor on the membrane of platelets, which is involved in the binding with various adhesive blood proteins, such as fibrinogen. The formation of the GpIIb–IIIa complex is an important step in the aggregation of platelets. On resting platelets GpIIb–IIIa is in an inactive conformation, whereas upon platelet activation the glycoprotein undergoes a measurable conformational change increasing its affinity for fibrinogen (Hetty 2002).

In this study the CD41 probe was used primarily to identify platelets, as it is only present on platelets and not any other circulating blood cell. However the values obtained from this probe may serve to give further insight regarding platelet physiology.

The CD41 MoAb reacts with GPIIb (CD41) in the intact complex with GPIIIa (CD61) but not with the separated subunits (Saboor et al., 2012).

According to our data there is significantly higher expression of the CD41/CD61 complex in the control group compared to the stroke patients as seen in figure 4.1.

This may be attributed to the fact that stroke patients may be given thrombolytic agents like tissue plasminogen activator (tPA), anti-platelet drugs (Aspirin) and anti-coagulants (blood

thinners). Furthermore it may be an indication that the platelet membrane of stroke patients has undergone conformational changes affecting the interaction of the subunits in the CD41/CD61 complex, resulting in fewer intact complexes.

The main role of the GPIIb-IIIa complex of activated platelets is to serve as a receptor for a number of adhesive proteins that mediate platelet adhesion and aggregation, therefore the primary effect of decreased GPIIb-IIIa will be defective platelet aggregation followed by adhesion and coagulation defects.

The CD42b MoAb used in this study specifically binds to CD42b also known as the Platelet glycoprotein Ib alpha chain (GPIbα). As mentioned previously GPIbα forms part of the GPIb-IX-V complex which is the receptor for von Willebrand's factor and is known as von Willebrand's factor-dependant adhesion receptor. GPIbα is the main binding domain of the GPIb-IX-V complex.

The GPIb-IX-V complex is a crucial platelet receptor for initial tethering and adhesion at sites of vascular injury. The adhesive function of GPIb-IX-V is mainly attributed to the interaction of GPIbα with its major ligand von Willebrand factor (VWF), exposed upon vascular damage.

According to Meyer *et al.* the importance of GPIbα far exceeds that of VWF in arterial thrombosis and GPIbα is a central receptor in different vascular processes of thrombosis and inflammation, all of which may contribute to the progression of ischemic stroke (De Meyer et al., 2011).

According to Mu *et al.* the engagement of GPIb-IX-V by von Willebrand factor (VWF) mediates platelet adhesion to damaged vessels and triggers platelet activation and thrombus formation in heart attack and stroke (Mu et al., 2008).

Prevention of early platelet adhesion to the damaged vessel wall by blocking platelet surface receptors GPIbα or GPVI protects from stroke without provoking bleeding complications. In addition, downstream signalling of GPIbα and GPVI has a key role in platelet calcium homeostasis and activation (Kraft et al., 2012).

We found a significant increase in GPIbα in stroke patients compared to the healthy control subjects as seen in figure 4.1.

Thus it seems as if the upregulation of GPIbα seen in the results confirms the pivotal role it plays in the development of thrombo-embolic ischaemic stroke as indicated by Kraft *et al*. The mechanisms by which the production of GPIbα is increased remains unknown and may

be a good topic to investigate further as it may be a useful target for stroke prevention therapies.

The CD62P-PE antibody binds to P-selectin and has been used in previous studies as a marker of platelet activation. Upon platelet activation P-selectin is phosphorylated and translocated from the α-granules of platelets and Weibel-Palade bodies of endothelial cells to the surface membrane.

As mentioned previously P-selectin is a thrombo-inflammatory molecule that plays a significant role in platelet function and thrombosis (Théorêt et al., 2011).

Flow cytometric analysis found a significant difference in platelet activation regarding P-selectin with stroke patients having a lower mean activation of 31.92% with a SD of 5% compared to an activation of 54.36% with a SD of 19% found in the control subjects as seen in figure 4.4.

This unexpected finding may be attributed to the fact that P-selectin can be cleaved from the membrane surface after activation releasing P-selectin into the plasma known as soluble P-selectin (sP-selectin). The exact mechanism of this shedding is unknown but several mechanisms have been suggested including cleavage by serum proteases or non-specific enzymes or by simple shedding (Blann and Draper, 2011).

As we did not analyse the plasma but strictly the platelets, only P-selectin present on the platelet membrane was measured. It appears that this shedding has taken place after activation occurred in the platelets of stroke patients, therefore this probe is not a suitable candidate to determine platelet activation in this instance however we can speculate that the platelets of the stroke patients were more activated at one stage as the platelets that shed their P-selectin can continue to circulate and function (Michelson *et al*, 1996).

There is agreement on the presence of elevated concentrations of platelet derived substances such as plasma P-selectin which may serve as the indicators of enhanced platelet activity in stroke patients.

It has been demonstrated that soluble P-selectin derived from activated platelets can be measured as a marker of platelet activation in platelet-poor plasma (Fijnheer *et al*, 1997).

The CD63 MoAb recognizes the activation-specific fusion of the lysosomal granule membrane with the plasma membrane, therefore it only binds on the surface of activated platelets and is a useful tool to use in the identification of activated platelets.

We found a significant difference in the activation of platelets determined by the CD63 MoAb (see figure 4.4). The stroke patients had a higher mean activation of 31.7% with a SD of 3% compared to the control subjects who had a mean activation of 15.54% with a SD of 6%.

Additionally we found no difference in the MCFI between the groups, indicating that the amount of lysosomal granules fusing with the cell membrane are equal as an average per cell in each group, however the amount of activated cell are higher in stroke patients.

These results are conclusive with the literature indicating that CD63 expression (platelet activation) is lower in control subjects than in stroke patients regardless of the treatments given (Grau et al., 2003).

Increased CD63 and CD62P (P-selectin) expression participate in the on-going inflammatory processes of atherosclerotic changes. According to Tsai *et al.* there is a trend of higher CD62P, CD63, and platelet-leukocyte expressions on presentation in patients with poor outcome in ischemic stroke (Tsai et al., 2009).

According to Marquardt the initial increase in both CD62p and CD63 expression by platelets is followed by a differential regulation of both parameters after stroke. The rapid decrease in CD62p expression may be caused by shedding from the cell surface as mentioned before. Its persistent elevation makes CD63 a good candidate for studies on predictors for stroke recurrence.

CD63 expression remains increased for at least 3 months after stroke. Therefore, CD63 could be an interesting parameter for future prospective studies that evaluate the role of platelet activation markers as potential predictors for first or recurrent ischemic events. A study by Grau *et al.* showed that increased expression of CD62p or CD63 after stroke was not associated with an increased proportion of circulating platelets with shape change (Grau et al., 1998).

The shape change is a very early and reversible event during platelet activation and a very sensitive marker of instantaneous platelet activation. Here Marquardt has made the interesting statement that this indicates that platelets after stroke are not particularly activated during their passage in peripheral venous blood. Thus, CD62p and CD63 are memory markers of platelet activation, and the increased expression of secretion-dependent antigens after stroke indicates that platelets had previously undergone activation. In a baboon model, degranulated platelets rapidly lost surface CD62p but continued to circulate and function (Michelson et al., 1996).

Platelets possess a lifespan of about 7 to 10 days. Therefore, increased CD63 expression in sub-acute stroke indicates continuously on-going platelet activation and likely systemic inflammation. Altogether, in the sub-acute stage after ischemic stroke, CD63 is a more sensitive marker of platelet activation than CD62p, most likely because of shedding of CD62p (Marquardt, 2002).

ROS Probes

The following probes were used to determine the presence of ROS; Hydroethidine (HE) was used to detect superoxide ($O_2^{\bullet -}$) Within the living cell, HE is enzymatically dehydrogenated, in part, to form ethidium, which becomes locked in the cell by virtue of its cationic nature. The ethidium intercalates into the DNA and is red fluorescent when excited by visible 535 nm light. Even though platelets are anucleate cells, the ethidium is able to intercalate into the DNA found in the mitochondria of platelets.

Hydrogen peroxide (H_2O_2) generation was assessed using 2,7-dichlorofluorescein diacetate (DCFDA), a non-fluorescent probe, which upon oxidation by ROS and peroxides is converted to the highly fluorescent derivative 2,7-dichlorofluorescein (DCF).

We found no significant difference in superoxide production in the platelets between the two groups however the whole blood levels of superoxide was elevated. To clarify a measurement was taken of HE and DCFDA specifically in the platelets and a second measurement was taken of the whole blood sample. The stroke patients had a significantly higher mean value of hydrogen peroxide in their platelets than the control group as seen in figure 4.5, while the whole blood hydrogen peroxide levels in the whole blood were higher than the control groups but not significantly.

To further explain the values obtained from the platelets ROS versus whole blood ROS it is important to note that values are given as the mean channel fluorescence intensity. Meaning the value represents the intensity of fluorescence of a group of cells or area gated. Therefore it would be possible for the ROS levels to be higher in the platelets than in the whole blood as the mean intensity for the group of platelets is higher than the entire gate of the whole blood.

When looking independently at superoxide one could speculate that the stroke group does not have increased superoxide levels in their platelets because of various reasons. One would expect an increase as stimulated platelets have been shown to produce superoxide (Caccese et al., 2000; Finazzi-Agrò et al., 1982; Krötz et al., 2004; Wachowicz et al., 2002).

A possible reason for the normal levels of superoxide in the stroke patients platelets is that the superoxide was only measured in the mitochondria of the platelets as that is where the probe is localized, as found by Joseph *et al.* fewer mitochondria were present in the platelets of stroke patients and might possibly indicate that the activated platelets of stroke patients release or consume mitochondria more readily than healthy individuals platelets (Joseph et al., 1989b). Furthermore it could also be indication that activated platelets release superoxide therefore mitochondria into the plasma as there is an increase in whole blood superoxide levels.

This increase could also be attributed to the production of superoxide from other blood cells found in their DNA as white blood cells are known to generate superoxide amongst other reactive oxygen species to kill bacteria (Vanaporn et al., 2011). Additionally superoxide can be generated by a variety of cells including phagocytes and non-phagocytic cells like fibroblasts, vascular smooth muscle cells and endothelial cells (Perner et al., 2003) which may all attribute to increased levels of superoxide in the plasma.

When looking at hydrogen peroxide independently the increase in the platelets of stroke patients can be attributed to an increased activation of oxidase enzymes or phagocytes at the site of inflammation which can lead to the production of hydrogen peroxide. As hydrogen peroxide is highly diffusible within and between cells it will be able to move into the platelets.

Furthermore activated platelets are known to produce hydrogen peroxide (Caccese et al., 2000; Finazzi-Agrò et al., 1982; Krötz et al., 2004; Wachowicz et al., 2002).

However superoxide and hydrogen peroxide are so closely related therefore they need to be analyzed together. Superoxide short lived compound and is readily converted to hydrogen peroxide via superoxide dismutase (SOD):

$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$

According to Aggarwal *et al*. ischemia can induce increased expression of enzymes like SOD and the endogenous antioxidant capacity can be overwhelmed, leading to increased superoxide and hydrogen peroxide concentrations (Aggarwal et al., 2010).

As hydrogen peroxide is poorly reactive in chemical terms it seems as if there may have been an increase in superoxide production by the platelets of stroke patients which were then converted to hydrogen peroxide via SOD. This would result in the concentration of superoxide returning to normal and an increased concentration of hydrogen peroxide as it does not react as readily except in the presence of transitional metal ions. Our results support this process as the stroke patients have significantly elevated hydrogen peroxide

levels in their platelets and normal superoxide levels compared to the healthy control subjects. Alternatively the increase in hydrogen peroxide in the platelets could be attributed to an increase production of superoxide by e.g. white blood cells in the plasma. The superoxide can then be converted to hydrogen peroxide by SOD and migrate into the platelets, explaining the normal level of hydrogen peroxide and increased level of superoxide in the whole blood and increased levels of hydrogen peroxide in the platelets as well as normal levels of superoxide. As superoxide is largely cell impermeable (Cai et al., 2007) it is more likely that superoxide will remain where it is synthesized and hydrogen peroxide produced via SOD will then move throughout different cells and areas.

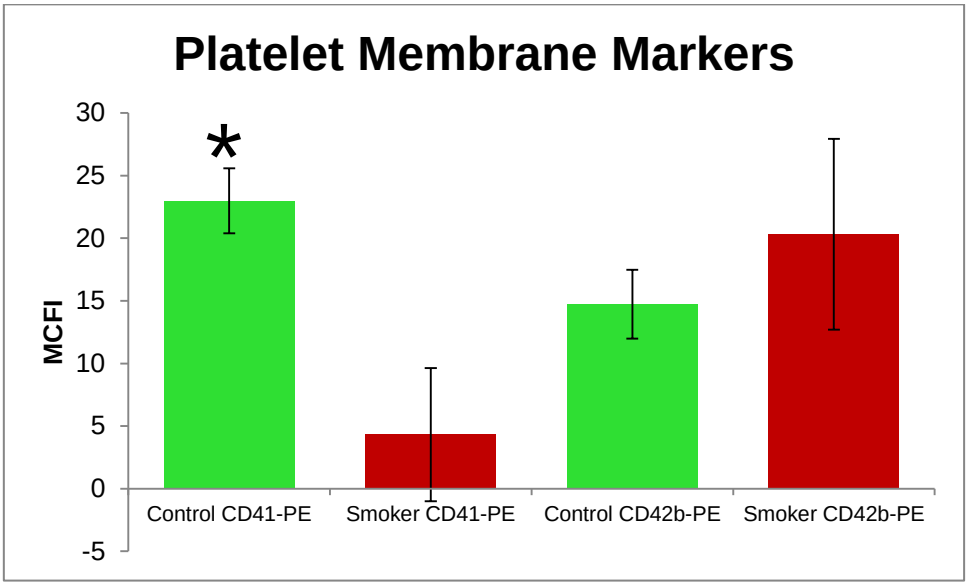
The question now arises whether in stroke patients superoxide production is increased in the plasma, where it is converted to hydrogen peroxide and transported into the platelets or if superoxide production within the platelet itself is at one stage elevated but then converted via SOD to hydrogen peroxide, decreasing superoxide levels in the platelet and increasing hydrogen peroxide levels in the platelet. Furthermore one can speculate that both these processes may occur simultaneously.

Hydrogen peroxide is able to either stimulate or inhibit platelet aggregation (Ambrosio et al., 1997) and in high concentrations is able to cause platelet aggregation (Canoso et al., 1974). In stroke patients this increased hydrogen peroxide may be indicative of a chronic inflammatory state as activated phagocytes produce hydrogen peroxide at the site of inflammation as well as a pro-thrombotic state as hydrogen peroxide is able to modify platelet activation.

4.5.2 Smoke versus Control

Results

Figure 4.9. Platelet membrane markers (CD41-PE and CD42b-PE) in smokers and control subjects. SD indicated and statistically significant differences with an asterisks*.



As shown in figure 4.9 there is a statistically significant difference in the MoAb CD41-PE MCFI between the control group and the smokers group ($p<0.05$), control subject having a higher MCFI (also see figure 4.2). Regarding MoAb CD42b-PE, there is no statistically significant difference between the control group and the smokers group ($p<0.05$) as seen in figure 4.8 and 4.2.

As seen in figure 4.10 and table 4.1 there is a statistically significant difference in MoAb CD62P-PE MCFI between the control group and the smokers group FL3 X-mean as well as the % activated platelets ($p<0.05$), control subjects having a higher MCFI and activation. In the smokers group 32.96% of the platelets were activated and 54.36% of the control subject's platelets were activated.

Regarding MoAb CD63-PE, there no significant difference between the control group and the smokers group FL3 X-mean however there is a statistically significant difference in the % activated platelets ($p<0.05$), smokers group having a higher activation of 38.84% compared to the 15.54% activation in control subjects as seen in figure 4.10 below. Also see figure 4.7.

Figure 4.10. Platelet activation markers in smokers and control subjects. SD indicated and statistically significant differences with an asterisks*.

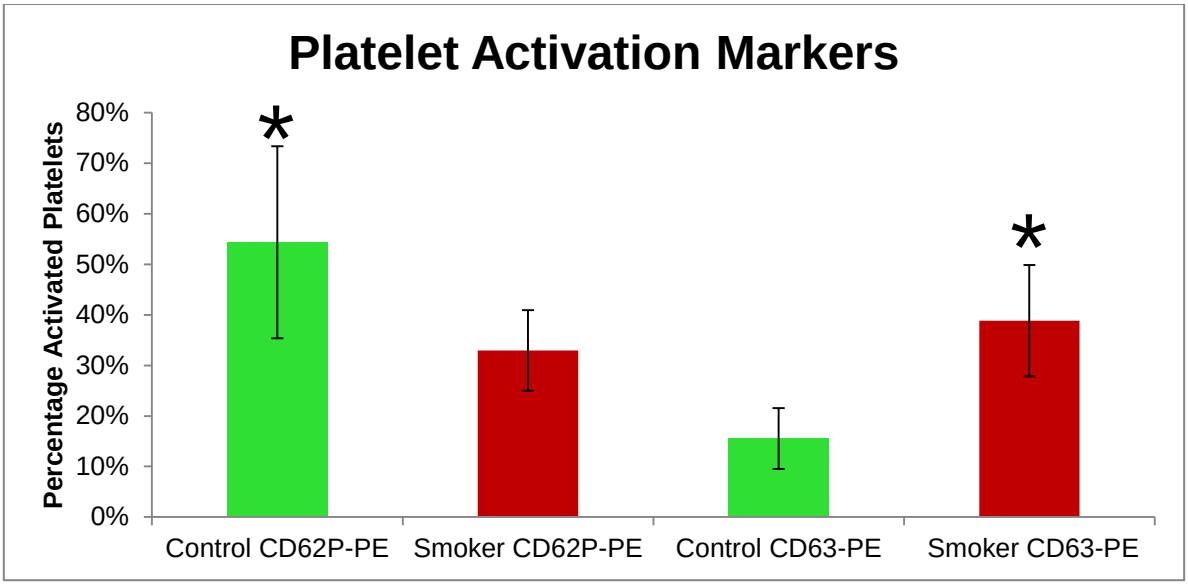


Figure 4.11. ROS levels in platelets (HE/Superoxide and DCFDA/Hydrogen peroxide) in smokers and control subjects. SD indicated and statistically significant differences with an asterisks*.

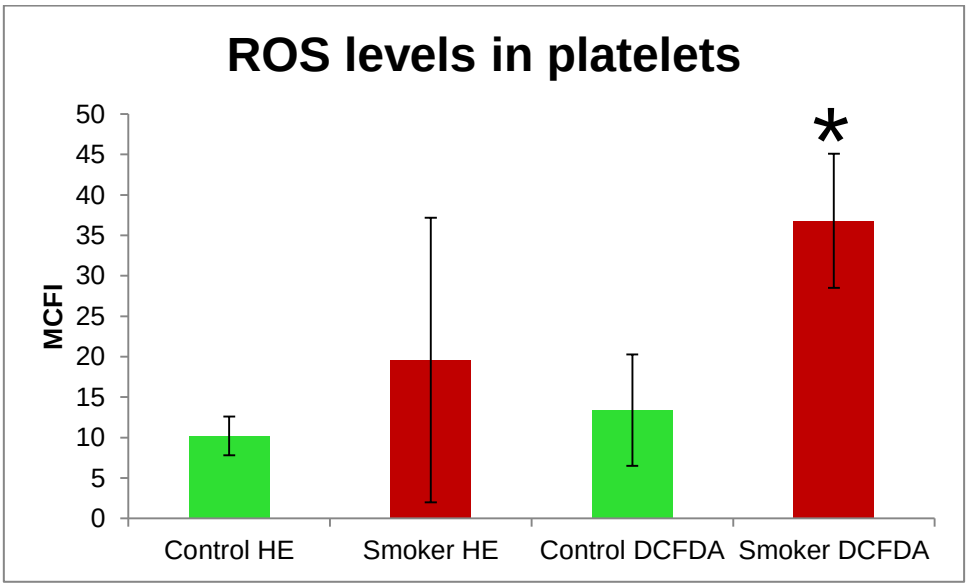
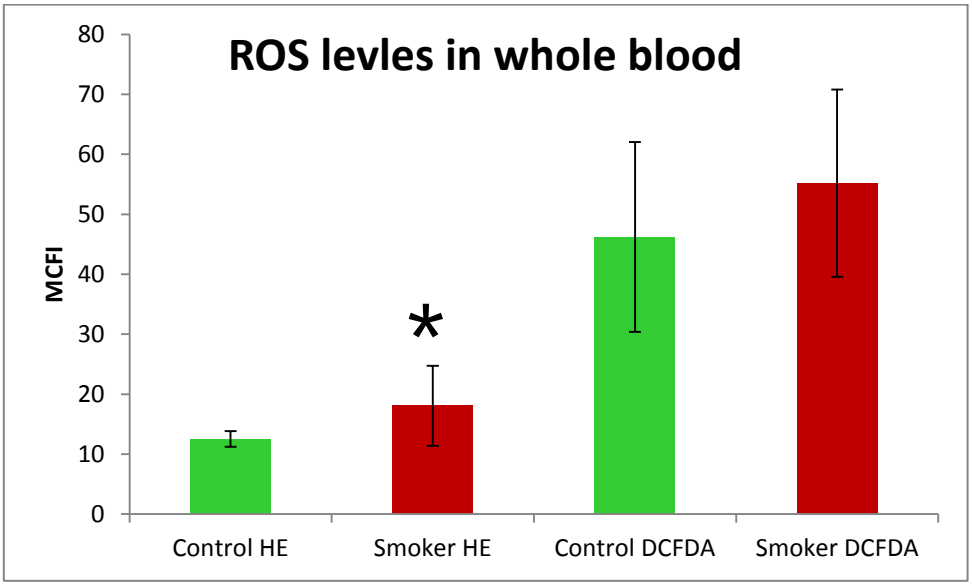


Figure 4.12. ROS levels in whole blood (HE representing superoxide and DCFDA representing hydrogen peroxide) in smoker's patients and control subjects. SD indicated and statistically significant differences with an asterisks*.



Regarding HE labelling, there was no significant difference between stroke patients and control subjects platelets, however there was a significant increase in superoxide levels in the whole blood of stroke patients compared to control subject (Fig 4.12)

As shown in figure 4.11 there is a statistically significant difference in DCFDA MCFI of the platelets between the groups. The smoker's group platelets having a higher MCFI than control subjects (see figure 4.8).

Figure 4.13 and 4.16 below shows the platelet ROS levels together to indicate the significant difference in HE between the stroke group and the smokers group. Figure 4.14 shows the different groups' whole blood ROS levels together.

Figure 4.13. ROS levels in platelets of stroke patients, smokers and control subjects. SD indicated and statistically significant differences with an asterisks*.

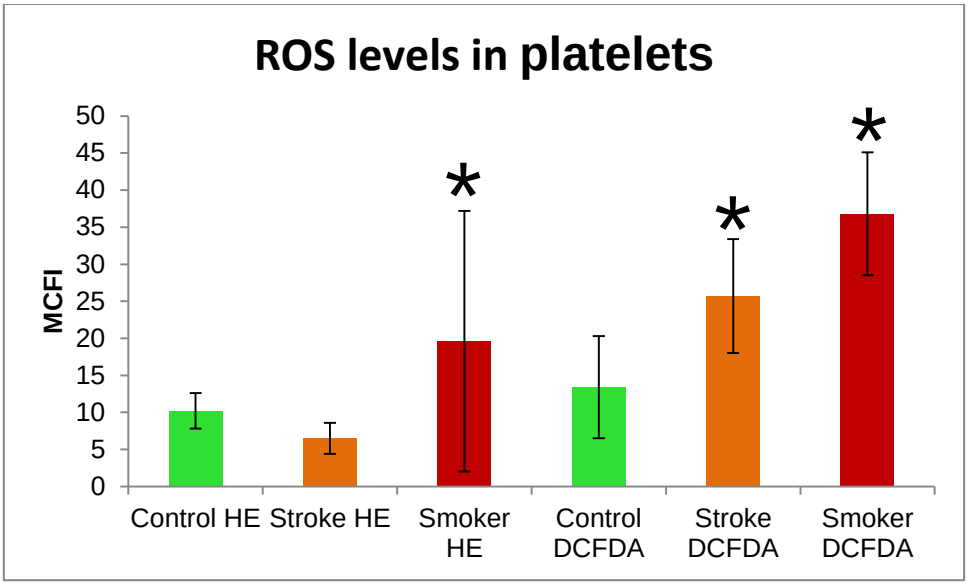


Figure 4.14. ROS levels in whole blood of stroke patients, smokers and control subjects. SD indicated and statistically significant differences with an asterisks*.

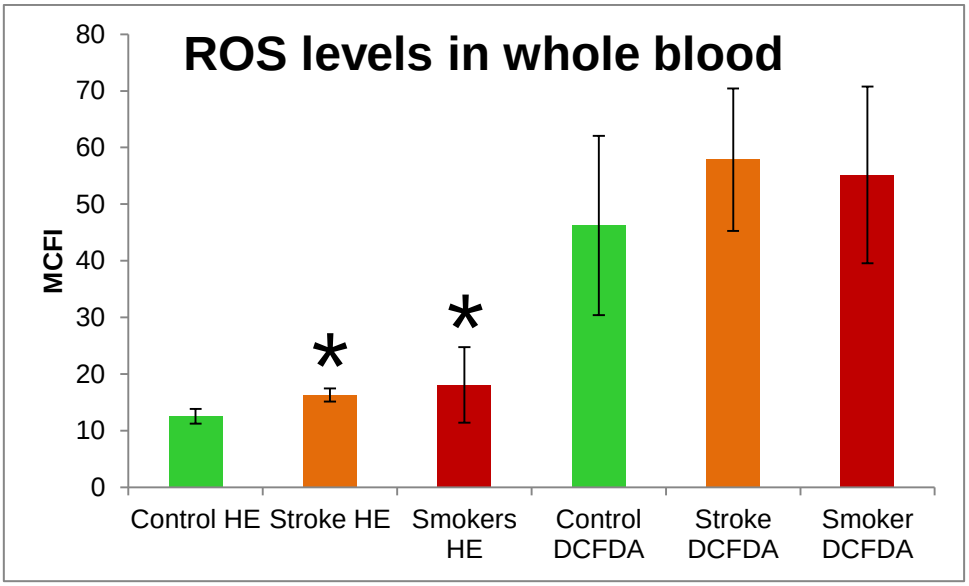
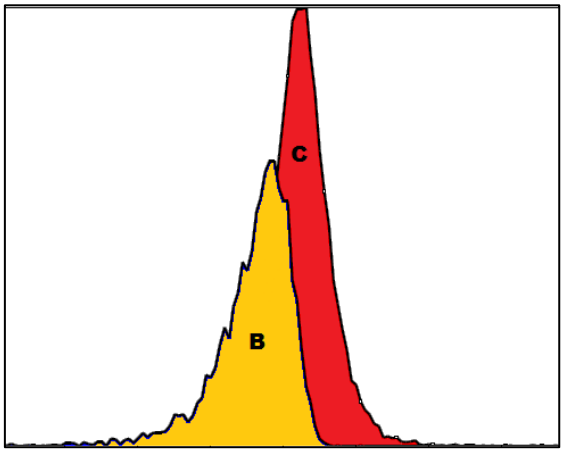


Figure 4.15. Histogram of Superoxide in platelets in B: stroke patients and C: smokers.



Discussion and Conclusion

Platelet Probes

Similar to the stroke data there is significantly higher expression of the CD41/CD61 complex in the control group compared to the smokers as seen in figure 4.9 and 4.2.

This may be due to experimental procedures however this is unlikely as the same protocol was followed for all samples. Previous research has shown that smoking causes changes in platelet membrane fluidity and Na^+/K^+ATPase activity (Padmavathi et al., 2010). Furthermore Pretorius confirmed this as she found that changes in membrane fluidity are structurally visible and translate into a more globular and bulbous appearance of the membrane surface in the platelets of smokers (Pretorius, 2012). Therefore the decreased amount of the CD41/CD61 complex in smokers could be further proof of changes occurring in the membrane fluidity as less intact complexes were measured.

There was no significant difference between the GPIbα sub-unit of the GPIb-IX-IV receptor determined by the CD42b MoAb as seen in figure 4.3 and 4.9. This may indicate that this receptor is not involved in the pathophysiological effects of smoking on haemostasis and thrombosis, therefore the increased platelet activation found in smokers may be attributed to other factors.

Flow cytometric analysis found a significant difference in platelet activation regarding P-selectin with smokers having a lower mean activation of 32.96% with a SD of 8% compared to an activation of 54.36% with a SD of 19% found in the control subjects (see figure 4.10 and 4.7).

Similarly to that of stroke patients this may be attributed to P-selectin shedding as described above and is not a reliable indicator of platelet activation in this instance.

However studies have shown an increase in both P-selectin on the surface of platelets and sP-selectin indicating that smoking causes chronic activation of platelets as P-selectin is being shed from activated platelets as new P-selectin is being expressed on recently activated platelets (Inoue, 2004).

CD63 expression was significantly higher in smokers with a 32.96% mean activation (SD:11%) compared to a 15.54% mean activation (SD:6%) in the control subjects (see figure 4.9 and 4.6). Therefore it is clear that this probe is a reliable marker of platelet activation as increased platelet activation has been established in smokers (Inoue, 2004).

ROS Probes

Similar to stroke patients there was no significant increase in superoxide production within the platelets of smokers compared to control subjects, but when compared to stroke patients there was a significant increase. There was however a significant increase in hydrogen peroxide in the platelets not only when compared to control subjects but also in comparison with stroke patients as seen in figure 4.13 showing all the group's results of both probes and figure 4.8 showing DCFDA. Also similar to stroke patients the whole blood tests showed in increase in superoxide levels and slightly elevated hydrogen peroxide levels but not significantly.

This may indicate that some of the same mechanisms found in stroke patients are responsible for this increase but additional factors might be involved.

Cigarette smoking is an independent risk factor for ischemic stroke in women and men (Cubrilo-Turek, 2004). One of the most important effects of smoking is that it exposes the user to excessive amounts of oxidants and toxins (Assinger et al., 2010), and a puff of cigarette smoke delivers up to 1018 free radicals to the human body (Padmavathi et al., 2010; Pryor and Stone, 1993; Smith and Fischer, 2001).

These toxins cause severe oxidative stress, resulting in inflammation (Assinger et al., 2010). Research has also shown that platelet function is affected by smoking (Barua et al., 2010). Although all cells in the body are influenced by these toxins, and homeostasis is disrupted in general, oxidative stress caused by ROS and inflammation-derived oxidants is able to modulate platelet function (Padmavathi et al., 2010) and therefore the coagulation system. The endothelium, which also plays an important role in haemostasis and in coagulation regulation, becomes activated and induces the intrinsic coagulation pathway.

This results in platelet activation and enhanced platelet aggregation, which in turn causes thrombin stimulation and fibrin formation (Padmavathi et al., 2010). In smokers, researchers have also found an increased platelet count (Roethig et al., 2010; Van Tiel et al., 2002).

In smokers the production of superoxide can occur via the oxidation of hydroquinone (QH_2) which is a constituent of cigarette smoke:

$$QH_2 + O2 \rightarrow Q^{-\bullet} + O_2^{-\bullet} + 2H^+$$

Spontaneous disproportionation of the superoxide radical ion, or catalytic disproportionation in vivo by SOD, generates hydrogen peroxide:

$$O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2$$

In the presence of transition metal ions, H_2O_2 can undergo dispropertionation via the Fenton reaction to generate the powerful hydroxyl radical.

$$H_2O_2 + Fe^{2+} \rightarrow \bullet OH + OH^- + Fe^{3+}$$

Cigarette smoke contains trace amounts of redox-active metals like copper and iron. Transitional metals in the tar of cigarette smoke are notable because of their capacity to promote the formation of the hydroxyl radical via the Fenton reaction in particular Fe^{2+} and Cu^{1+} (Halliwell and Poulsen, 2006; Wooten et al., 2006).

Given the above mentioned information one would suspect that the Fenton reaction is going to occur readily as hydrogen peroxide as well as the required metals are present derived from cigarette smoke.

However it seems as if hydrogen peroxide accumulates in the platelet of smokers, almost as if the Fenton reaction is being inhibited or extremely high concentrations of superoxide results in the excess hydrogen peroxide. Mechanisms exist in the blood plasma to sequester transition metal ions into protein-bound forms however it is unknown if the same mechanisms exist in platelets. Also the Fenton reaction may be dose dependant and the concentrations of the transitional metal may not be optimal for the reaction to occur.

In Figure 4.11 it is clear that there is a large SD in the HE results of smokers, in other words there is a large variation in the values. This may indicate that factors like age and lifestyle e.g. consumption of anti-oxidants through diet, play an important role in the production of ROS and its effect on smokers.

The drastic increase in hydrogen peroxide compared to the control group as well as the stroke patients may be because of the immense oxidative stress cigarette smoke causes in

the vascular system. This increased hydrogen peroxide may be responsible for the chronic activation found in the platelets of smokers.

In conclusion the flow cytometric data obtained in this study shows that the membrane of platelets in both stroke patients and smokers are altered in some way. Similarly both stroke patients and smokers platelets appear to be in an activated state. The production and subsequent reactions of reactive oxygen species appear to be influential in both groups and may likely be a crucial factor in the development of a pro-thrombotic, pro-inflammatory state which may prove to be a hallmark in the pathophysiology of stroke and smoking.

CHAPTER 5: A COMPARISON OF PLATELET ULTRASTRUCTURE IN THROMBO-EMBOLIC ISCHEMIC STROKE AND SMOKERS USING CONFOCAL MICROSCOPY AND SCANNING ELECTRON MICROSCOPY

5.1 CHAPTER OBJECTIVES

This chapter aims to examine the morphology of the platelets found in the blood of stroke patients and smokers compared to healthy individuals using confocal microscopy and scanning electron microscopy (SEM).

5.2 INTRODUCTION

Confocal microscopy is a useful tool as it enables us to study and analyse cells in their living, non-fixed state. In particular Hermann *et al* has developed a technique to assess platelet vitality using confocal fluorescence microscopy (Hermann et al., 2010). SEM has been well established in the use of evaluating platelets in various disease conditions including thrombo-embolic stroke and in people who smoke (Pretorius, 2012; Pretorius et al., 2012).

Resting platelets exhibit a smooth tightly-packed discoid shape. On activation at sites of vascular injury they undergo rapid activation and dramatic shape change, becoming spherical, extending pseudopodia and spreading, and recruiting other incoming platelets from the blood stream to form a haemostatic plug at the injured site.

The cell shape changes from flat discs of ± 2.5 µm in diameter to the fully spread platelets of ± 8–10 µm in diameter. The final extensive spreading enables the platelet to flatten over the damaged area and ensures firm adhesion to an irreversible mode and is essential in generating a greater thrombogenic base that favours the capture of adhesive proteins and platelets in a flowing system, leading to platelet aggregate accumulation on the surface.

During their activation a drastic reorganization of their cytoskeleton, secretion of intracellular granules contents, expression of the receptors for fibrinogen and coagulation factors, and aggregation occur (Jen et al., 1996; Kukharenko et al., 2006; Shiraki et al., 2004).

5.3 MATERIALS AND METHODS

Similar to the methods in chapter 3 blood will be collected from the 3 groups, here it is necessary to obtain whole blood samples in citrate tubes.

5.3.1 Confocal Microscopy

The blood obtained in the citrate tube from stroke patients, smokers and control subjects was centrifuged at 6500 rpm for 8 minutes to obtain platelet rich plasma (PRP). The PRP was then transferred to an eppendorf tube and centrifuged at 8000 rpm for 8 minutes to obtain a platelet pellet.

The pellet was resuspended in 10µl of the residual PRP using a vortex. 20µl CD41-FITC and 1µl HE was then added to the sample and incubated at room temperature in the dark for 20 min. These are the same probes that were used for flow cytometry.

5µl of the sample was then mounted on a glass slide and covered with a coverslip. The sample was viewed using a Zeiss LSM 510 META confocal microscope with a Plan-Apochromat 63x/1.4 Oil DIC objective. The following filters were be used: Ch3-1: BP 505-550 and Ch3-2: LP 560 and wavelengths of 488 nm and 514 nm.

A fair amount of technique development had to be done here as a protocol did not exist using these probes together in this manner. I firstly experimented with a few of the flow cytometry probes, including CD41-PE, CD41-FITC, HE and DCFDA. It was found that using CD41-PE and DCFDA proved ineffective as the hydrogen peroxide measured by DCFDA was not localized within the platelets, therefore it was not possible to view the samples in this way. However HE and CD41-FITC proved useful as the product of HE is localized within the DNA of the mitochondria of the platelets and CD41-FITC enabled me to visualize the platelet membrane.

Furthermore I experimented with various fluids to resuspend the platelet pellet in. Here I used sheath fluid used in flow cytometry as well as phosphate buffered saline but I found that using the residual plasma left after centrifugation proved to be optimal. Regarding the plasma I also had to determine how much to add to the platelet pellet for optimal visualisation of the platelets and after many experiments this was found to be a very small amount of 10 µl.

Lastly I had to determine how much of the platelet probe mixture had to be put on the glass slide as this also seemed to have an effect on visualization. This was found to be the small amount of 5µl.

5.3.2 Scanning Electron Microscopy (SEM)

On day of admission, 5ml of blood was drawn from each stroke patient in a citrate tube; the same was done for smokers and healthy control subjects. The blood was then centrifuged at 6500 rpm for 8 minutes to obtain PRP.

20µl of the PRP was transferred directly to a 0.2 m 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 phosphate buffered saline (PBS) to create a humid environment and placed at 37ºC for 10 minutes. Following incubation the millipore membranes with the coagula was placed in PBS and placed on a shaker 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 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 serially 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.

5.4 Results

CD41-FITC results in the green colour seen in the figures, showing the platelet membrane. HE stains the DNA of cells red, therefore in this case since platelets lack a nucleus the DNA found in the mitochondria of platelets will be stained red. HE is a superoxide marker, thus the superoxide localized in the mitochondria of platelets will be stained.

Table 5.1. Comparison of confocal images of control subjects, stroke patients and smokers.

	Control	**Stroke**	**Smokers**
Clot Formation	Yes, loosely arranged clot (Fig 5.12 & 5.13)	Yes, tight clot (Fig 5.8 & 5.9)	Yes, tight clot (Fig 5.10 & 5.11)
Platelet Shape	Spherical	Irregular with few Spherical (Fig 5.2)	Irregular, but some are Spherical (Fig 5.18)
Platelet Spreading	No	Yes, prominent (Fig 5.2, 5.5 & 5.14)	Yes, less prominent (Fig 5.6, 5.15 & 5.21)
Pseudopodia	Few (Fig 5.7, 5.12, & 5.13)	Yes (Fig 5.2)	Yes, most prominent (Fig 5.3 & 5.22)
Smooth membrane	Overall Yes (Fig 5.1)	Overall No (Fig 5.2)	Overall No (Fig 5.3)
Platelet-Platelet interaction and membrane fusion	Slight fusion of Spherical platelets and interaction (Fig 5.4)	Prominent interaction and fusion (Fig 5.14)	Prominent interaction and fusion (Fig 5.11)
HE (Superoxide) present	Superoxide in platelet membrane and inside platelets (Fig 5.1)	Prominent, on membrane and inside platelets (Fig 5.14)	Prominent on membranes and inside platelets (Fig 5.6)
HE (Superoxide) Intensity	Low	High, specifically in clots (Fig 5.8)	High, specifically in clots (Fig 5.10)

Table 5.2. List of diagram labels.

A	Focus point on platelet membrane
B	Spherical platelet
C	HE localized in the platelet membrane
D	HE localized within the platelet
E	Pseudopodia
F	Platelet spreading
G	Irregular shaped platelet
H	Platelet-Platelet interaction
J	Platelet aggregate/clot formation

Figure 5.1. Control subject platelets. A: Focus on level of platelet membrane, B: Spherical platelet, C: HE/Superoxide localized in the mitochondria in the platelet membrane, D: HE/Superoxide localized in the mitochondria inside the platelet.

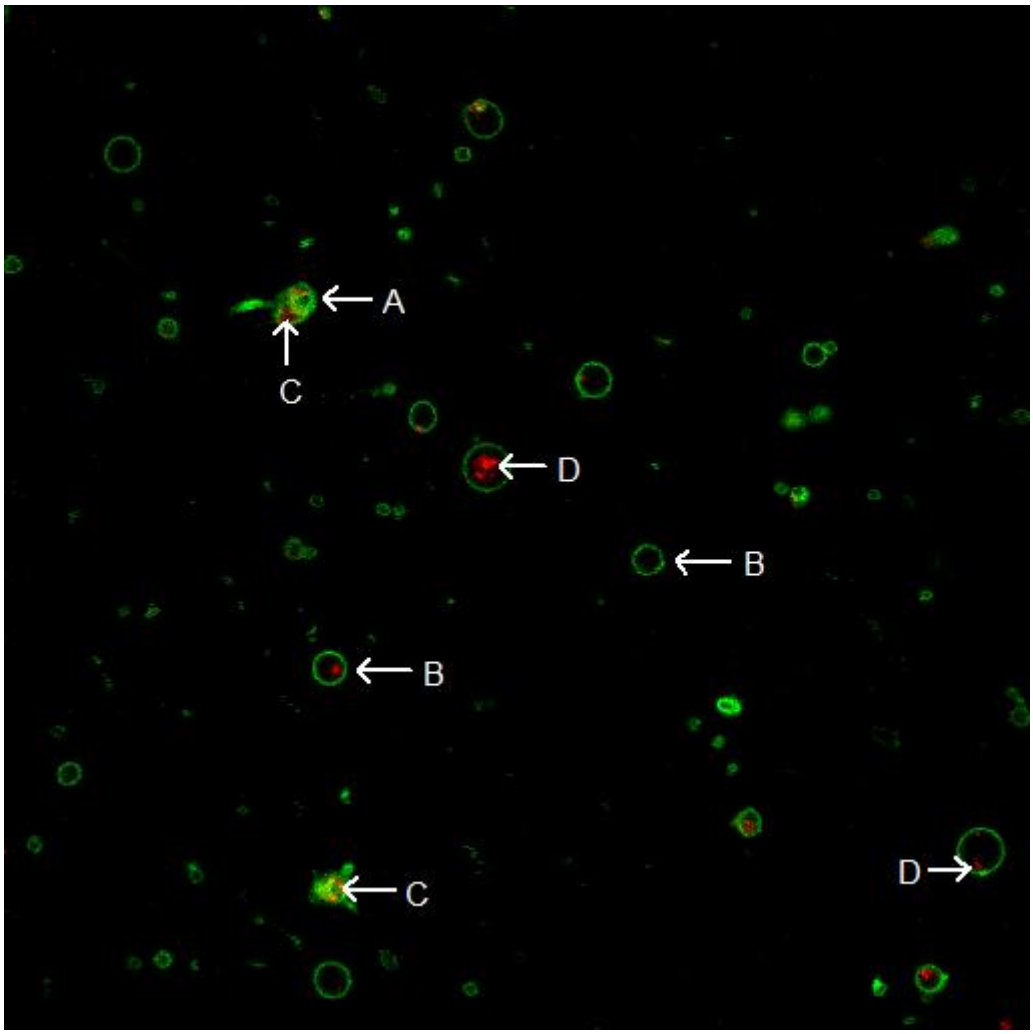


Figure 5.2. Stroke patient's platelets. B: Spherical platelet, C: HE/Superoxide localized in the mitochondria in the platelet membrane, E: Pseudopodia, F: Platelet spreading, G: Irregular shaped platelets, H: Platelet-Platelet interaction.

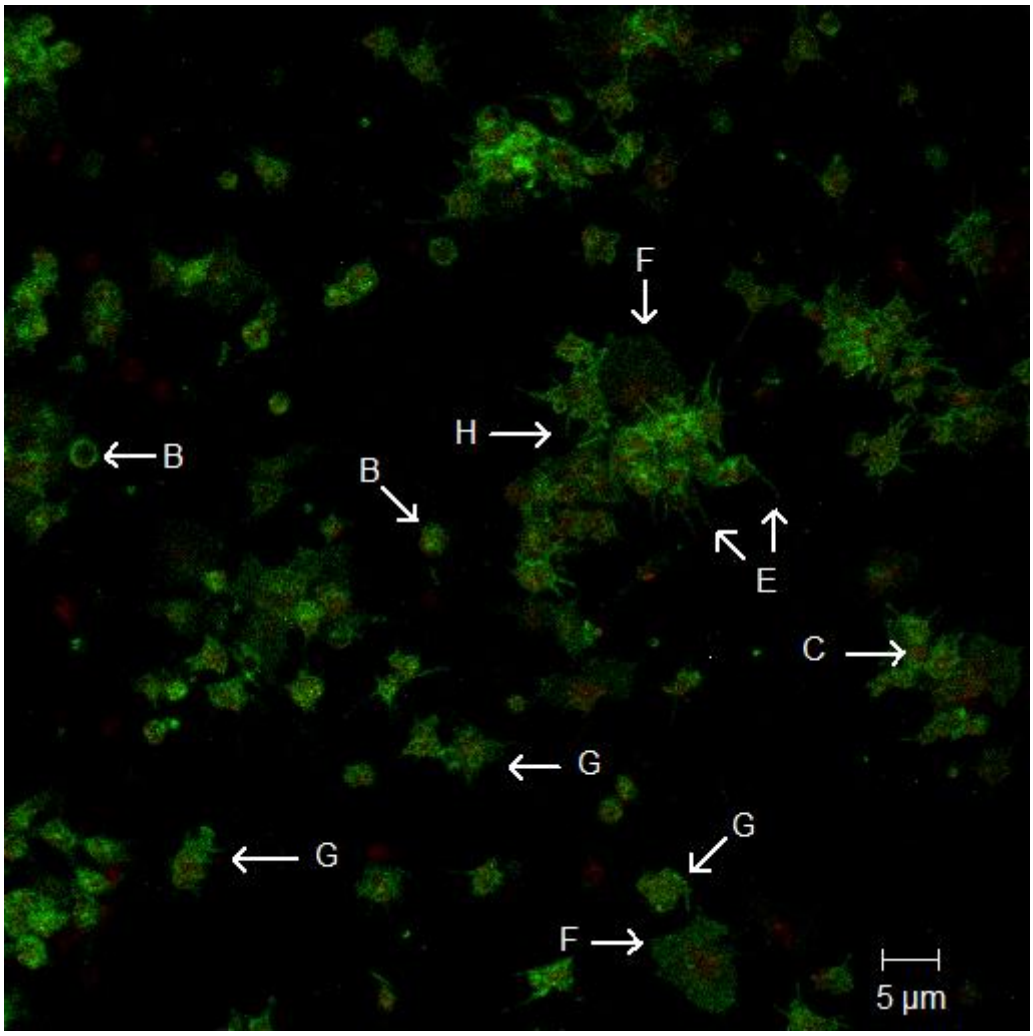


Figure 5.3. Smoker's platelets. C: HE/Superoxide localized in the mitochondria in the platelet membrane, E: Pseudopodia, G: Irregular shaped platelets.

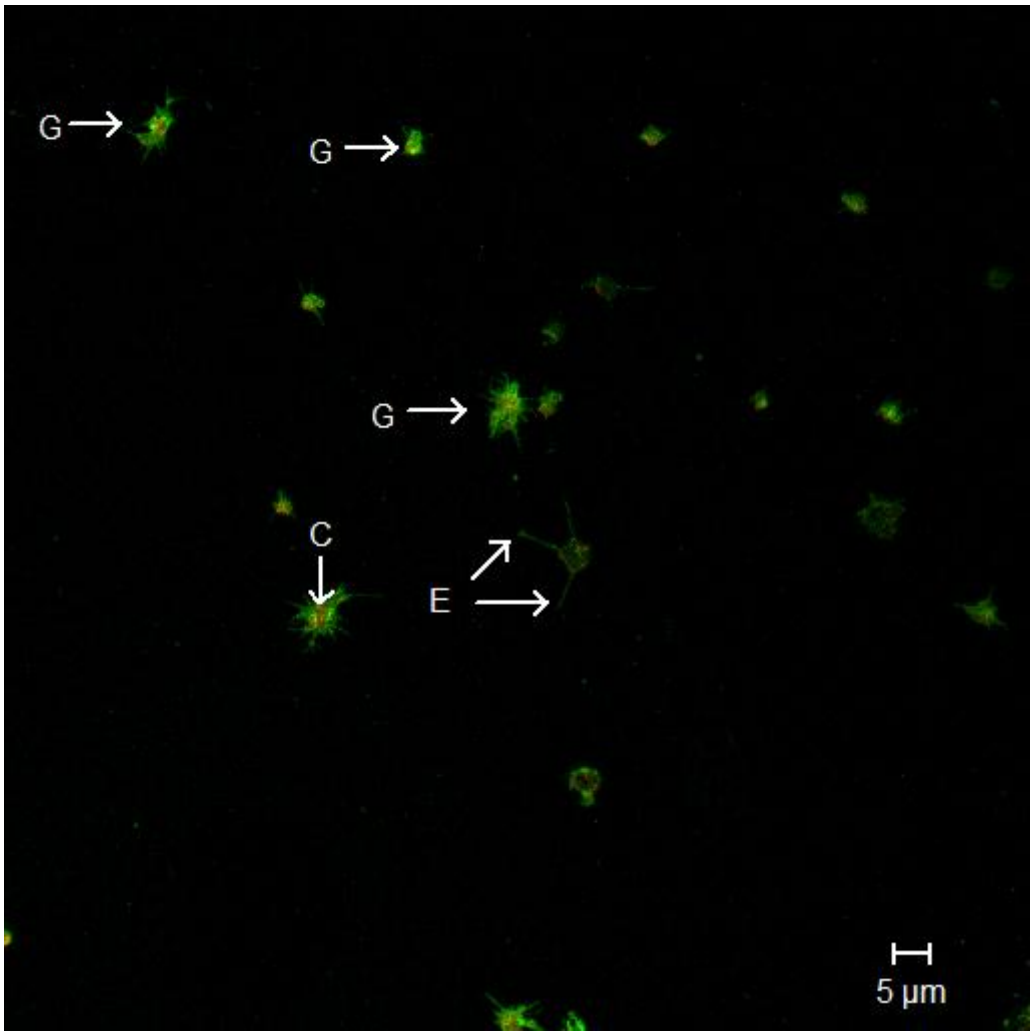


Figure 5.4. Control subject platelets. B: Spherical platelet, D: HE/Superoxide localized in the mitochondria inside the platelet, H: Platelet-Platelet interaction.

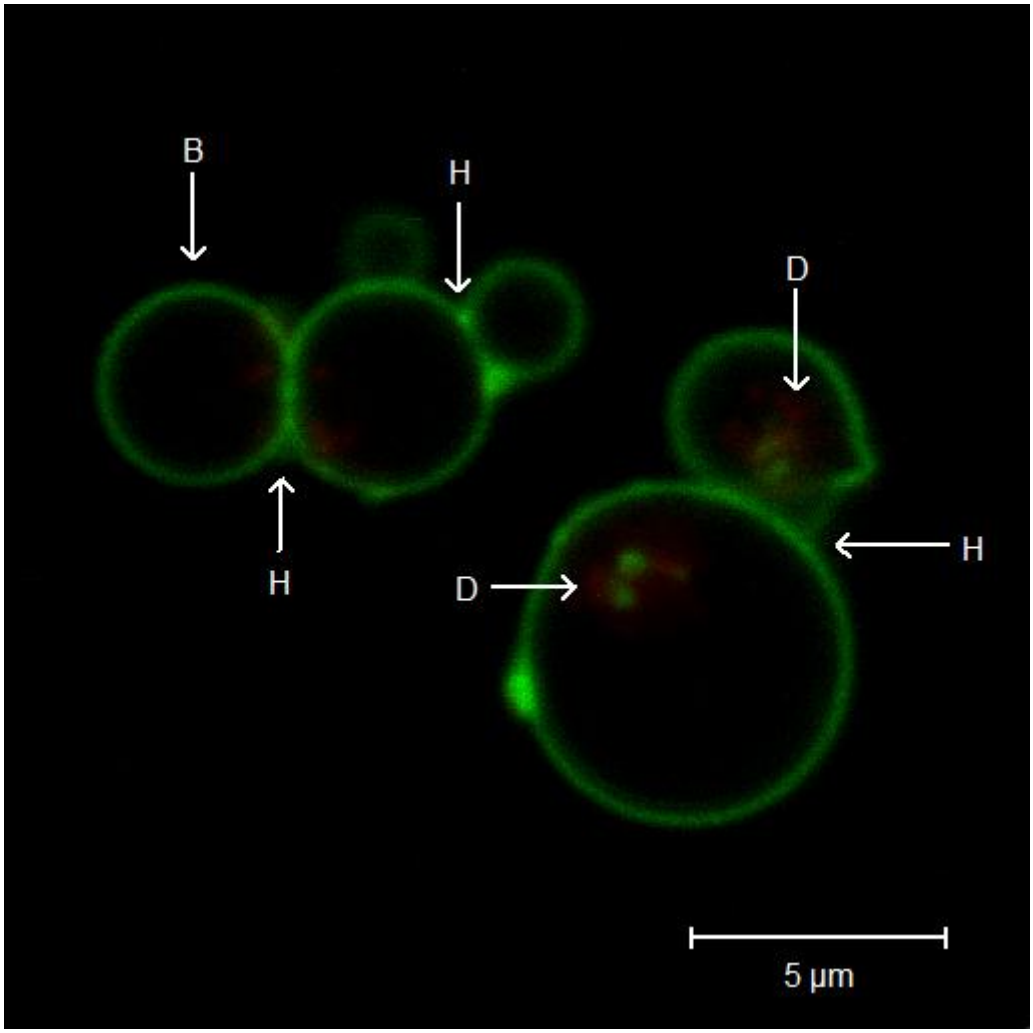


Figure 5.5. Stroke patient's platelets. F: Platelet spreading.

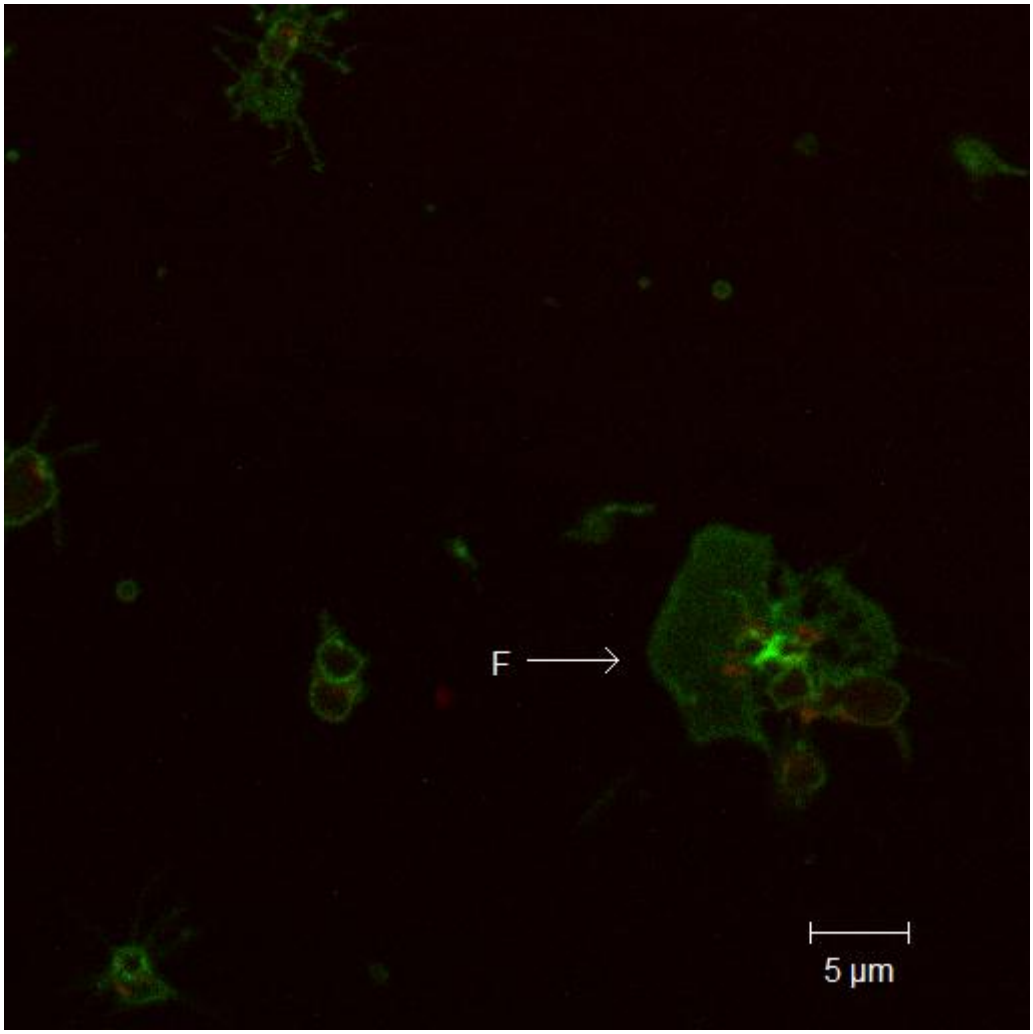


Figure 5.6. Smoker's platelets. B: Spherical platelet, C: HE/Superoxide localized in the mitochondria in the platelet membrane, D: HE/Superoxide localized in the mitochondria inside the platelet, E: Pseudopodia, F: Platelet spreading, H: Platelet-Platelet interaction, J: Platelet aggregation/clot formation.

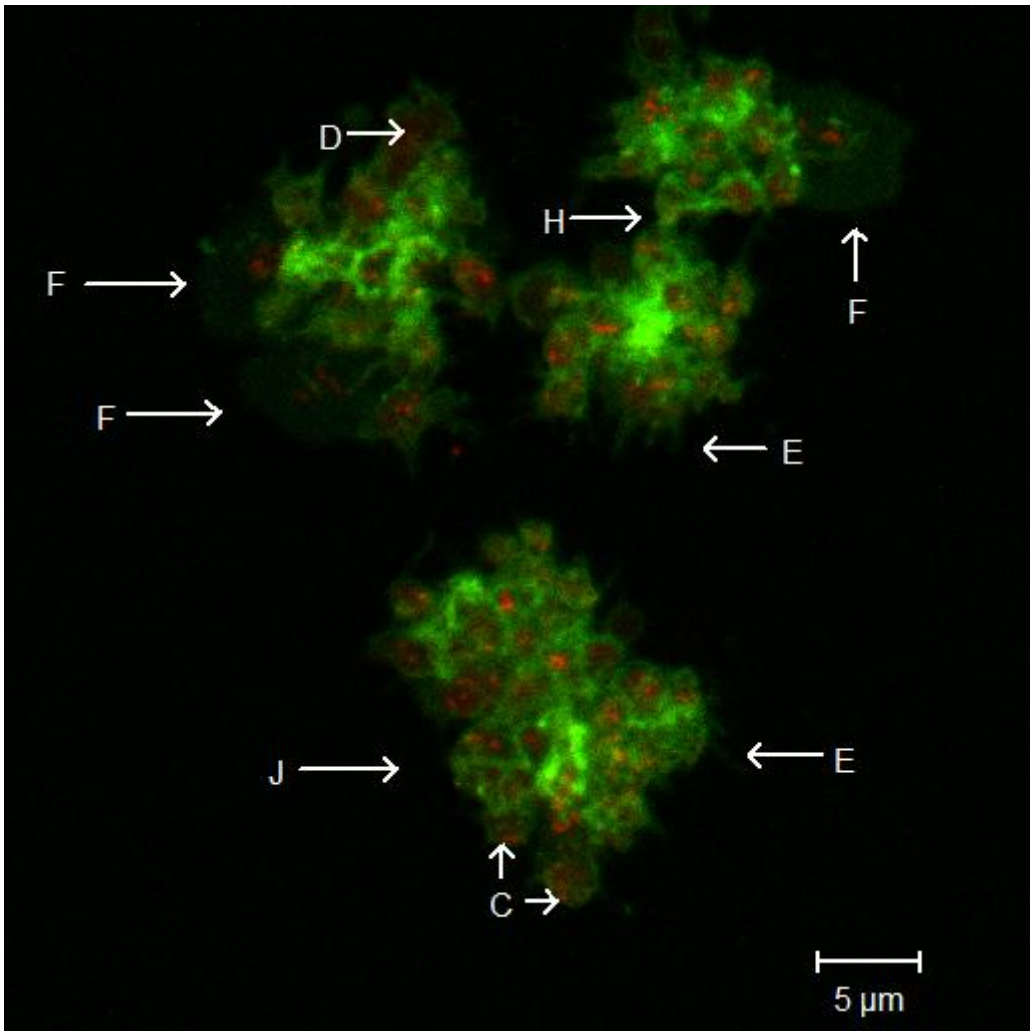


Figure 5.7. Control subject platelets. B: Spherical platelet, D: HE/Superoxide localized in the mitochondria inside the platelet, E: Pseudopodia, H: Platelet-Platelet interaction, G: Irregular shape.

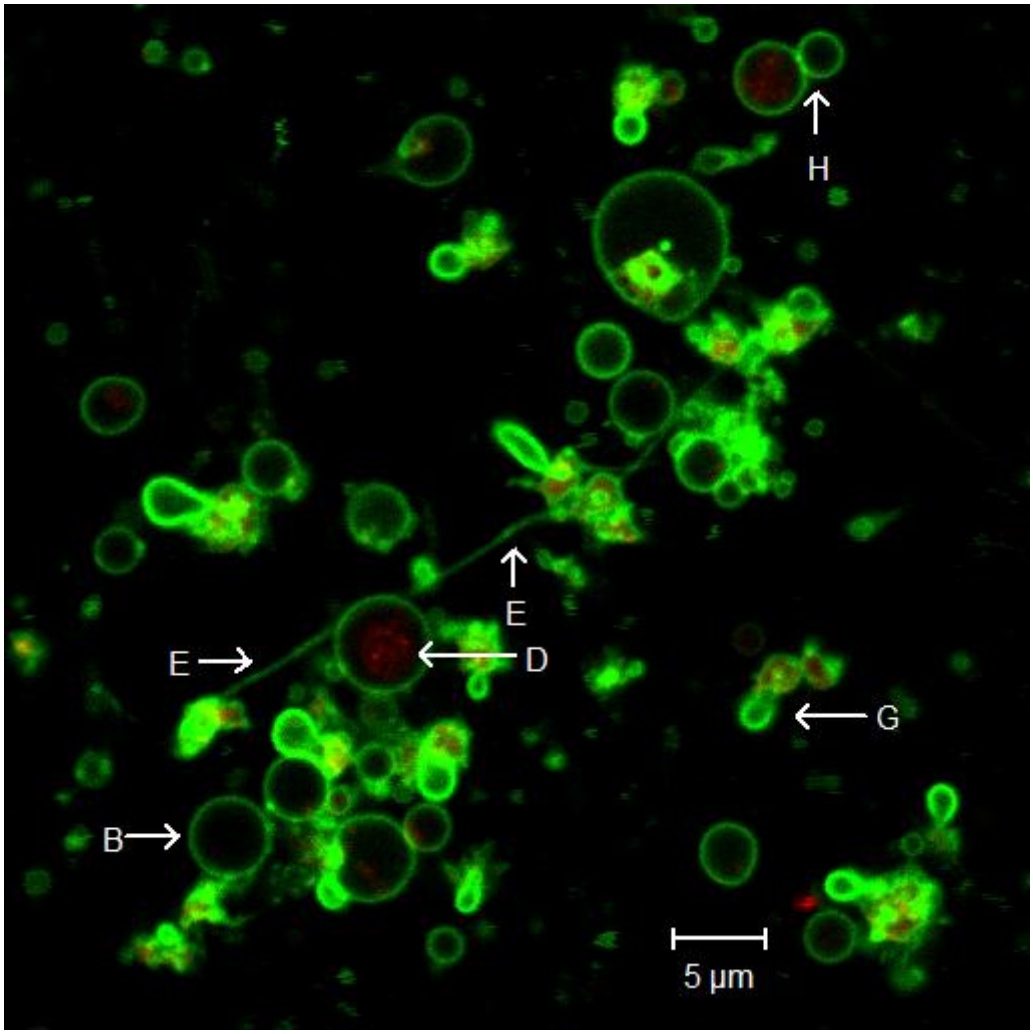


Figure 5.8. Stroke patient's platelets. Tight/Dense clot formation with apparent platelet membrane fusion and aggregation of HE/Superoxide in the centre of each clot. Arrows indicating platelets migrating toward the clot and interacting with other platelets.

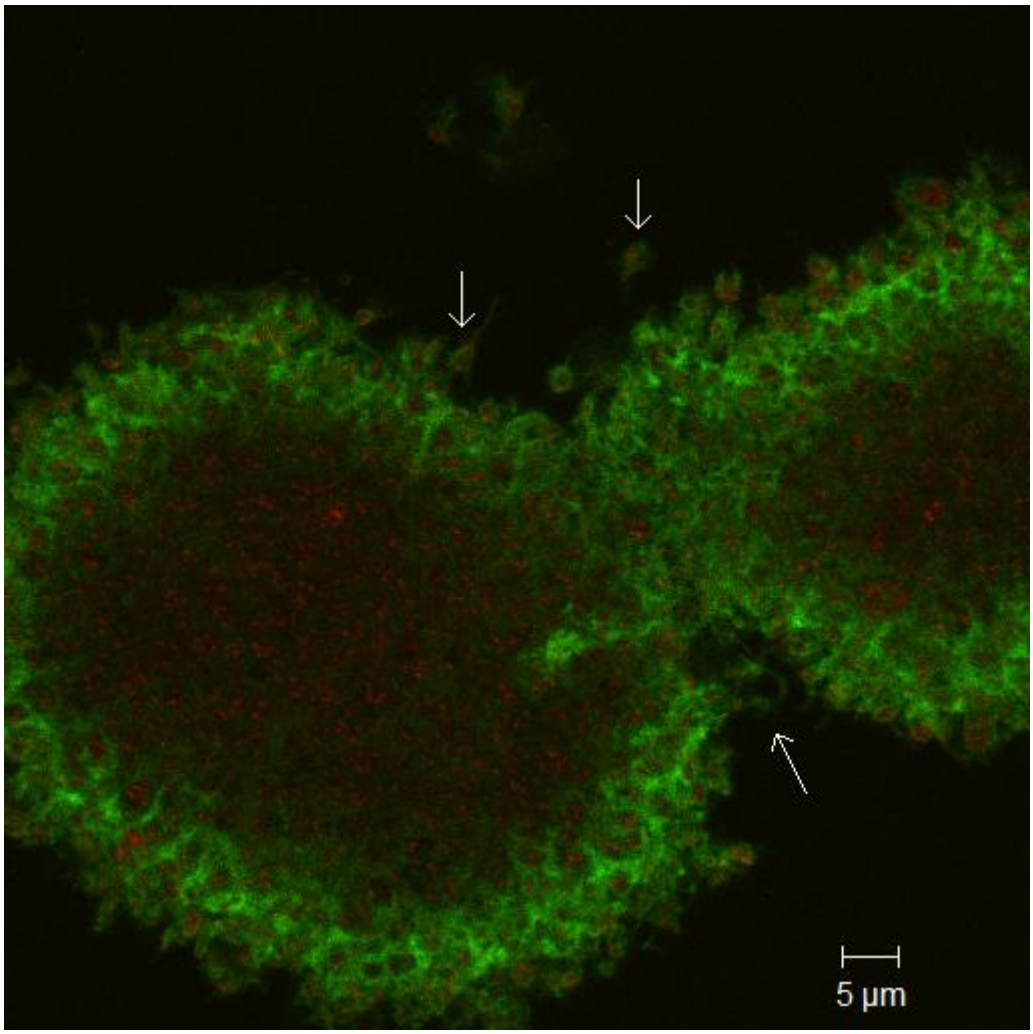


Figure 5.9. Stroke patient's platelets. Tight/Dense clot formation with apparent membrane fusion and spreading of HE/Superoxide throughout, the arrow indicates platelet-platelet interaction

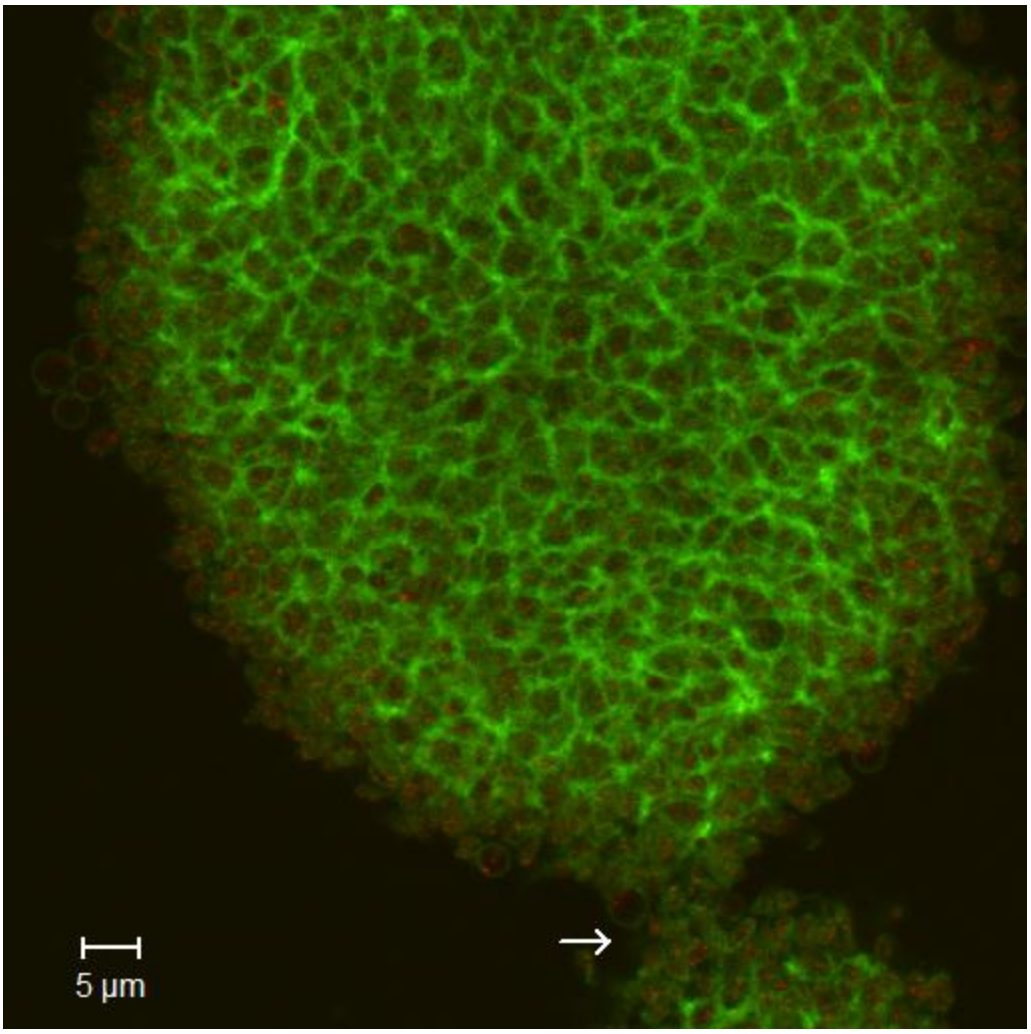


Figure 5.10. Smoker's platelets. Tight/Dense clot formation with apparent platelet membrane fusion and aggregation of HE/Superoxide in the centre of the clot as indicated by the arrow.

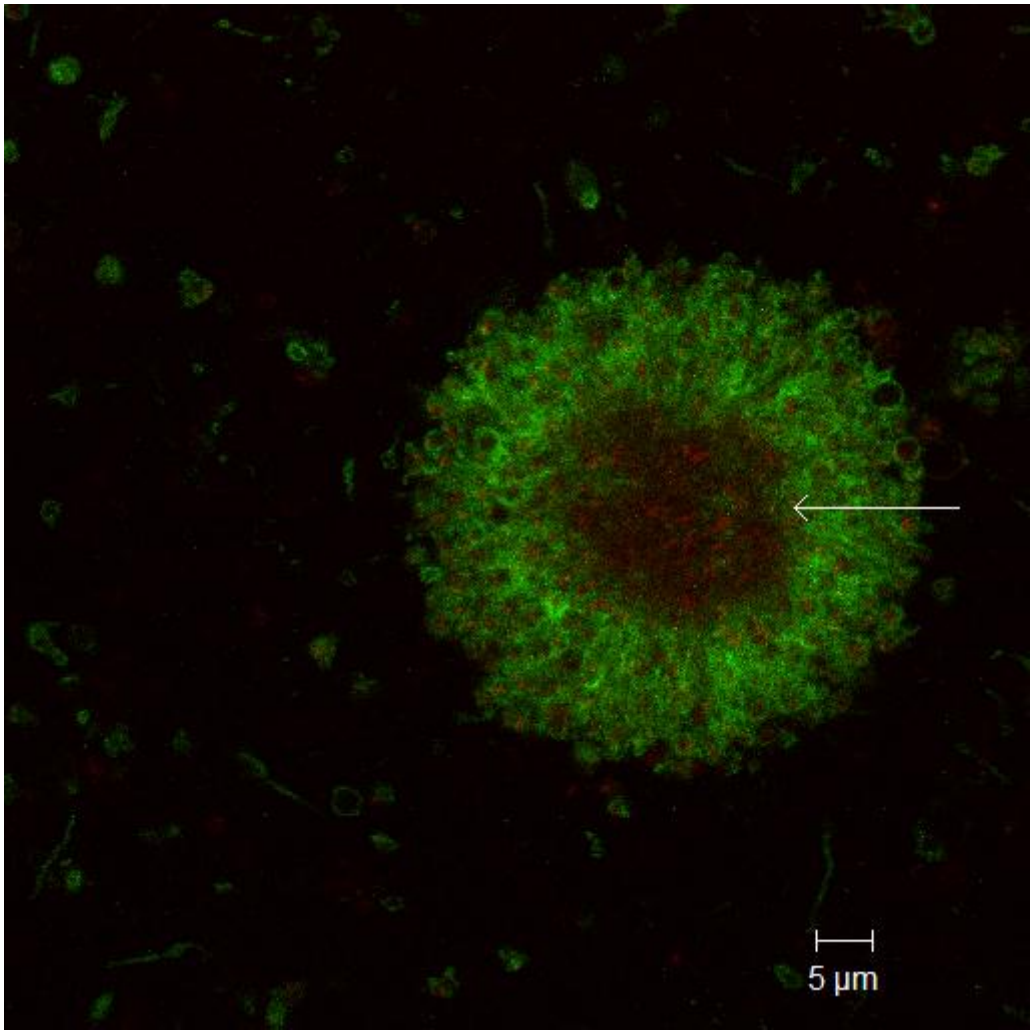


Figure 5.11. Smoker's platelets. Tight/Dense clot formation with apparent platelet membrane fusion indicated by the arrow and HE/Superoxide distributed throughout.

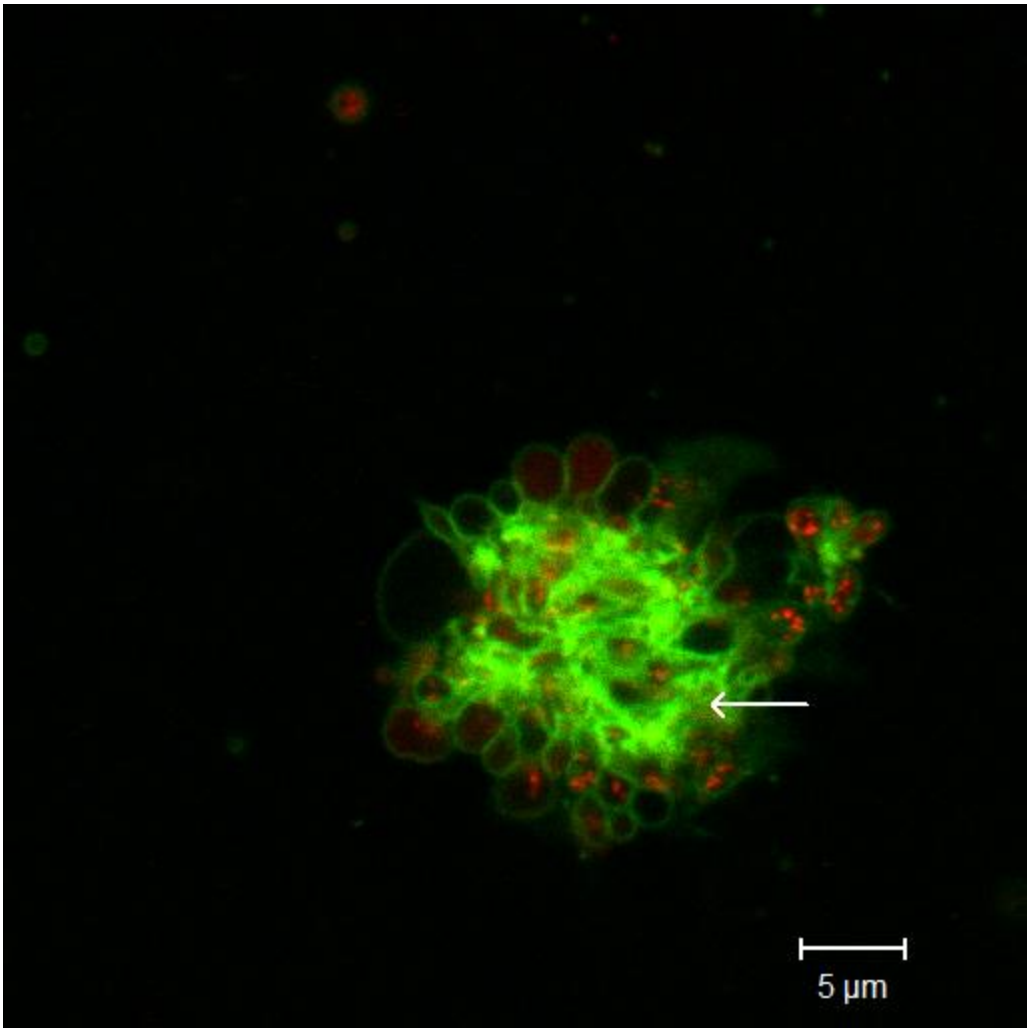


Figure 5.12. Control subject platelets. Loose clot formation with platelets mostly remaining in the Spherical shape, E representing formation of pseudopodia possibly to anchor the clot.

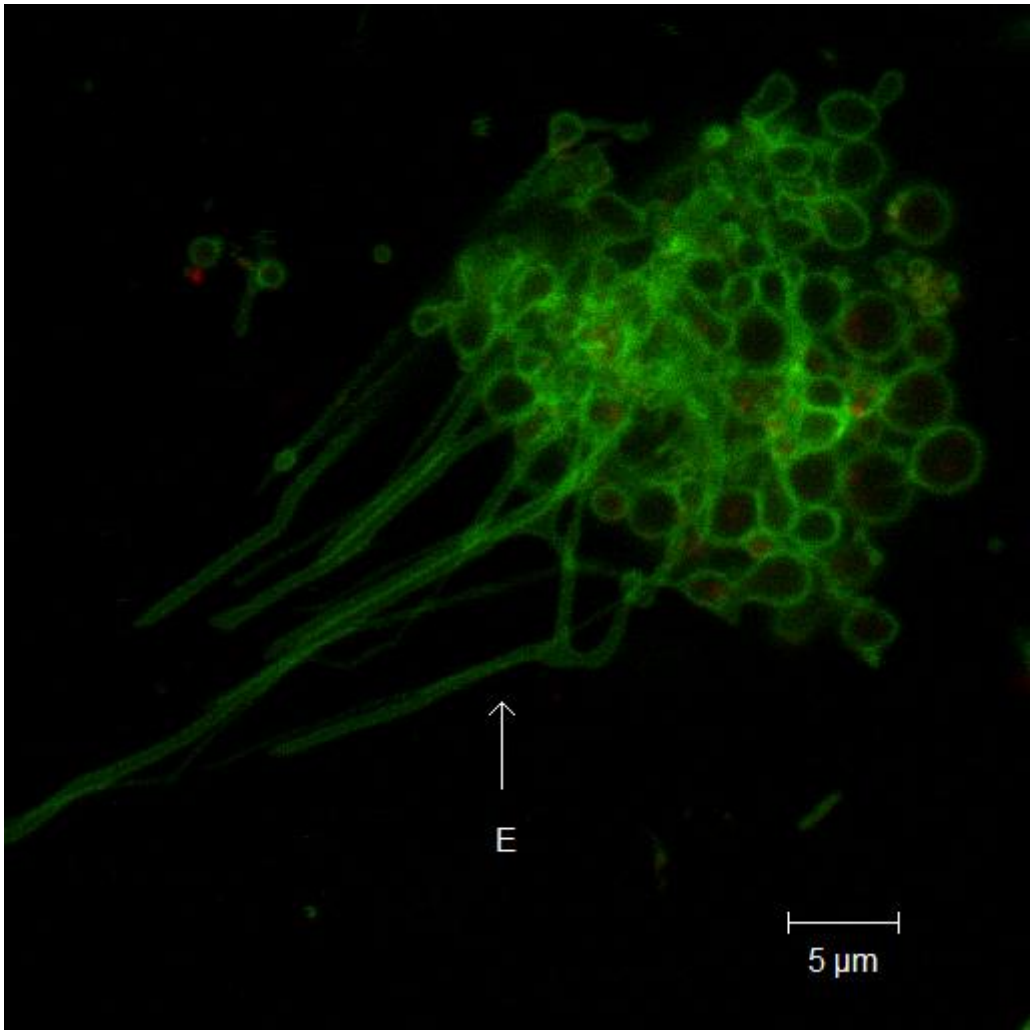


Figure 5.13. Control subject platelets. Loose clot formation with platelets mostly remaining in the Spherical shape,

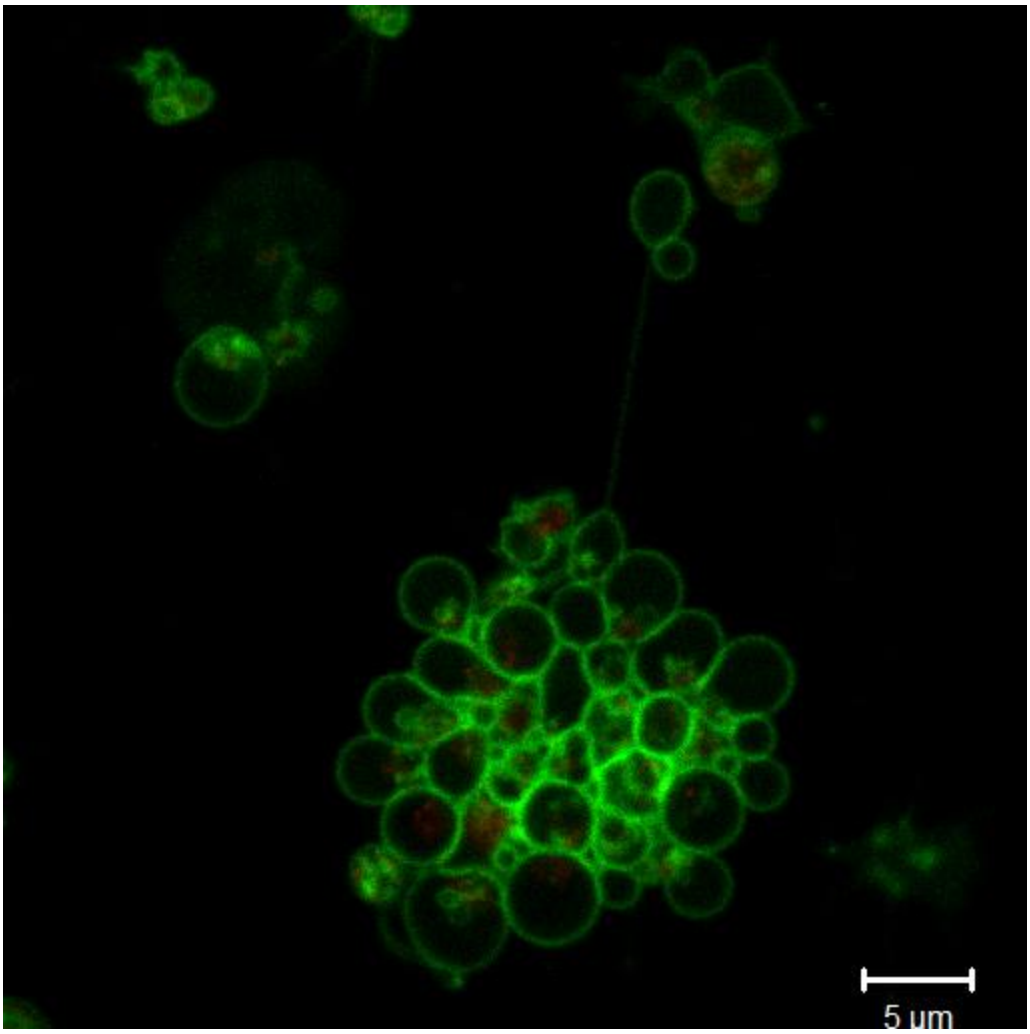


Figure 5.14. Stroke patient's platelets. C: HE/Superoxide localized in the mitochondria in the platelet membrane, D: HE/Superoxide localized in the mitochondria inside the platelet, E: Pseudopodia, F: Platelet spreading, H: Platelet-Platelet interaction with membrane fusion.

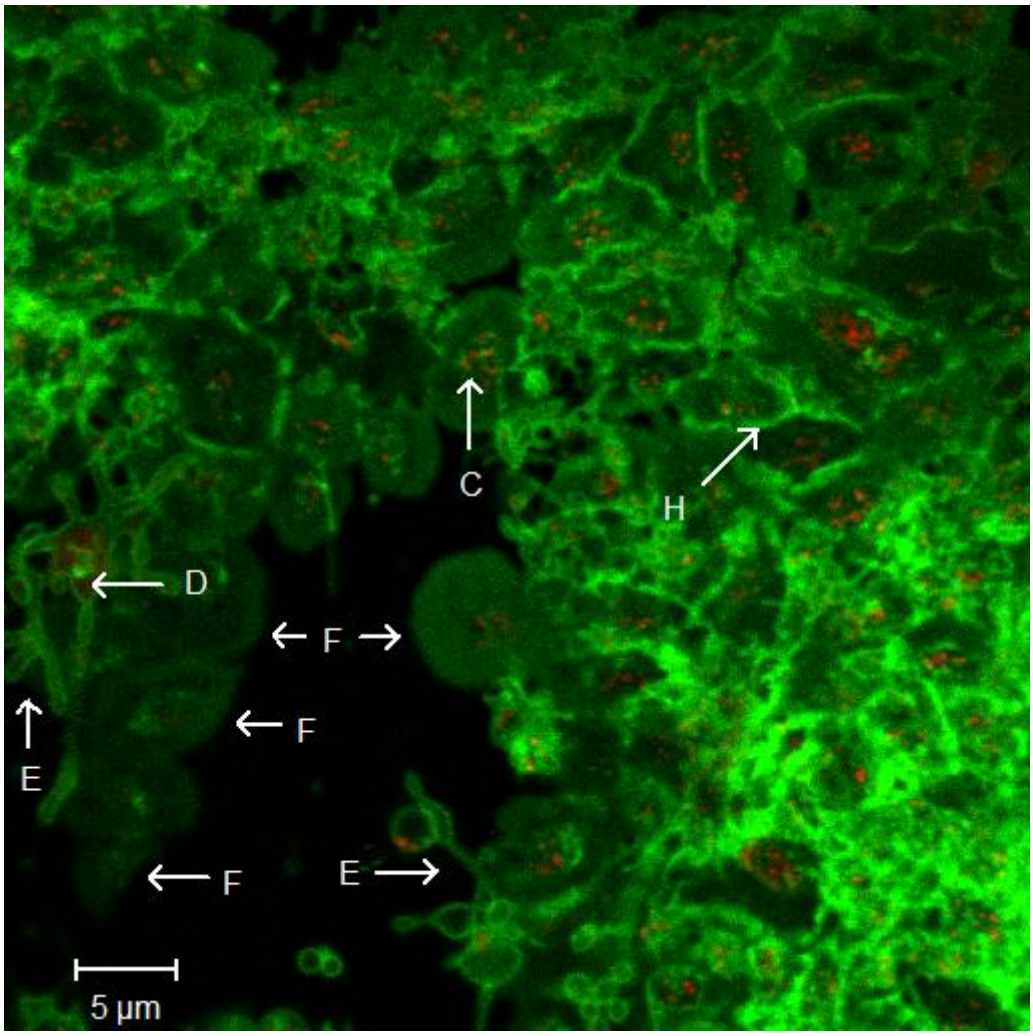


Figure 5.15. Smoker's platelets showing Spreading (F).

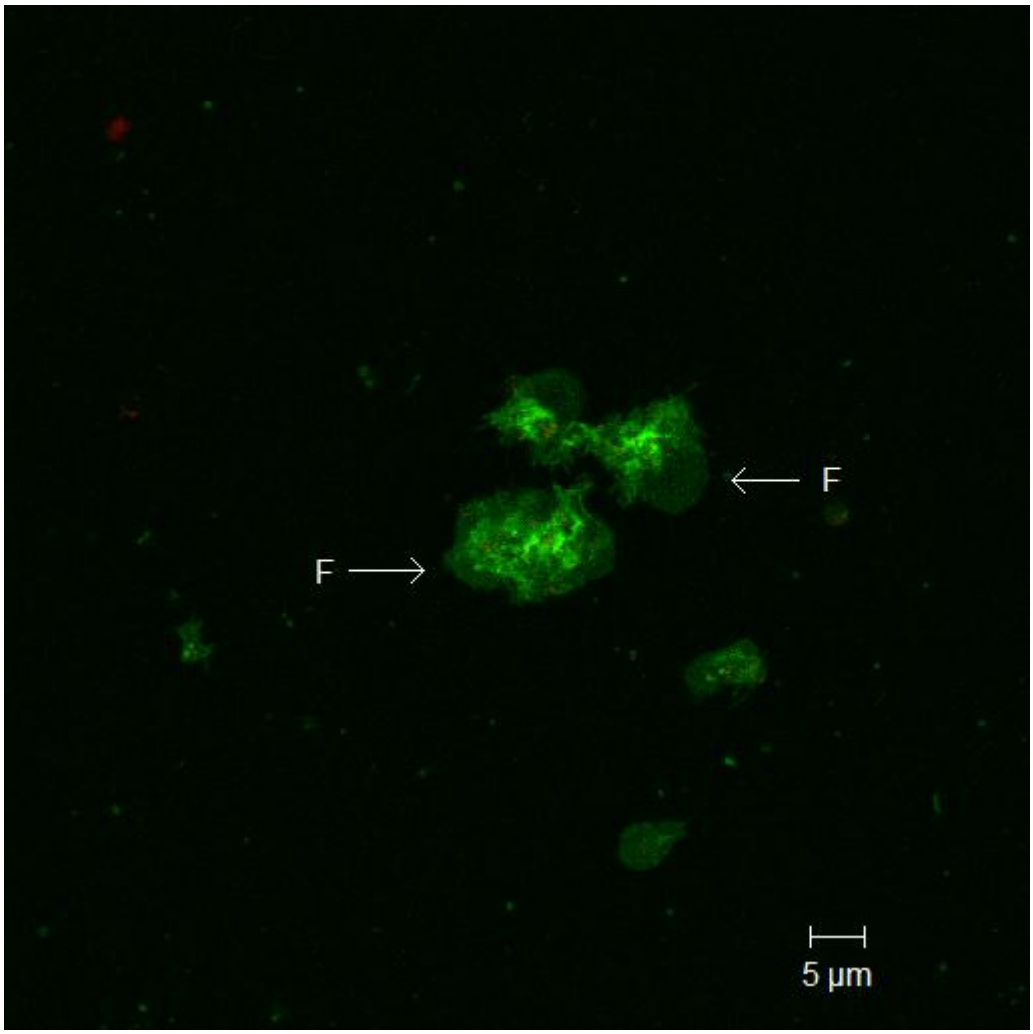


Figure 5.16. Control subject platelets with arrows indication the beginning of the formation of pseudopodia.

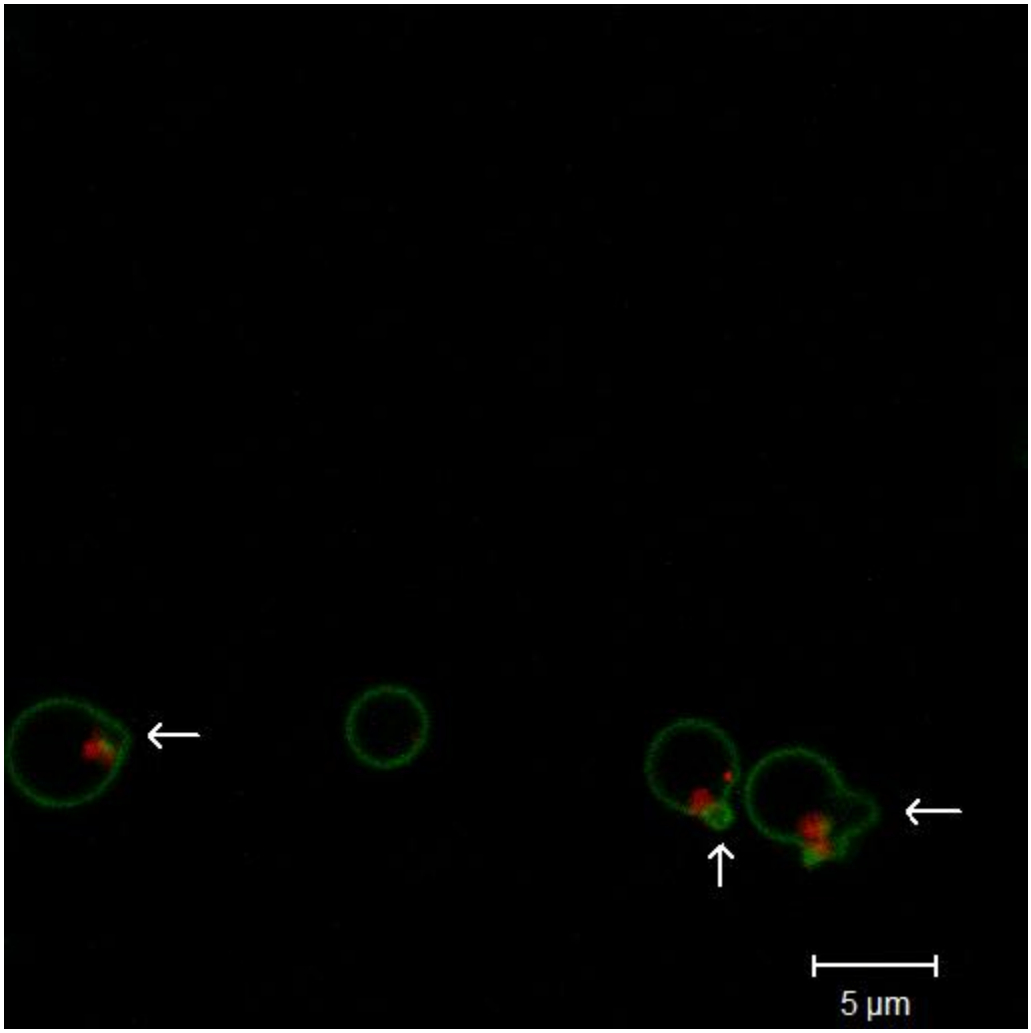


Figure 5.17. Stroke patient's platelet. Beginning of formation of pseudopodia with HE/Superoxide migrating to the membrane.

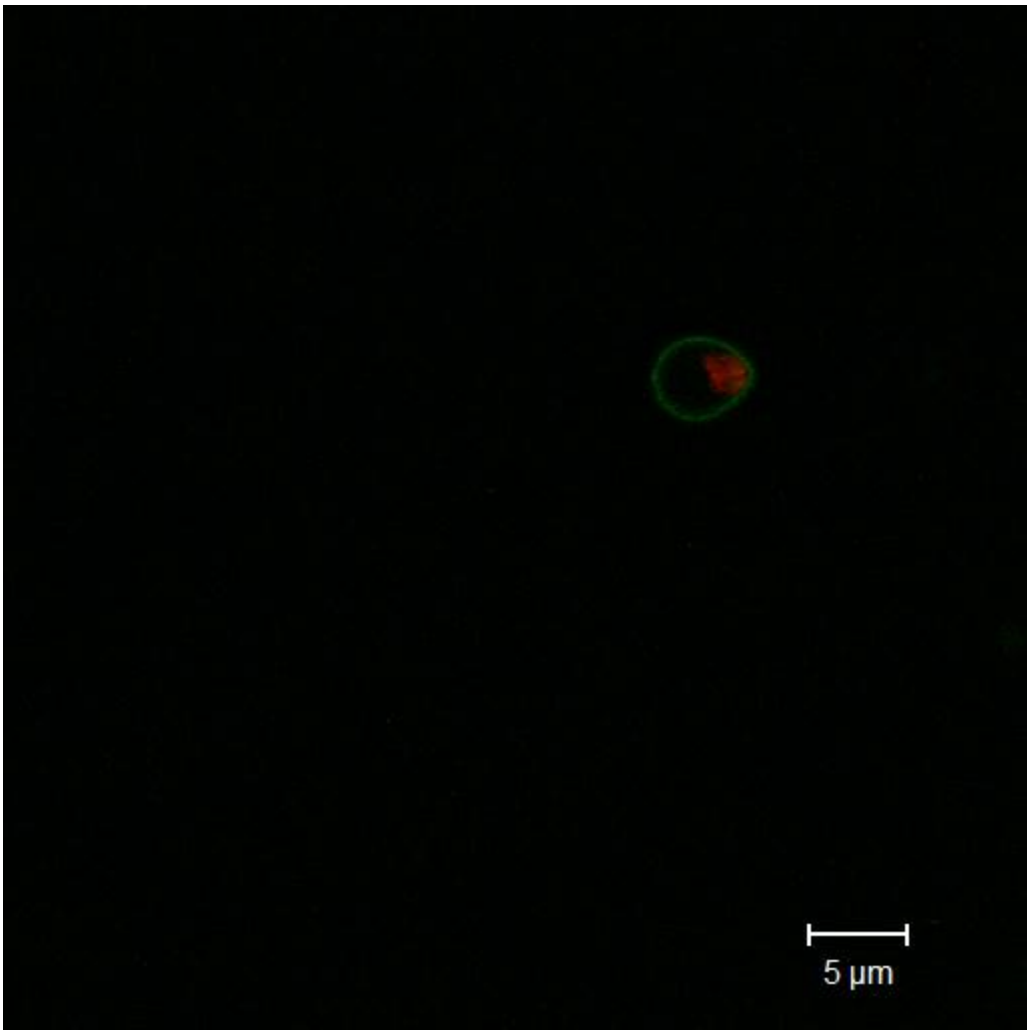


Figure 5.18. Platelets of a smoker with initiation of formation of pseudopodia.

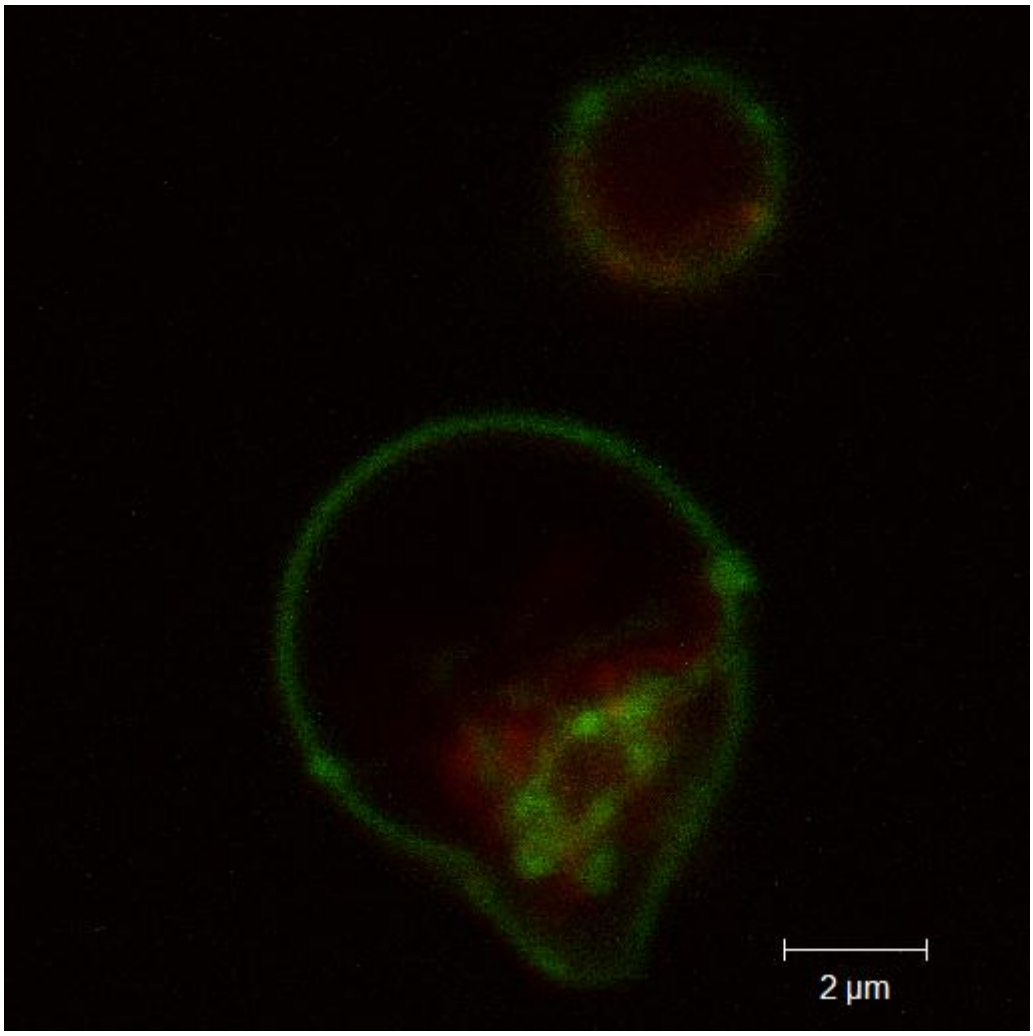


Figure 5.19. Control subject platelet showing pseudopodia. B: Spherical platelet, E: Pseudopodia.

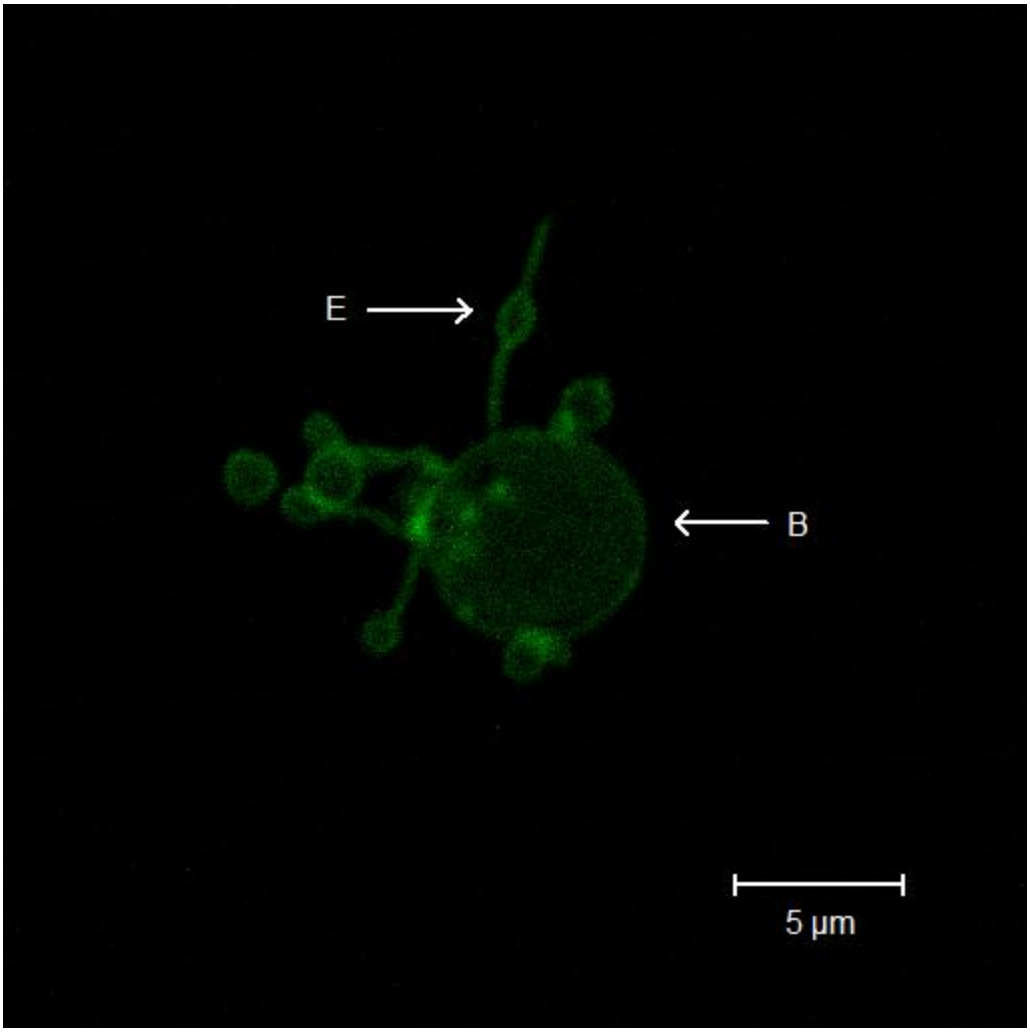


Figure 5.20. Platelets of a stroke patient, the majority of platelets appear to have an irregular shape and HE/Superoxide localized within.

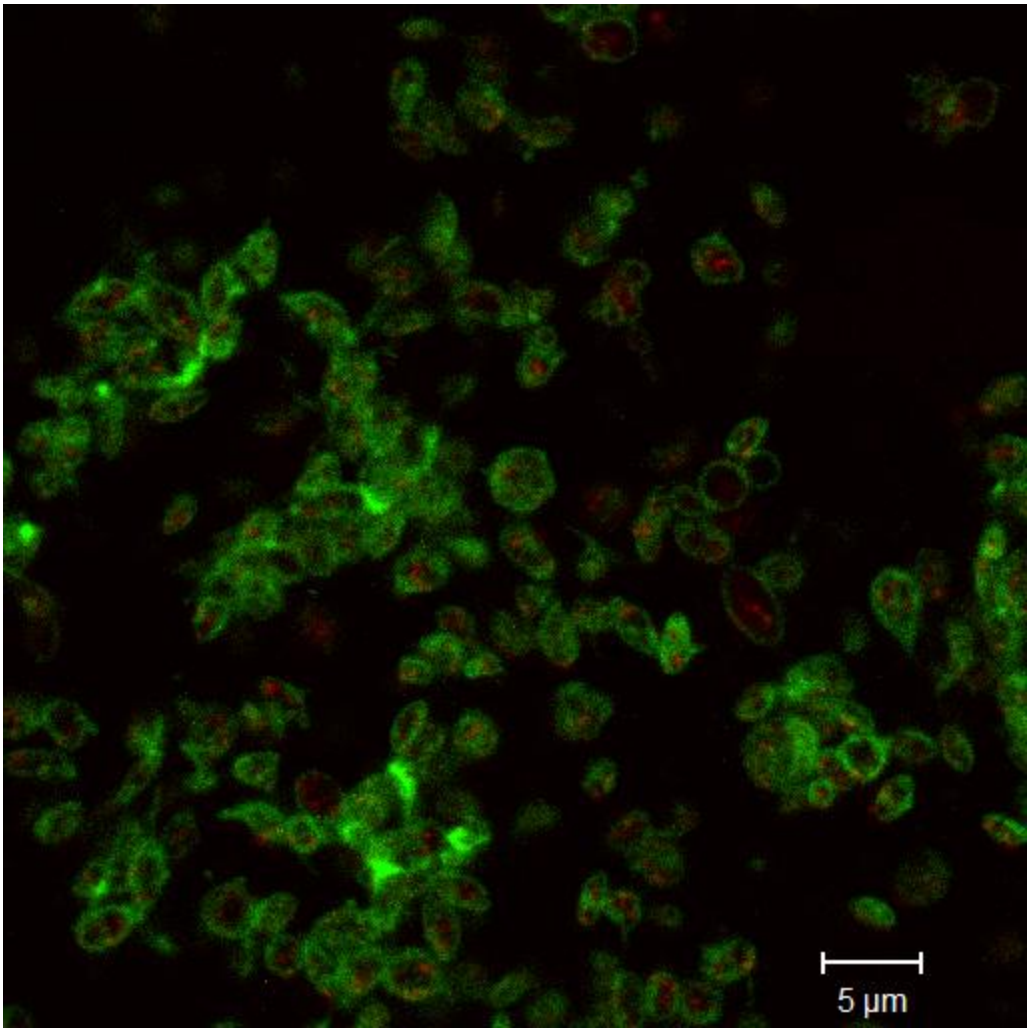


Figure 5.21. Smoker's platelets showing spreading (F).

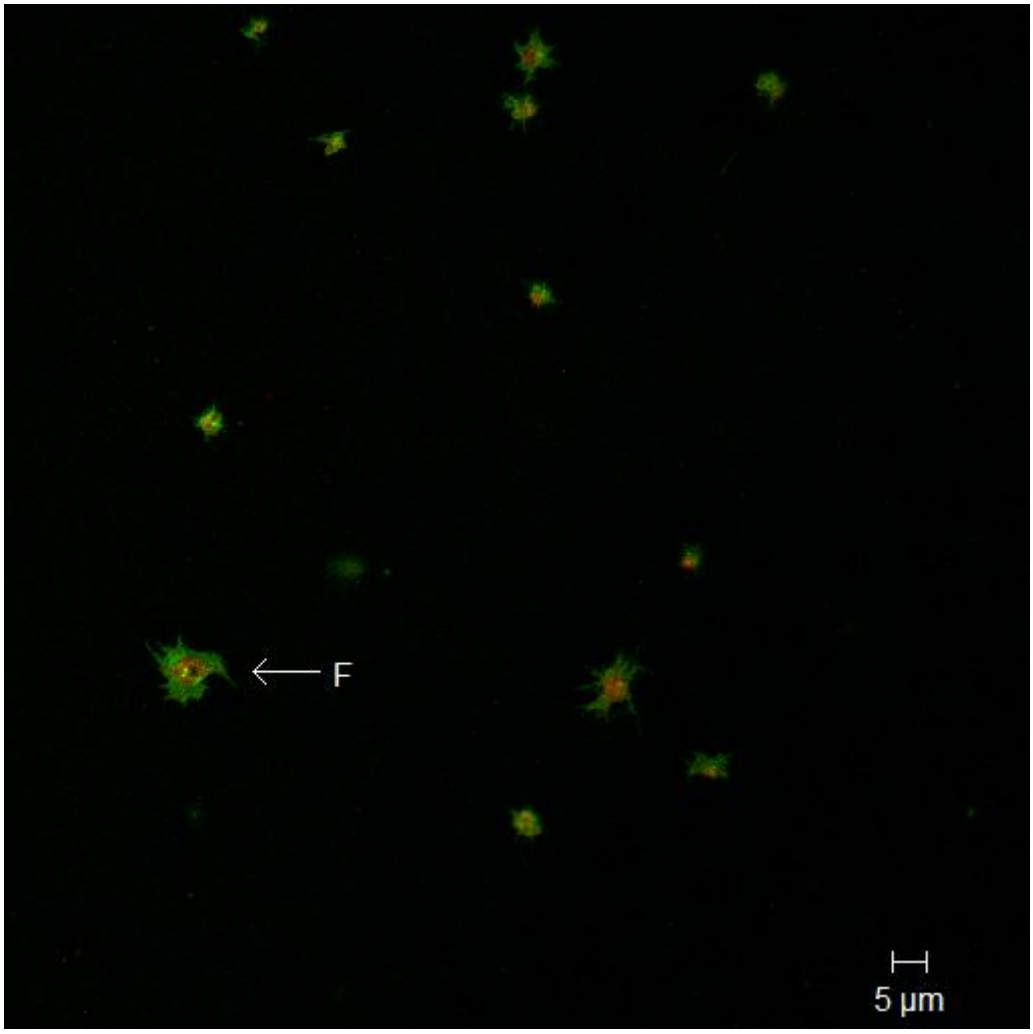


Figure 5.22. Smoker's platelets with activated platelets presenting with extensive pseudopodia almost covering the entire membrane of the platelets. High consentreations of HE/Superoxide present as the red fluorescence.

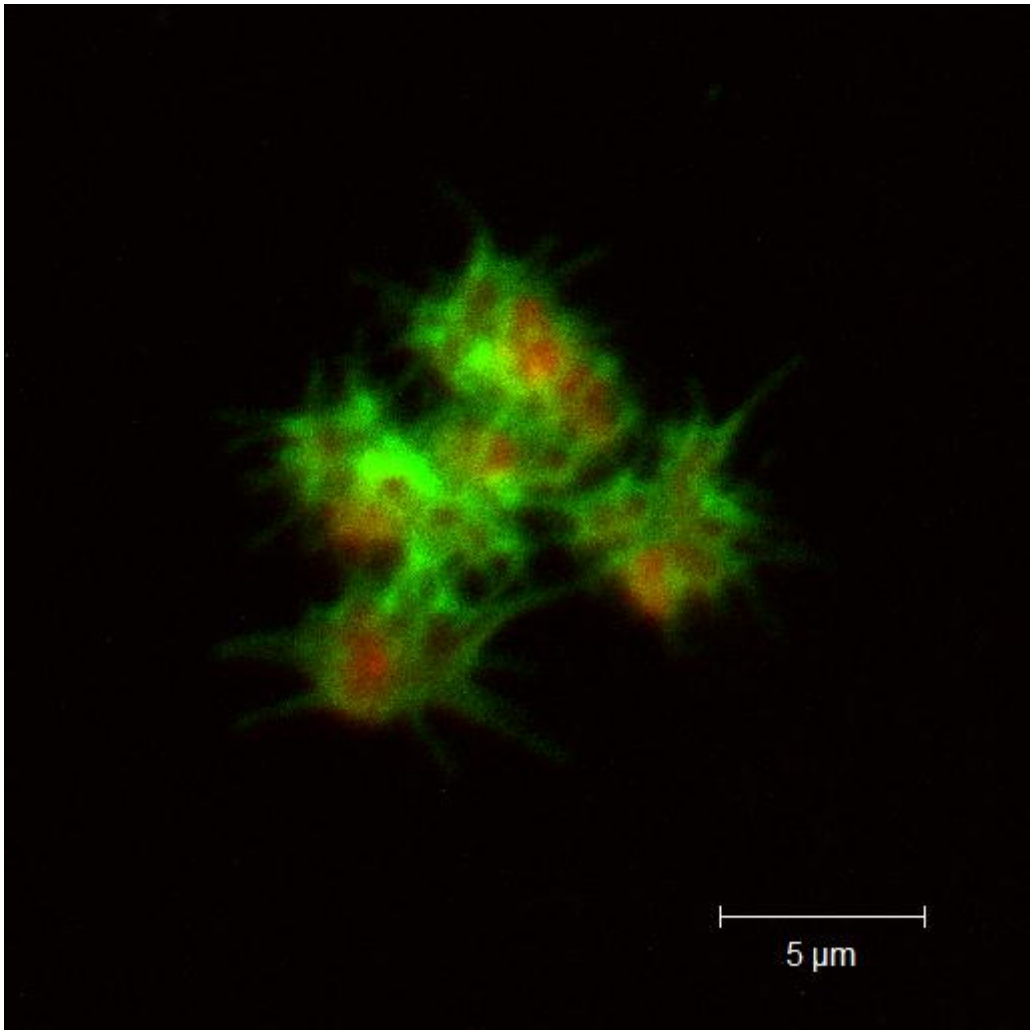


Figure 5.23. Control subject platelets. The red objects may be a kind of white blood cell's nucleus, therefore showing platelet interaction with white blood cells.

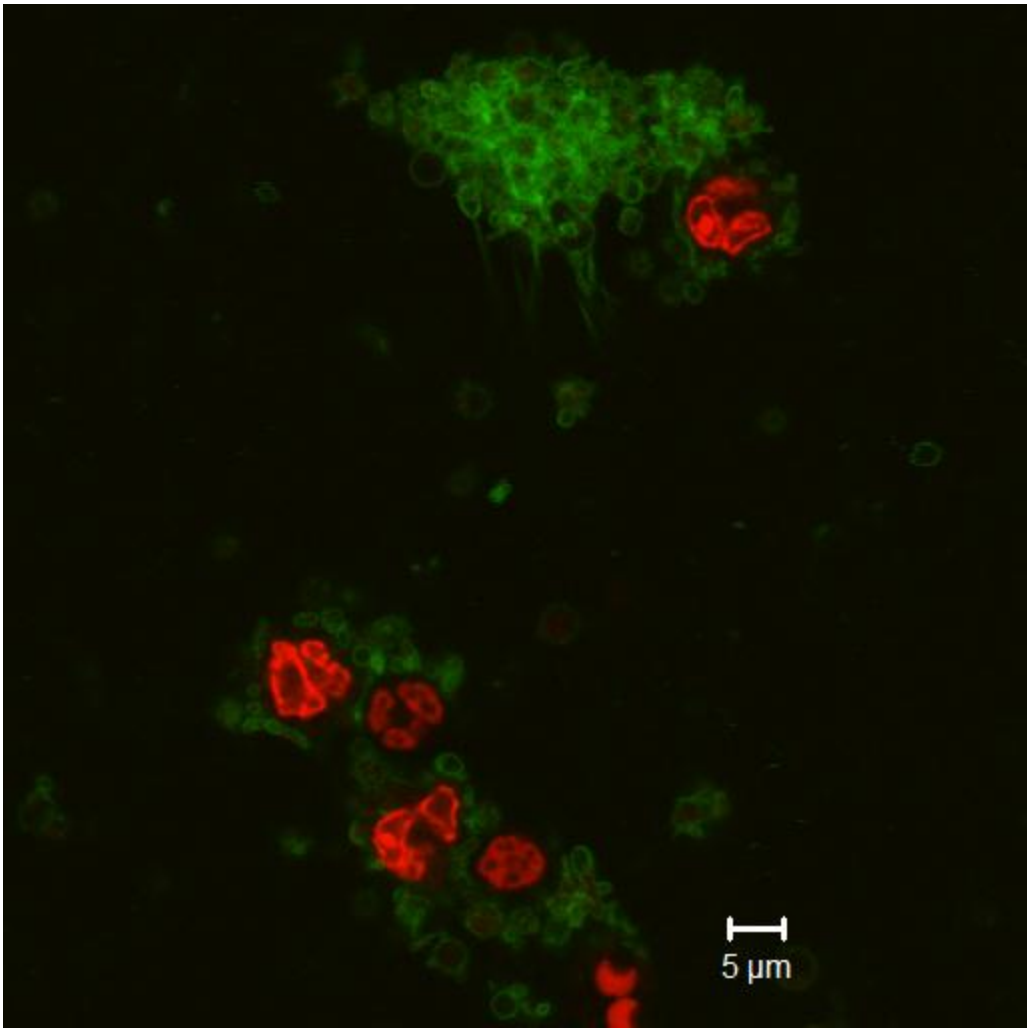


Figure 5.24. SEM of control subject platelets. Red arrow indicating formation of pseudopodia compared to similar confocal microscopy fig 6.25.

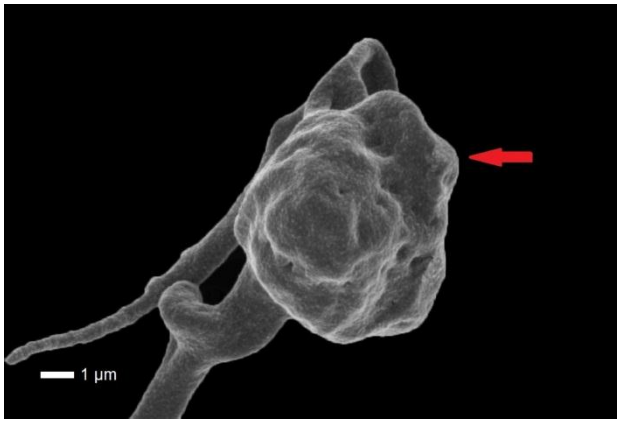


Figure 5.25. Confocal microscopy of control subject platelets. Red arrow indicating formation of pseudopodia.

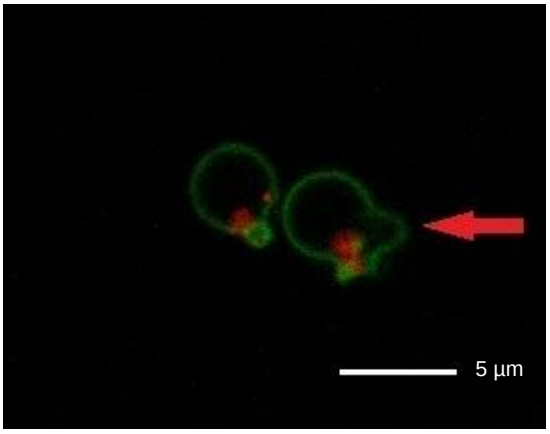


Figure 5.26. SEM of stroke patient's platelets

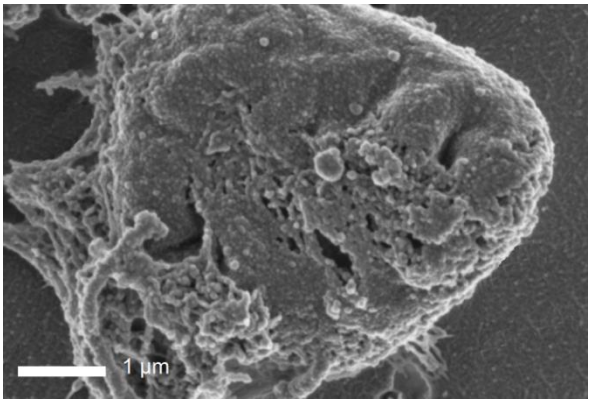


Figure 5.27. Confocal microscopy of stroke patient's platelets

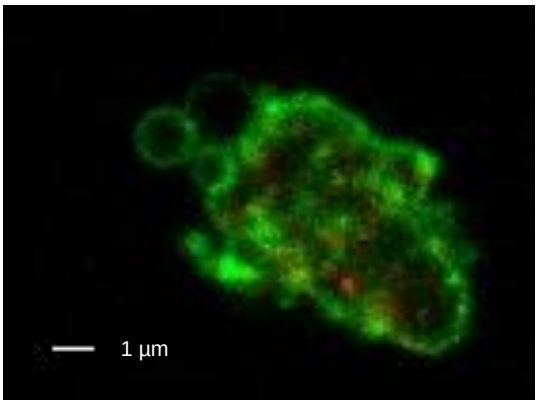


Figure 5.28 SEM of smoker's platelets showing pseudopodia and spreading.

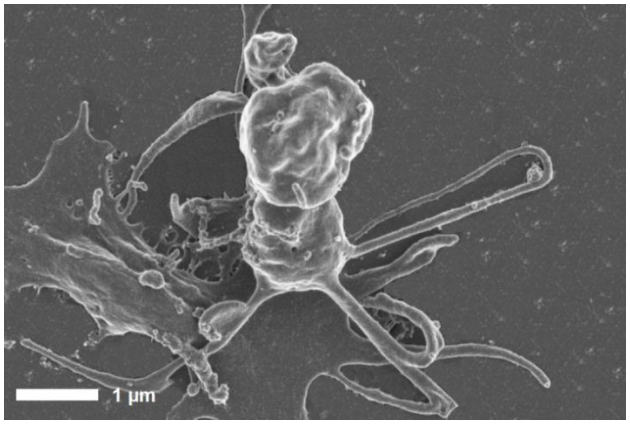
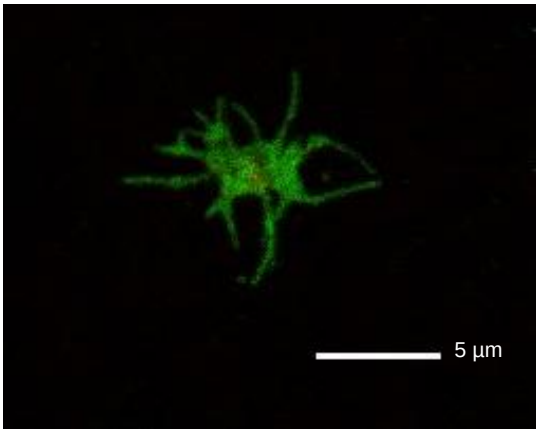


Figure 5.29. Confocal Microscopy of smoker's platelets showing pseudopodia and spreading.



5.5 Discussion and Conclusion

Firstly it is important to know what one is looking at, as mentioned before the CD41-FITC stains the membrane of platelets green, therefore used to identify platelets. HE as mentioned previously intercalates DNA, in this case the DNA found in the mitochondria of platelets, indicating the presence of superoxide.

Furthermore it is important to note that different focus points were looked at as the platelets were in an aqueous solution between two glass slides. When looking at figure 5.1, B points to a Spherical platelet, the focus point of it being deeper than the platelet membrane. These Spherical platelets were classified as being normally shaped platelets. Then when looking at A, it shows the focus point being on the actual platelet membrane.

Confocal microscopy has amongst other things been used to determine platelet vitality in humans (Hermann et al., 2010) and proved useful in various animal studies relating to platelets (Signorello et al., 2009). However its use to evaluate platelets in an unfixed state of stroke patients and people who smoke has not been explored.

5.5.1 Control Platelets

When looking at the control groups platelets it is apparent that the shape of the platelets is spherical, therefore normal. Very little pseudopodia were seen with no platelet spreading.

There was some interaction between the platelets with slight fusion of the platelet membranes (Fig 5.4). Some clot formation was seen and the clot arrangement was loose (Fig 5.12). As the platelets are viewed when alive using confocal microscopy and are mounted on a glass slide, some platelet activation is to be expected. This would explain the formation of pseudopodia and clots in the control group.

Superoxide was present near the platelet membrane as well as within the platelets, this was to be expected as normal platelet functioning requires mitochondria to generate energy.

Figure 5.24 & 5.25 is a comparison between SEM and Confocal microscopy, here the formation of pseudopodia indicated by the red arrow is shown similarly by both techniques even though the kind of image differs greatly. As a point of interest figure 5.23 shows the possible interaction of a white blood cell/cells with platelets, indicating the role of white blood cells in clot formation.

5.5.2 Stroke Platelets

Various studies have been done on the ultrastructure of platelets as well as platelets of stroke patients throughout the years using techniques like SEM and transmission electron microscopy (TEM). In 2012 Pretorius *et al*. conducted a study on the platelets of thrombo-embolic stroke patients using SEM. They found that the platelets from these stroke patients have a typical necrotic, swollen morphology where the membranes have tears and the platelets appear more balloon-shaped or rounded, they concluded that this necrotic appearance may be due to the pro-coagulant activity found in stroke as well as the inflammatory profile typical of ischemic stroke (Pretorius et al., 2012).

In this study the stroke platelets appeared to be more irregular shaped with few spherical ones. Pseudopodia and platelet spreading (fig 6.2) was present and prominent, the platelets seem to be in a more activated state.

Platelet interaction was abundant with the fusion of membranes being prominent (fig 5.14). Very tight and numerous clot formation was observed. Superoxide was prominent near the platelet membrane and inside the platelets but was most intense in the platelet aggregates or clots. When looking at a clot of a stroke patients like fig 5.8 it seems as if the superoxide accumulates in the centre of the clot, indicating the mitochondria condenses in the centre of the clot.

Fig 5.26 and 5.27 is a comparison between SEM and Confocal microscopy, the necrotic appearance of stroke patients can be seen in the SEM photo with the confocal photo resembling a similar appearance at a much lower magnification.

5.5.3 Smokers Platelets

Padmavathi *et al.* showed that smoking directly influences platelets by increasing adhesiveness, as well as aggregation (Padmavathi et al., 2010). Furthermore alterations in platelet membrane fluidity have been found, decreased platelet membrane fluidity may lead to increased platelet activation and aggregation (Padmavathi et al., 2010). ROS and NOS generation is a major role player in these changes as they are associated with an increase in lipid peroxidation and carbonyl groups (Padmavathi et al., 2010; Terassa et al., 2008).

The sticky fibrin phenomenon was found by Pretorius and co-workers in 2010 which showed that fibrin network ultrastructure is changed due to smoking (Pretorius et al., 2010).

Furthermore in 2012 Pretorius and co-workers found that changes in membrane fluidity is structurally visible and translates into a more globular and bulbous appearance of the membrane surface using SEM. They also found increased pseudopodia formation in smokers and did not see typical apoptotic body formation (Pretorius, 2012).

In the current study the platelets of smokers appear irregular in shape with some being spherical. Platelet spreading is present but to a lesser extent than the stroke group. Pseudopodia were most prominent in the platelets of smokers (fig 5.22). This may be indicative of hypercoagulability.

There appears to be prominent interaction between these seemingly “sticky” platelets as well as fusion of the platelet membranes (fig 5.11). Similar to the stroke patients, clot formation was common and the clots were tight clots (fig 5.10). This indicates that platelets are prone to clot in dense masses similar to the dense matted deposits that fibrin is changed to as found by Pretorius *et al.* in 2010 (Pretorius et al., 2010).

Superoxide was prominent near the platelet membranes and inside the platelets and similar to the stroke group superoxide accumulates in the centre of some clots (fig 5.10).

Figure 5.28 and 5.29 is a comparison between SEM and Confocal microscopy. In the photos of both techniques it is clear that the platelets of smokers are irregular shaped with numerous pseudopodia forming as well as platelet spreading.

Figure 5.16 to 5.18 shows the formation of pseudopodia, in all the photo’s it appears as if the mitochondria that are stained red are migrating towards where the pseudopodia is being formed. This could possibly be because the platelet requires more energy to generate the pseudopodia, therefore the mitochondria moves closer to the area where more energy is required as it is the supplier of energy.

In conclusion the platelets of stroke patients and smokers appear to be more activated and more prone to form tight clots. Furthermore an increased amount of superoxide is present in stroke patients and smokers, specifically in the centre of clots. This may be an indication that once platelets have aggregated and started to fuse together, the mitochondria are expelled from the platelets and “trapped” within the clot.

The platelets of smokers seem to have more pseudopodia than the stroke patients and less spreading when compared to the stroke patients. This could indicate that the platelets of

smokers are in a type of early activated state while the platelets of stroke patients are in a more advanced state of activation. This could also testify to the fact that stroke patients platelets have been found to be necrotic with swollen platelets and membrane tears as found by Pretorius *et a.l* in 2012 (Pretorius et al., 2012), while smokers platelets are not apoptotic or necrotic but have a more bulbous appearance indicating changes in the platelet membrane fluidity (Pretorius, 2012).

When comparing SEM with confocal microscopy there are some similarities between the images obtained however the high resolution attainable in SEM makes it an excellent technique to examine the platelet membrane where confocal microscopy falls short. Confocal microscopy on the other hand proves useful in its own way as platelets can be visualized in an unfixed state, allowing the viewing of platelet interaction and clot formation to a greater extent. Also the ability to incorporate markers like superoxide presents the opportunity to widen the range of data that can be obtained from the images. There is no doubt that previous research done on stroke patients and smokers using SEM has set a "golden standard" to which one can compare data obtained via other techniques as seen by the confocal microscopy images from this study.

Smoking is an important risk factor for developing of thrombotic stroke. Here we confirm that platelets of smokers show the same morphological trends as platelets of stroke individuals, albeit more subtle.

CHAPTER 6: A COMPARISON OF PLATELET ULTRASTRUCTURE AND LOCAL ELASTIC PROPERTIES IN THROMBO-EMBOLIC ISCHEMIC STROKE AND SMOKING USING ATOMIC FORCE MICROSCOPY

6.1 CHAPTER OBJECTIVES

The chapter objective is to compare atomic force microscopy images of healthy control subjects, smokers and stroke patients as well as the quantitative determination of the local elastic properties of the platelets in the groups.

6.2 INTRODUCTION

Atomic force microscopy (AFM) invented in 1986 (Binnig et al., 1986) is a unique technique which enables the quantitative determination of microelastic properties of living cells in an aqueous environment (Radmacher et al., 1996) as well as in fixed and dehydrated cells (Yang et al., 2010). Recent AFM techniques enables the solving of a number of problems in cell biomechanics due to the simultaneous evaluation of local mechanical properties and topography of living cells at a very high spatial resolution and force sensitivity (Kuznetsova et al., 2007). Imaging of elastic properties with the AFM is possible using force modulation (Radmacher and Tillmann, 1992), which has previously been applied to platelets (Lam et al., 2011; Radmacher et al., 1992). Quantitative analysis of elastic and other sample properties can be obtained using AFM by taking force curves (Burnham, 1989).

It is generally well accepted that cellular function is essentially determined by structure (Kuznetsova et al., 2007). At various hierarchy levels found, the structural organization present in cells is characterized by mechanical properties. It is expected that cellular structure should vary, both in a variety of physiological processes (such as cell growth, differentiation, adhesion) and under pathogenesis (thrombosis, oxidative stress, inflammation) (Kuznetsova et al., 2007)

Three types of filamentous proteins are found in the cytoskeleton of cells; intermediate filaments, actin filaments (microfilaments), microtubules, and a wide variety of associated proteins. Together all these proteins are responsible for the general shape of the cell, the movement of the cell, and notably the generation of force. Additionally they can have other functions for example the transport of vesicles (Radmacher et al., 1996).

Platelets shape change and activity after activation depends on the stimuli/agonists that are involved. The reorganization of the cytoskeleton in platelets is an important factor in the complex mechanisms found in thrombosis and haemostasis. The cytoskeleton is primarily responsible for regulating platelet shape (Fox, 2001). The main cytoskeletal component in platelets is actin (Bearer et al., 2002; Hartwig and DeSisto, 1991). Activation of platelets results in rapid changes in the amount of actin that is polymerized into actin filaments as well as the organization of them. This process is essential for the start of platelet aggregation (Bearer et al., 2002). Various proteins are also involved in the reorganization of the cytoskeleton.

Clearly the cytoskeleton plays a pivotal role in many aspects of the cell including; cell division, cell shape, cell adhesion and cell motility, therefore additional information about the stiffness of the cytoskeleton may prove useful in elucidating pathological conditions (Radmacher et al., 1996). Platelets activation and spreading have been extensively investigated using AFM (Agnihotri et al., 2009; Hussain and Siedlecki, 2004; Lee and Marchant, 2000), however the platelets found in pathological conditions like thrombo-embolic ischemic stroke and tobacco smokers have not yet been thoroughly studied in this field.

6.3 MATERIALS AND METHODS

6.3.1 Sample Preparation

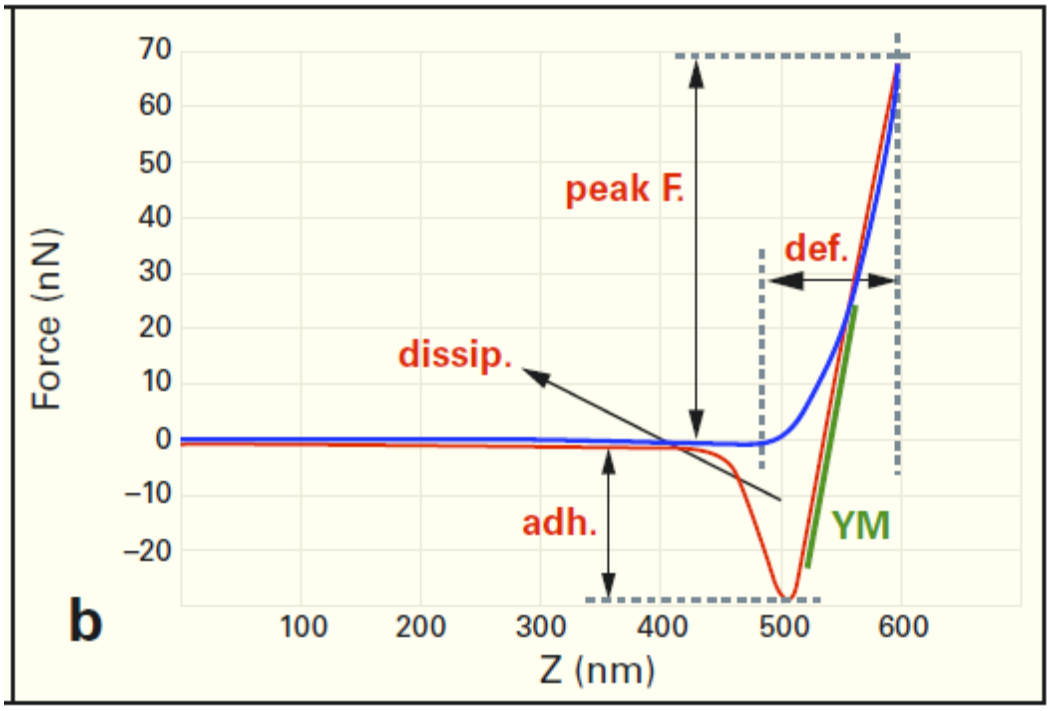
Blood was collected in citrate tubes; a drop of the blood was placed on a glass cover slip, and spread out using Spherical motions for 30 second to allow activation and adhesion of the platelets to the glass cover slip. The coverslip was then washed with Dulbecco's Phosphate buffered saline (DPBS) buffer on a micro-plate shaker to separate and remove all other blood cells and plasma proteins from the glass cover slip and the platelets.
Washed samples were then fixed in 2.5% glutaraldehyde DPBS with a pH of 7.4 for 15 minutes, followed by rinsing with DPBS and post-fixation with 1% osmium tetraoxide (OsO_4.) The samples were then again rinsed with DPBS and dehydrated with a series of ethanol. The procedures were completed by the dehydration of the material in hexamethyldisilazane (HMDS).

6.3.2 AFM Imaging and Measurement

AFM imaging and quantitative determination of the local elastic properties of blood platelets was carried out using the Dimension Icon system manufactured by Bruker, operating in Peak Force™ QNM™ tapping mode. This mode operates by applying a controllable, constant force at each data point and using the resulting force-distant curve for the formation of a

series of images; topography or height images are extracted from the cantilever deflection signal at the maximum contact force, modulus images are calculated from the slope of the retract curve near zero separation, adhesion images from the minimum of the retraction curve, deformation from the variation between the peak force point and the point where the force is zero and energy dissipation is calculated from the area between the approach and retract curve (Berquand, 2011) (see Figure 6.1).

Figure 6.1. Schematic representation of force / separation plot illustrating the type of the information that can be obtained (Berquand, 2011).



Additionally, and more importantly this mode is able to store the quantitative data of each force curve, enabling accurate modulus value acquirement at any preferred area.
Silicon Nitride probes (ScanAsyst Air, Bruker, USA) with a force constant of 0.4 N/m (newton metre), a resonant frequency of 70 kHz (kilohertz) and a nominal tip radius of 9nm (nano meter) was employed in all AFM measurements.

Six randomly selected cells of each sample was scanned at the following fields; 10µm by 10µm (this is similar to a ±25000 - 30000 times magnification) and 1µm by 1µm. A field of 0.25µm by 0.25µm was also scanned on the central (highest) region of the cell where three random scan lines of force-distance curves was recorded, an average of 1000 force curves was taken per cell.

Only force curves with a goodness of fit of 0.85 and above was used for elasticity measurements. The statistical significance of the difference between calculations was determined using one-way analysis of variance. A 2-tailed P value of less than 0.05 was considered to be significant.

The elastic Young's modulus of the cells was calculated from the force-distance curves using the Derjaguin–Muller–Toporov (DMT) theory (Derjaguin et al., 1975), which is based on the well-known Hertz models geometry, but considers that additional attractive interactions occur (Berquand, 2011).

6.4 RESULTS

The Young's modulus for the fixed and dehydrated control platelets were in the range of 30-130 MPa (megapascal), which is comparable to results found in similar studies (Shamova et al., 2011) and approximately a thousand times higher than living "wet" platelets, 1-50 kPa (kilopascal) (Radmacher et al., 1996). Blood platelets obtained from patients who has suffered stroke, elastic modulus is decreased by roughly 40 %, whereas platelets from individuals who smoke cigarettes modulus is decreased by approximately 20 %, which indicate a biophysical alteration of the platelets making them "softer".

Table 6.1. Average values of Young's modulus (E, MPa).

	Mean	**Standard Deviation**	**Median**	**Standard Error**
Control	57.56	27.63	53.6	0.23
Stroke	34.91	20.40	31.6	0.23
Smoker	44.65	14.43	43.8	0.16

Figure 6.2. Distribution of Young's moduli obtained from control platelets (blue), stroke platelets (red) and cigarette smoke platelets (green).

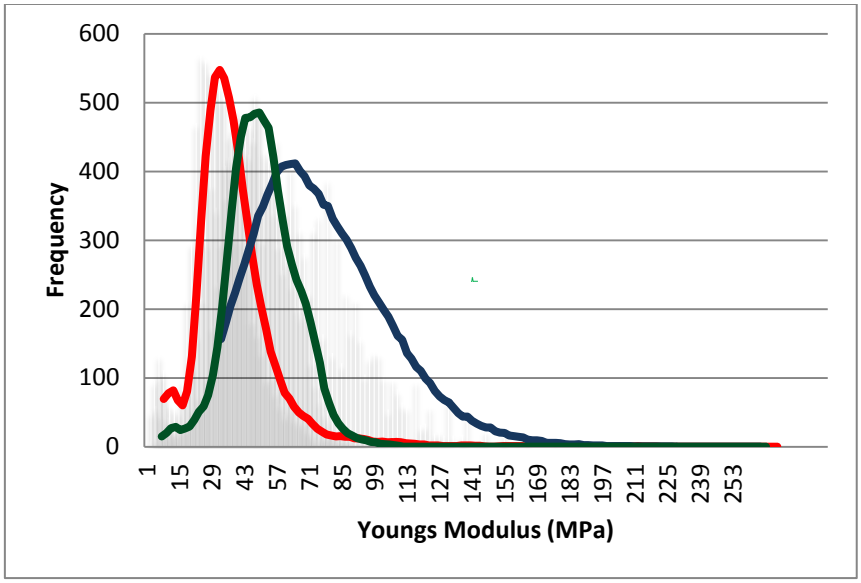


Figure 6.3. Force-Distance curves obtained on control platelets (blue), stroke platelets (red) and cigarette smoke platelets (green).

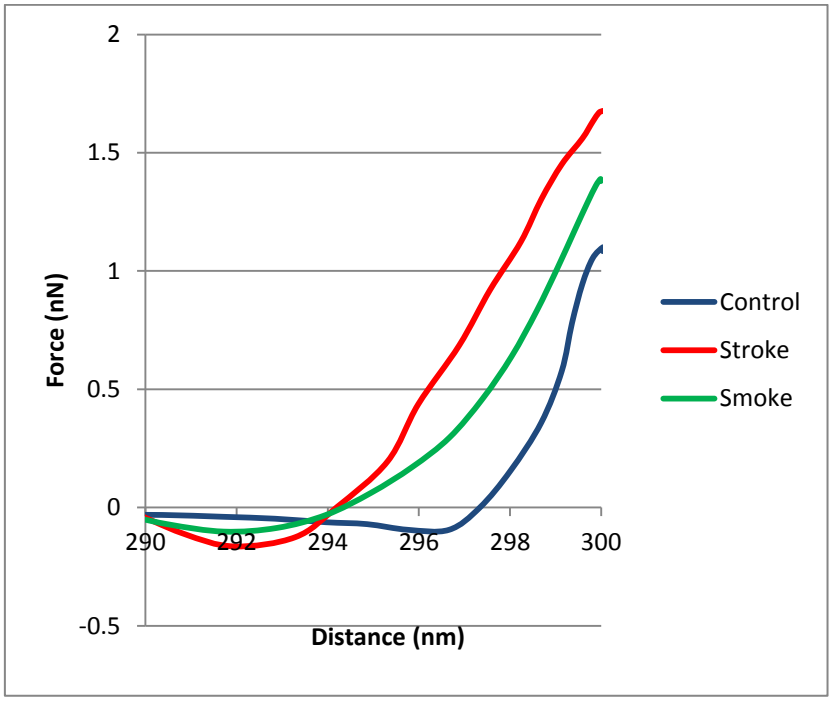
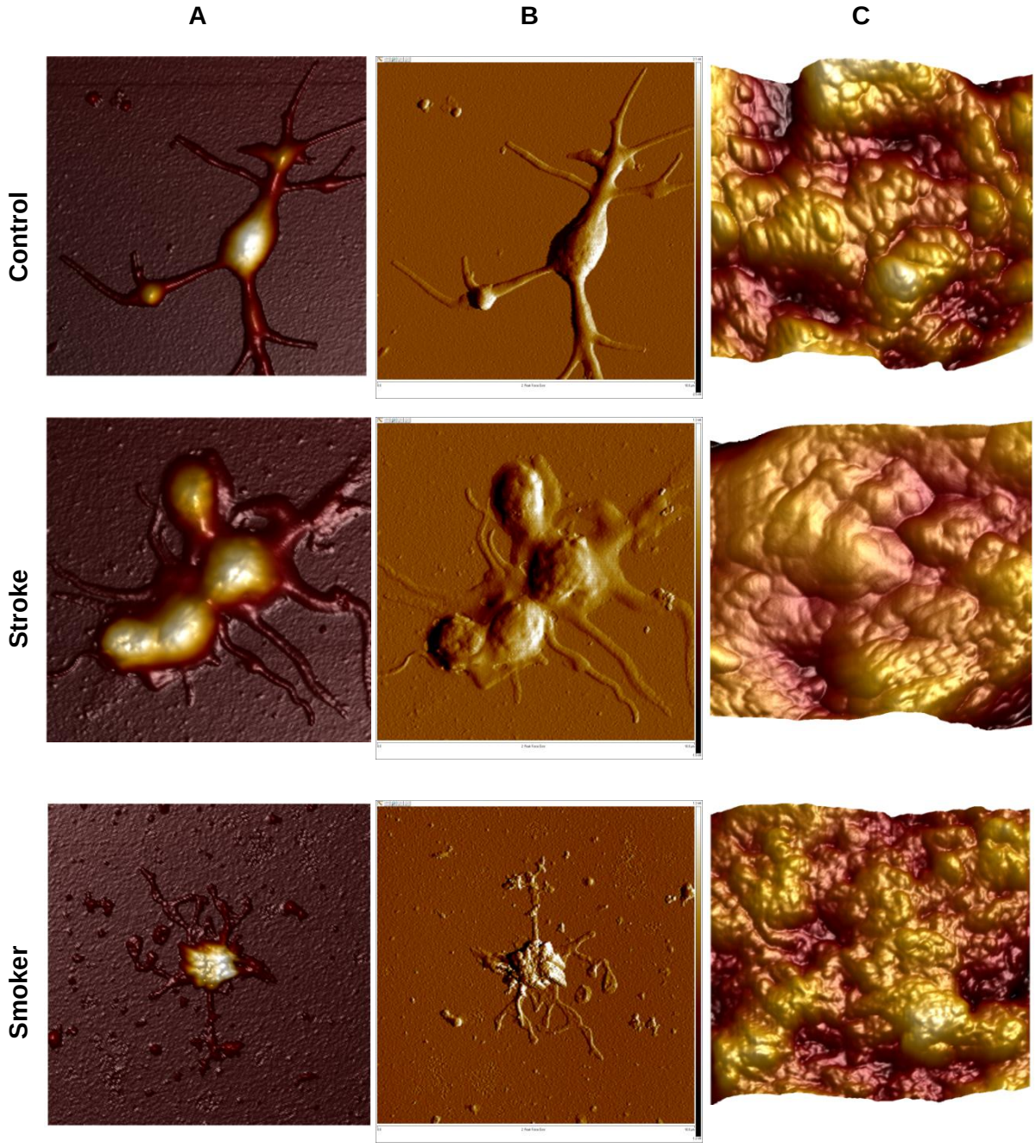


Figure 6.4. AFM images of a platelets from a control subject, stroke patient and smoker, a) Height image, x-y scale: 10µm by 10µm, z scale: 1µm, b) Error image, c) Height image of the platelet membrane, x-y scale: 1µm by 1µm, z scale: 0.2µm.



6.5 DISCUSSION AND CONCLUSION

From the quantitative data obtained conclusions regarding activation can be made. The platelets obtained from patients who has suffered stroke, elastic modulus is decreased by roughly 40 %, whereas platelets from individuals who smoke cigarettes, modulus is decreased by approximately 20 %, which indicate a biophysical alteration of the platelets making them “softer”. This “softer” appearance is indicative of cytoskeletal rearrangement.

Seeing as platelet activation is partly initiated and continued via various forms of cytoskeletal rearrangement one would expect both stroke patients’ and smokers’ platelets to be in an altered state. What is interesting here is the fact that the modulus of stroke patients is decreased approximately by double that of the smokers group. This could be an indication that the platelets of stroke patients may be in a more advanced activated state than the smokers group.

Regardless the smokers group decreased modulus once again points out the negative effects of tobacco smoke as this 20% decrease when compared to healthy individuals is indicative of irregular platelet morphology and function.

The difference between the groups may be because of platelets of stroke patients being activated, translocating P-selectin to the surface, shedding P-selectin where after platelets are able to circulate and function. These platelets that where once activated could possibly carry a “memory” of activation, leading to a population of activated platelets, previously activated platelets and resting platelets circulating, resulting in a significantly decreased modulus indicative of cytoskeletal rearrangement.

Electron microscopy has proven useful to study the organization of activated platelets, a strong correlation has been found in the topography and the underlying cytoskeleton (Albrecht et al., 1989; Caine et al., 1986; Loftus and Albrecht, 1984). According to Radmacher *et al.* AFM figures obtained of platelets can be discussed using the underlying organization of cellular features. They made use of the following nomenclature; pseudonucleus, inner filamentous zone, outer filamentous zone, and the cortex (Radmacher et al., 1996).

The pseudonucleus consists primarily of small vesicles filled with proteins, granules and cytosol. This area is known to be the softest part of the cell. The inner filamentous zone contains the contractile apparatus of the platelet. Stiffer parts found may correspond to the areas with a dense network of filamentous actin and myosin adjacent to softer regions where a lower concentration of the polymers is present. The outer filamentous zone consists

primarily of microtubules and bundles of actin filaments. A dense homogeneous network consisting of short actin filaments is found in the cortex (Radmacher et al., 1996).

Deduced from the above mentioned information and the appearance of "softer" platelets found in the results in smokers and stroke patients, it seems as if the softer appearance of the platelets is likely due to the release or secretion of granules, (Alpha-granules, dense granules) lysosomes etc. by the activated platelets. Radmacher *et al.* described the pseudonucleus as the softest part of the platelet, therefore the presence of granules, vesicles and cytosol is responsible for this. Platelet secretion or exocytosis releases molecules at the site of injury to activate other cells or to facilitate cellular adhesion. Platelets secrete molecules from intracellular granules. These molecules play central roles in haemostasis, thrombosis, and vascular remodelling (Rendu and Brohard-Bohn, 2002; Roberts et al., 2012).

Therefore the "softer" appearance of the platelets of stroke patients and smokers may be indicative of an increased production and release of granules as well as a breakdown in the organization of the cytoskeleton/contractile apparatus.

Figure 6.4 shows images obtained from the different groups using AFM. When looking at the stroke patient's platelet in figure A, it appears as if the platelet is activated as well as appearing to be more swollen when compared to the control platelet. This swelling may indicate the presence of necrosis as cell swelling is a characteristic of necrosis. Pretorius et al. in 2012 found that necrotic platelets are present in stroke patients (Pretorius et al., 2012).

When looking at the platelet of a smoker in figure 6.4 A, the platelet appears to be activated however contrasting to the stroke patients platelet it seems as if shrinkage of the platelet has occurred and the membrane appears irregular. Cell shrinkage is characteristic of apoptosis however no blebbing is apparent therefore one cannot conclude that apoptosis is taking place. As mentioned previously Padmavathi and co-workers found in 2010 that smoking induces alterations in platelet membrane fluidity and NA^{+}/K^{+}-ATPase activity, this was confirmed ultrastructurally by Pretorius *et al.* in 2012 (Padmavathi et al., 2010; Pretorius, 2012).

Figure 6.4 C is a high magnification of the platelet membrane, here the membrane of the stroke patients platelet appears more "stretched" where the smokers appears to be more "shrivelled" when compared to the control.

In conclusion both the stroke patients and smokers' platelets appear to be in a more activated state than the control group with some form of cytoskeletal rearrangement involved

to a more severe extent in the stroke group than in the smokers. Necrosis may be present in the platelets of stroke patients while neither apoptosis nor necrosis can be identified in the platelets of smokers however some form of membrane alteration may be present.

CHAPTER 7: CONCLUSIONS AND FUTURE WORK

7.1 CHAPTER OBJECTIVES

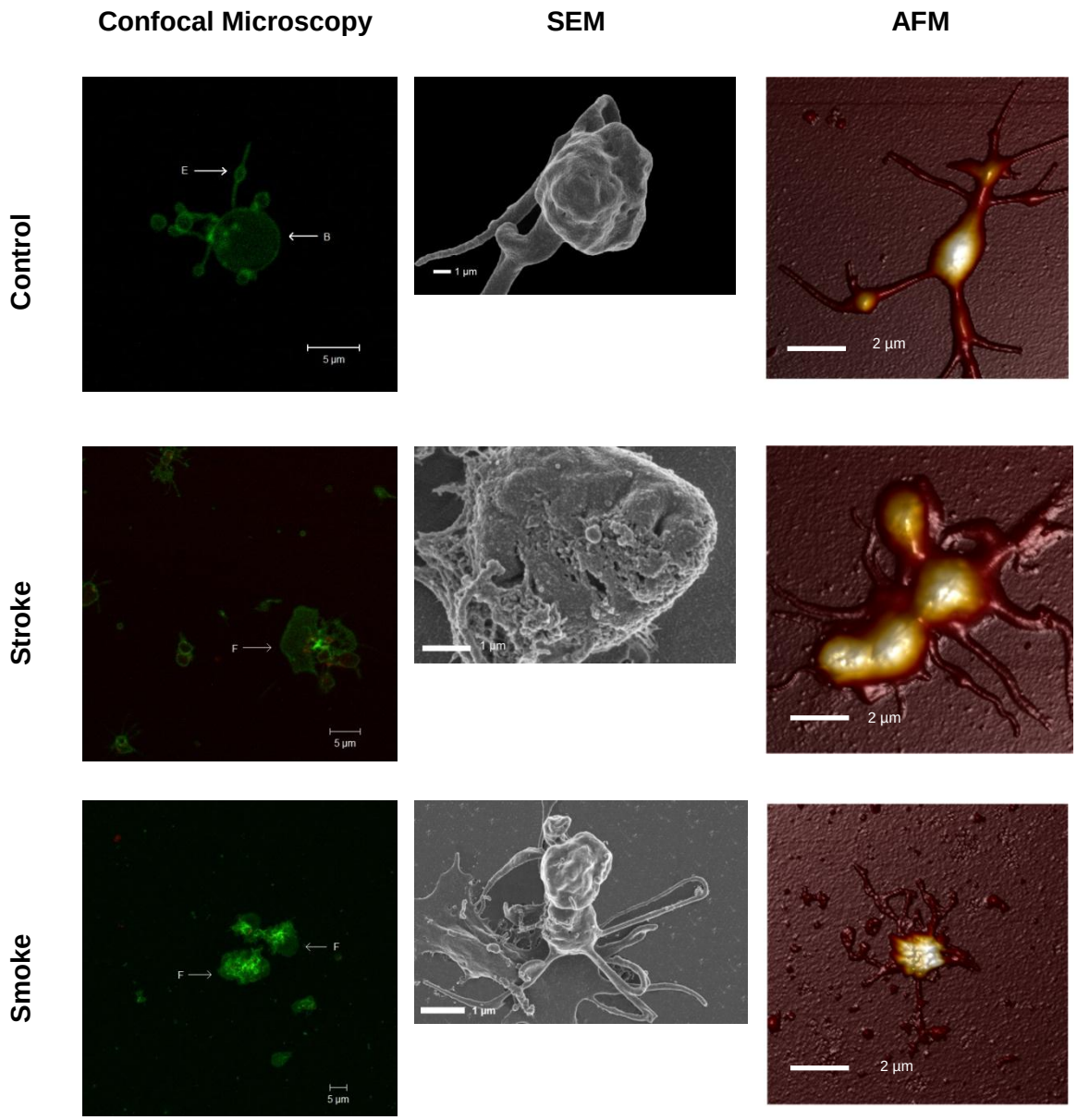
In this chapter all the data obtained in the previous chapters using the primary techniques will be compared and discussed

7.2 RESULTS

Table 7.1. Comparison of results from various chapters.

	Flow Cytometry	**Confocal Microscopy**	**Atomic Force Microscopy**
Platelet Activation	Stroke patients & Smokers have increased platelet activation	Stroke patients & Smokers have increased platelet activation	Stroke patients & Smokers have increased platelet activation
Platelet Membrane	Platelet membrane of Stroke patients and smokers seem to be altered in some way	Platelet membrane of stroke patients especially seem to be necrotic in nature	Stroke patients' platelet membrane appears swollen. Smokers platelets membrane seem altered (shrivelled)
Superoxide	Superoxide concentrations of stroke patients and smokers are normal compared to control subjects while smokers have increased superoxide levels compared to stroke patients	Superoxide concentrations appear higher in stroke patients and smokers, specifically in clots	NA
Hydrogen Peroxide	Hydrogen peroxide increased in stroke patients and smokers	NA	NA
Signs of Cellular Damage	NA	Yes stroke patients platelets appear necrotic while smoker platelets appear to be neither apoptotic or necrotic	Yes stroke patients platelets appear necrotic while smoker platelets appear to be neither apoptotic or necrotic

Figure 7.1. Images of platelets from control subjects, stroke patients and smokers using 3 different microscopy techniques.



7.3 DISCUSSION AND CONCLUSION

In Table 7.1 the results obtained from the primary techniques in this thesis are compared and in Figure 7.1 the images obtained from confocal microscopy, SEM and AFM are compared. Platelet activation is increased in stroke patients and smokers in all techniques. Flow cytometry made use of membrane receptors to confirm this while in the microscopy techniques this was apparent by the presence of the irregular formation of pseudopodia and platelet spreading.

Regarding the platelet membrane irregularities were also present throughout. Flow cytometric analysis indicated significant differences in both platelet membrane receptors used for the stroke group while a significant difference was only found in one of the receptors in the smokers group. Confocal microscopy and SEM showed that the membranes of smokers and stroke patients were less smooth when compared to healthy individuals. AFM showed that the membranes of the platelets of stroke patients appeared to be swollen while the smokers group appeared altered (shrivelled).

Both confocal microscopy and AFM showed signs pointing towards the presence of necrotic platelets in stroke patients while the smokers platelets were neither apoptotic nor necrotic but it seems as if the platelet membrane is altered.

Superoxide was only evaluated using flow cytometry and confocal microscopy, the only difference found here was when smokers were compared to stroke patients they had significantly higher amounts of superoxide present. Confocal microscopy showed a type of aggregation of superoxide within clots of platelets that formed. Hydrogen peroxide was found to be significantly elevated both stroke patients and smokers. It is apparent that ROS has a role to play in the behaviour of platelets but the exact mechanism remains unknown.

Regarding ROS, oxidative stress and anti-oxidants some controversy exists in the scientific community at present. Lipinski wrote in 2012 that insufficient scientific evidence exist that oxidative stress bears serious consequences for human health as is widely accepted. Even though many studies have shown the excessive generation of ROS in degenerative diseases, Lipinski notes that not all ROS are oxidants and it is possible for biomolecules to be enriched in oxygen atoms by different reactions than oxidation (Lipinski, 2012).

Recent studies have shown that hydrogen peroxide can prevent the formation of dense matted deposits and the same can be done by other oxidizing agents (Pretorius et al., 2013). The most effective antioxidant known as ascorbic acid, is in fact a reducing agent, resulting in the alleviation of oxidative stress, and also the potentiation thereof by contributing to the

generation of hydroxyl radicals (Gutteridge and Halliwell, 2010; Sies, 1996). Traditional definitions and accepted thought processes are being questioned and challenged making this an interesting and daunting field to examine. However in this thesis ROS was examined on a basic level, further research regarding ROS and platelets on an in depth chemical level will most definitely prove useful.

In conclusion all the techniques used showed an increase in platelet activation in stroke patients and smokers, necrotic platelets may be present in the stroke patients while the platelet membrane of smokers seems to be altered. ROS is present and alters the platelet function of smokers and stroke patients in some way. It appears as if thrombo-embolic stroke patients and smokers platelets have similar trends in activation but the processes involved to achieve this differ as there are structural differences present. These differences may prove a useful tool to further understand the pathophysiology behind thrombo-embolic ischemic stroke as well as to discover new therapeutic pathways to target.

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