Inflammatory markers and ultrastructure of the coagulation profile of diabetes mellitus

by

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Summary

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Diabetes mellitus has emerged as a major public health problem with pandemic growth as the International Diabetes Federation estimates that there were 415 million diabetics in 2015 with that number reaching 642 million by 2040, affecting all regions of the world. Globally we are all interconnected when we deal with problems of climate change, water shortage, HIV or Ebola. The war against type 2 diabetes and other non-communicable diseases should be no different, as effective solutions will need expanded global engagement in science to win it.

The risk of cardiovascular events in type 2 diabetes remains unchanged despite good control of diabetes and other cardiovascular risk factors. A better understanding of thrombogenicity in diabetes may help to identify novel therapeutic agents and a starting point would be to identify ultrastructural changes in diabetic erythrocytes, platelets and fibrin networks. In diabetes, thrombogenicity is enhanced and is characterised by: hyperactive platelets, higher levels of clotting factors and impaired fibrinolysis. Thus, in this research study, the technique of scanning electron microscopy (SEM) was used to identify ultrastructural abnormalities in erythrocytes, platelets and fibrin networks of diabetic subjects. Distinct abnormal morphological findings were observed in the erythrocytes, platelets and fibrin fibres of diabetic subjects in comparison to the controls. Physiological parameters such as platelet markers and tissue factor levels were also assessed. Flow cytometric analysis revealed hyperactive platelets in the diabetic subjects. The measurement of tissue factor in plasma was completed by using an ELISA. Tissue factor levels in the diabetic subjects were markedly elevated when compared to controls.

Biomedical research has provided evidence that has led to the hypothesis that inflammation is the culprit behind almost most chronic illnesses. Hyperglycaemia, a key feature of diabetes, is known to promote a state of low-grade chronic inflammation. A natural method that can resolve acute and chronic inflammation is earthing. Earthing involves coupling your body to the Earth's surface energies by

simply walking barefoot or being connected to a conductive device. When earthed, the electrons are conducted into the human body at the same electrical potential as the earth. It is also suggested that free electrons from the earth neutralize the positively charged free radicals that are the hallmark of chronic inflammation. In this study, earthing was accomplished with conductive adhesive patches placed on the sole of each foot and palm of each hand. An earthing cord was connected to the patches and led outdoors to be connected to a stainless-steel rod driven into the ground. Diabetic subjects were earthed for a session of two hours. Bloods were drawn before and just prior to the end of the two-hour session. Morphological SEM findings of the erythrocytes, platelets and fibrin networks at two-hours showed a remarkable difference when compared to findings at baseline. More importantly, the erythrocytes, platelets and fibrin findings revealed that they all almost reverted to looking like control erythrocytes, platelets and fibrin networks. It remains to be seen if earthing will reduce cardiovascular events in diabetics by improving morphology of cells involved in coagulation.

Key words: Diabetes mellitus, erythrocytes, platelets, fibrin networks, coagulation, thrombosis, hypofibrinolysis,

Declaration

I, Prashilla Soma hereby declare that this research dissertation is my own work and has not been presented for another degree at another university.

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List of Abbreviations

%	Percentage
µL	microlitre
µm	Micrometre
ACEI	Angiotensin converting enzyme inhibitor
ACS	Acute coronary syndrome
AD	Alzheimer's disease
AFM	Atomic force microscopy
AIDS	Acquired immunodeficiency syndrome
Ca-antagonist	Calcium antagonist
CABG	Coronary arterial bypass grafting
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
CD	Cluster of Differentiation
CPK	Type of three-dimensional molecular model
CVD	Cardiovascular disease
Da	Dalton is the unit to indicate atomic mass
DMD	Dense matted deposits
DNA	Deoxyribonucleic acid
DTS	Dense tubular system
ECG	Electrocardiogram
ELISA	Enzyme-linked immunosorbent assay
ETD	Everhart-Thornley detector
Fe^{2+}	Ferrous ion
Fe^{3+}	Ferric ion
FEG-SEM	Field emission gun scanning electron microscope
FFA	Free-fatty acids
FITC	Fluorescein isothiocyanate
fl	Femtolitre
FVII, FX, FXIII	Coagulation factors
FXa	Activated FX
GP	Glycoprotein
H_2O_2	Hydrogen peroxide

H_2SO_4	Sulphuric acid
HBA_{1c}	Glycated haemoglobin
HDL	High density lipoproteins
HH	Hereditary hemochromatosis
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
IL	Interleukin
IRE	Iron responsive elements
IRP	Iron responsive protein
kg	Kilogram
LDL	Low density lipoprotein
log	Logarithm
MCFI	Mean channel fluorescence intensity
MCHC	Mean cellular haemoglobin concentration
mg	Milligrams
MI	Myocardial infarction
min	minutes
mL	millilitre
MoAbs	Monoclonal antibodies
mPas	milliPascal seconds
mRNA	Messenger RNA (Ribonucleic acid)
Na^+/K^+ ATPase	Sodium-Potassium adenosine triphosphate
nm	Nanometre
ng/ml	Nanograms per millilitre
nm^3	Cubic nanometre
NTBI	Non-transferrin bound iron
$O_2^{\cdot -}$	Superoxide
ºC	Degrees celsius
OCS	Open canalicular system
OH^-	Hydroxide
OsO_4	Osmium tetroxide
$P2Y_{12}$	G_i-coupled platelet receptor for adenosine diphosphate
PAD	Peripheral arterial disease

PAI-I	Plasminogen activator inhibitor I
PAR 1,2,3,4	Protease-activated receptors
PE	Phycoerythrin
pg/mL	Pictograms per millilitre
RBC	Red Blood cell
ROS	Reactive oxygen species
rpm	Revolutions per minute
SA	Sialic acid
SEM	Scanning electron microscopy
-SH	Sulphydryl group
sP-selectin	Soluble platelet selectin
TAFI	Thrombin-activatable fibrinolysis inhibitor
TAT	Thrombin-antithrombin
TF	Tissue factor
TFMV	Tissue factor microvesicles
TFPCA	Tissue factor procoagulant activity
TFPI	Tissue factor pathway inhibitor
TNF-α	Tumour necrosis factor alpha
t-PA	Tissue plasminogen activator
u-PA	Urokinase-type plasminogen activator
u-PAR	Urokinase-type plasminogen activator receptor
α	Alpha
β	Beta

1. CHAPTER 1: INTRODUCTION

1.1 Aims and outline of the thesis

Diabetes mellitus has emerged as a major public health problem with pandemic growth as the International Diabetes Federation estimates that there were 415 million diabetics in 2015 with that number reaching 642 million by 2040, affecting all regions of the world (Narayan KM, 2016). Type 2 diabetes mellitus is a metabolic disorder characterized by chronic hyperglycaemia as a result of a defect in insulin secretion and a major contribution from insulin resistance (Saha A, 2005). Among the array of pathophysiological features for atherosclerosis, chronic low-grade inflammation features strongly and is a part of plaque initiation, progression and thrombosis (Pradhan AD, 2002). Cross-sectional studies favour the hypothesis that chronic subclinical inflammation may be associated with insulin resistance and precede the development of clinically overt type 2 diabetes (Pradhan AD, 2002). By assigning the title of an inflammatory disease to type 2 diabetes provides a logical framework which offers unique opportunities for both primary prevention and the alleviation of significant diabetes-related cardiovascular complications (Pradhan AD, 2002). Thus, a new, simple and alternative therapeutic technique is adopted in this study, based on its anti-inflammatory mechanism of action to investigate it effects on the coagulation profile of diabetic subjects.

The main focus of this manuscript is to describe the ultrastructural morphological findings in the erythrocytes, platelets and fibrin networks in subjects with diabetes mellitus. The findings will be compared using three groups: a healthy group, a group of diabetic subjects with cardiovascular disease (CVD) and a group of diabetic subjects without CVD. The inflammatory marker measured in this study is serum ferritin. In addition, the effect of earthing (alternative, anti-inflammatory therapeutic technique) on the morphological changes will be reported and these are novel findings.

An extensive detail of the structure and function of platelets, fibrin networks and erythrocytes forms the basis to better understand increased morbidity and mortality caused by accelerated atherosclerosis in diabetes mellitus. This is

explored in **Chapter 2**, the Literature Review. The alterations in the morphology of platelets, fibrin network and erythrocytes in diabetic subjects is described and mechanisms causing the abnormalities are defined. Previous research investigating the ultrastructural changes in platelets, fibrin and erythrocytes is also highlighted. It is envisaged that a better understanding of the mechanisms causing accelerated atherosclerosis in diabetic subjects will lead to better and more effective therapeutic strategies as cardiovascular complications in diabetes remain a global problem. It is thus imperative that alternative methods of therapy be explored to curtail diabetic complications.

Chapter 3 reviews briefly the physiology of ferritin, the formation of free radicals and its consequences when their levels are excessive. Previous research shows profound morphological changes in erythrocytes, platelets and fibrin networks in many clinical diseases as a result of high iron levels. There is also emerging evidence that lowering iron levels has beneficial effects including improving glycaemic control in diabetic subjects. This may have huge public health implications. The morphological findings in erythrocytes, platelets and fibrin networks caused by elevated ferritin in diabetic subjects are also highlighted here.

The participation of platelets in the coagulation pathway is of vital importance. Flow cytometry thus provides a numerical technique which is both objective and quantitative to assess platelet function. Specific glycoproteins on the platelet membrane will be detected by monoclonal antibodies. In addition, flow cytometry facilitates the identification of molecular transfer to the surface from the interior platelet granules, which are suggestive of platelet activation. The predominance of specific glycoproteins relevant in diabetic subjects is discussed in **chapter 4**. Statistical analysis confirms significant increases in platelet markers and is in keeping with previous research that platelets are hyperactivated in diabetes.

Tissue factor is a key initiator of the coagulation cascade, leading to the ultimate formation of fibrin. Furthermore, tissue factor is recognised to be involved in the pathogenesis of cardiovascular disease. It is also known that in type 2 diabetics, higher levels of circulatory tissue factor are modulated by both glucose and insulin and the two appear to have an additive effect. An enzyme-linked immunosorbent assay (Elisa) was performed to measure tissue factor in a representative sample

containing both groups of diabetic subjects. Results as shown in **chapter 5** indicate that plasma tissue factor levels in both diabetic groups, with and without CVD are increased when compared to the controls.

Chapter 6 explores and describes the characteristic morphological findings in healthy and diabetic erythrocytes, platelets and fibrin networks using scanning electron microscopy (SEM). Remarkable and distinct differences are observed between the diabetic and healthy groups. This study reveals that more profound morphological changes are seen in the diabetic group with CVD. Fibrin diameters and axial ratios of erythrocytes were measured in the two diabetic groups. Differences observed were not statistically significant. Of note, is that there were remarkable changes in the morphology (of erythrocytes, platelets and fibrin) following the two-hour earthing session. This constitutes novel findings and are described in chapter 7.

Chapter 7 explores the topic of earthing. Simply stated, earthing research offers insight into the clinical potential of barefoot contact with the earth, or simulated barefoot contact via simple conductive systems, on the stability of internal bioelectrical function and human physiology. Reduction in inflammation as a result of earthing has been documented with infrared imaging. This phenomenon of anti-inflammatory effect can be viewed as earthing allows negatively charged antioxidant electrons from the earth to enter the body and neutralize positively charged free radicals at sites of inflammation. The physiological effects of earthing are also briefly described. The effects of a two-hour earthing session were documented by viewing morphological changes in erythrocytes, platelets and fibrin networks. Significant changes with observed when comparing SEM images at baseline and at 120 min. Earthing may just yet prove to be an effective simple and natural intervention against chronic stress, autonomic dysfunction, inflammation, pain, hypercoagulable blood and many other health disorders, including cardiovascular disorders.

Chapter 8 will place abnormalities in perspective and collate all current findings with previous research. The way forward and recommendations based on the evidence unfolded in this thesis, will conclude the thesis in **the conclusion** section.

In summary, research questions that originate from this study include:

- What is the reason for accelerated atherosclerosis in diabetes?
- Have we identified all risk factors for atherosclerosis?
- Is the coagulation process altered in the diabetic subject?
- Why are platelets in diabetes mellitus identified as ‘angry’?
- Previous research highlighting morphological abnormalities adds to a better understanding of pathogenesis, should microscopy techniques be included as routine practice in clinical medicine?
- Closer inspection of thrombosis, reveals that tissue factor plays a key role and measuring tissue factor may assist to identify another risk factor. More importantly, will the inclusion of pharmacological treatment for high tissue factor improve thrombosis in the diabetic patient?
- Is there perhaps an alternative therapy, which is simple, safe, accessible and inexpensive that we have neglected? The beneficial effects of earthing are now emerging. Thus, an important objective of the thesis is to investigate the effects of earthing on the morphology of diabetic erythrocytes, platelets and fibrin networks. These are novel findings.

2. CHAPTER 2: LITERATURE REVIEW - THE ULTRASTRUCTURAL ABNORMALITIES IN PLATELETS, FIBRIN NETWORKS AND ERYTHROCYTES CONTRIBUTING TO ACCELERATED ATHERO-SCLEROSIS IN TYPE 2 DIABETES MELLITUS

2.1 Abstract

Accelerated atherosclerosis is the main underlying factor contributing to the high risk of atherothrombotic events in patients with diabetes mellitus. Like with many bodily systems, pathology is observed when the normal processes are exaggerated or uncontrolled. This applies to the processes of coagulation and thrombosis as well. In diabetes, in fact, the balance between prothrombotic and fibrinolytic factors is impaired and thus the scale is tipped towards a prothrombotic and hypofibrinolytic milieu, which in association with the vascular changes accompanying plaque formation and ruptures, increases the prevalence of ischaemic events such as angina and myocardial infarction. Apart from traditional, modifiable risk factors for cardiovascular disease like hypertension, smoking, elevated cholesterol; rheological properties, endogenous fibrinolysis and impaired platelet activity are rapidly gaining significance in the pathogenesis of atherosclerosis especially in diabetic subjects. Blood clot formation represents the last step in the athero–thrombotic process and the structure of the fibrin network has a role in determining predisposition to cardiovascular disease. It is no surprise that just like platelets and fibrin networks, erythrocytes have been shown to play a role in coagulation as well. This is in striking contrast to their traditional physiological role of oxygen transport. In fact, emerging evidence suggests that erythrocytes enhance functional coagulation properties and platelet aggregation. Among the spectrum of haematological abnormalities in diabetes, erythrocyte aggregation and decreased deformability of erythrocytes predominate. More importantly, they are implicated in the pathogenesis of microvascular complications of diabetes. The morphology of platelets, fibrin networks and erythrocytes are thus essential role players in unravelling the pathogenesis of cardiovascular complications in diabetic subjects.

2.2 Introduction

Clinically, diabetes mellitus is characterized by polyuria, polydipsia, polyphagia and hyperglycaemia. Similarly, haematologically it is known that type 2 diabetes

mellitus is associated with abnormalities in platelet function, coagulation and fibrinolysis, all of which contribute to vascular complications in diabetes (Kluft & Jesperson, 2002). Evidence shows that diabetes has been considered to have a prothrombotic status. Characteristic findings in type 2 diabetes includes: increased coagulation, impaired fibrinolysis, endothelial dysfunction and platelet hyper-reactivity (Creager et al. 2003).

In particular, the hyperglycaemia causing the platelet activation, the increase in fibrinogen and hypofibrinolysis related to insulin resistance, all play a significant role in the development of angiopathy (Kluft & Jesperson 2002). A detailed description of the ultrastructure of platelets, fibrin networks and erythrocytes, which follows, will thus lead to a better understanding of the pathogenesis of angiopathy in diabetes. The outline of the review is highlighted in Figure 2.1.

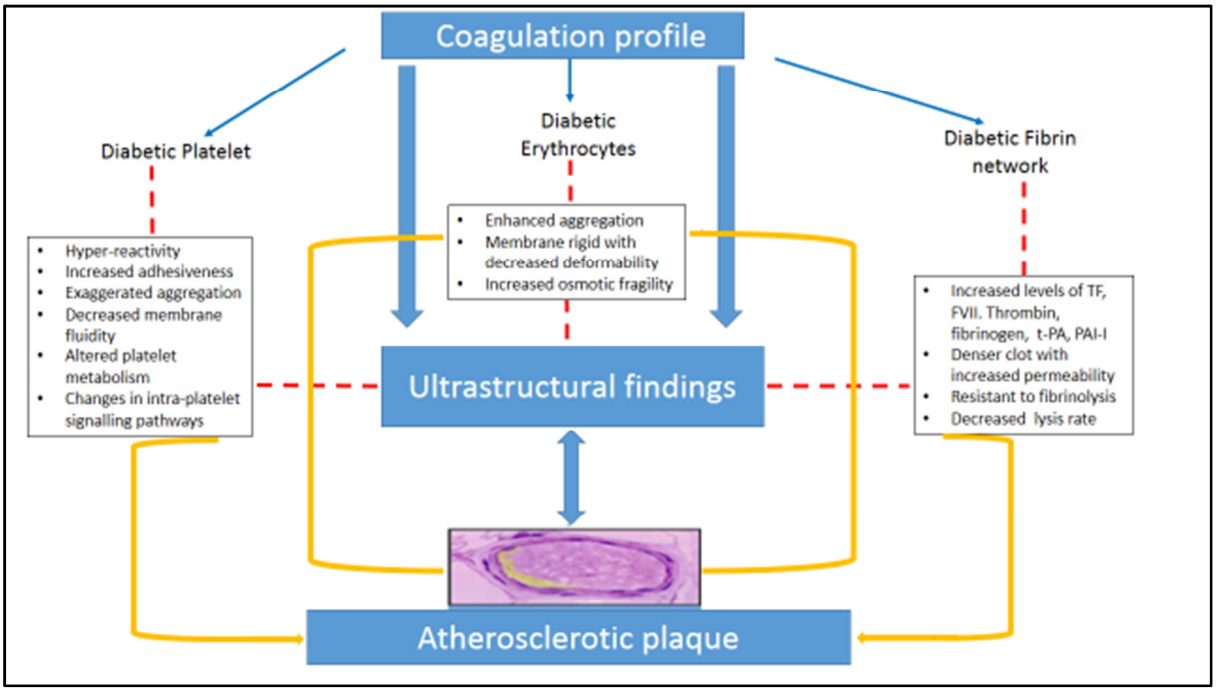


Figure 2.1: **Outline of the review: cells involved in coagulation are described with characteristics thereof which contribute to atherosclerosis. Ultrastructural findings in erythrocytes, platelets and fibrin fibres are then reviewed. (TF tissue factor, FVII factor VII, t-PA tissue plasminogen activator, PAI-I plasminogen activator inhibitor I).**

2.3 Platelets

An important element postulated in the pathogenesis of the prothrombotic state in diabetic patients is platelet hyper-reactivity (Ferreiro, Gómez-Hospital & Angiolillo 2010). The prothrombotic condition that is characteristic in diabetes, in turn, can

be ascribed to the following factors: (i) increased coagulation, (ii) impaired fibrinolysis, (iii) endothelial dysfunction and (iv) platelet hyper-reactivity (Creager et al. 2003). In diabetes, several mechanisms contribute to platelet dysfunction, such as those due to hyperglycaemia, insulin deficiency and insulin resistance, associated metabolic conditions and other cellular mechanisms (Ferreiro, Gómez-Hospital & Angiolillo, 2010). Each of the latter four factors will be discussed in this section. A review of the normal platelet structure and function will allow a better analysis of the ultrastructural abnormalities.

2.3.1 Brief review of platelet structure and function

Human platelets form part of the cellular component of plasma. They are anucleated, small and discoid in shape with a diameter of 2.0 - 4.0 µm and mean cell volume of 7-11fl. Their normal count is in the range of 150 - 450 x 10^9 platelets per litre of whole blood, each with an average lifespan of 8 -10 days. The precursor cell for the platelet is the megakaryocytes which are constituents of the bone marrow. Even though platelets perform multiple functions, one of their primary functions is to respond to blood vessel injury by utilizing some of its unique characteristic like changing shape, secreting granule contents and aggregating to form a platelet clot. Secondary functions include: maintenance of vascular tone, inflammation, host defence and tumour biology (George 2000) (Avecilla et al. 2004).

A structure that plays a crucial role in platelet function is the glycoprotein (e.g. GP IIb-IIIa, GP IX-V, and GPIV) layer. This glycoprotein layer forms the outermost layer of the platelet and contains various receptors which bind to a variety of agents like adhesive agents, aggregating agents, inhibitors and procoagulant factors. This feature contributes to the vital haemostasis function of platelets (Vizcaino-Salazar 1994) and (McEver 1990). The most abundant glycoprotein GP IIb-IIIa binds to fibrin and fibrinogen thus facilitating platelet-platelet interaction. Whilst GP Ib contributes to the binding of platelets to von Willebrand factor and GP Ia-IIa facilitates adhesion to collagen (Kainoh et al. 1992), (Suzuki, Yamazaki &Tanoue 1992) and (Moroi & Jung 1998). Another example of an adhesive agent is P-selectin, which when activated redistributes from the membrane of the granules (storage site of P-selectin) to the platelet plasma membrane (Johnston,

Kurosky & McEver 1989). P-selection is only expressed by activated platelets and resting platelets on their plasma membrane, thereby posing as receptors for neutrophils and monocytes (Crovello, Furie & Furie 1993).

Like with all cells, the plasma membrane lies beneath the outermost layer and its main component is a phospholipid bilayer in which cholesterol, glycolipids and glycoproteins are embedded. Unlike erythrocytes, platelets present these molecules on their surface. The organization of the phospholipids between the inner and outer leaflets is asymmetrical and this regulates coagulation. There is an abundance of negatively charged phospholipids in the inner leaflet of the plasma membrane which maintains the platelet surface in a non-procoagulant state (Heemskerk, Bevers & Lindhout 2002). The phospholipids contribute to coagulation by stimulating the coagulation factor X to Xa and prothrombin to thrombin, both integral steps in the coagulation cascade (Rosing et al. 1985). Other protein components of the resting platelets include CD36, CD63, CD9 and GLUT-3. A cross section of the discoid platelet also reveals that the platelet membrane is densely packed with highly specific surface receptors that tightly controls signal-dependent platelet activation and may modify α-granule release to coagulation, inflammation, atherosclerosis, antimicrobial host defence, angiogenesis, wound repair or malignancy (Blair & Flaumenhaft 2009).

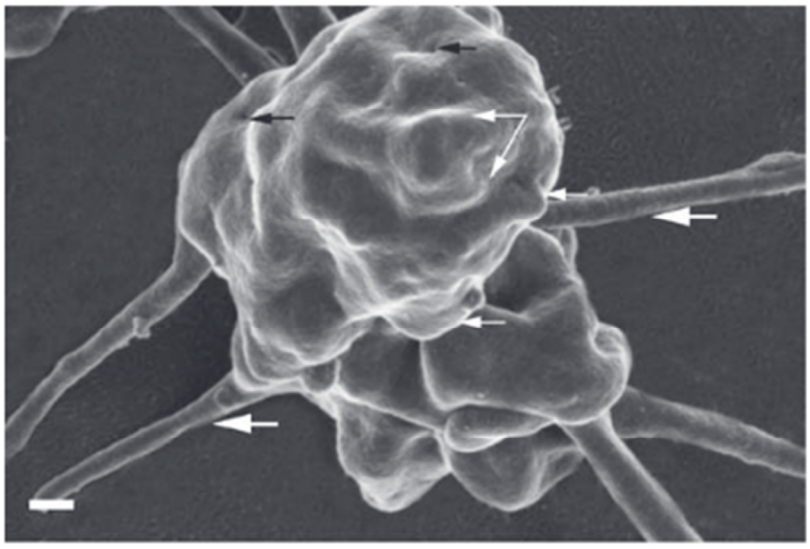


Figure 2.2: **SEM of a healthy subject, prepared as smear from platelet-rich plasma at 100 000 X magnification with thick white arrows indicating extended pseudopodia, thin white arrow representing the smooth platelet membrane and the thin black arrows the OCS. Taken from (Du Plooy et al. 2013). Scale = 200nm.**

One distinguishable structural element that platelet develop during maturation is invaginations of the surface membrane that form the open canalicular system (OCS) as illustrated in Figure 2.2. The OCS is made up of an elaborate series of plasma membrane-contiguous indentations and channels that tunnel throughout the interior of the platelet (Behnke 1970). Three important functions performed by the OCS include: (1) external elements gain entry into the platelet, as well as a route for the release of granule contents to the outside of the platelet, (2) features like filopodia and spreading following platelet adhesion to an activating surface can be ascribed to the OCS, (3) it serves as a storage site for plasma membrane glycoproteins (Michelson 1992). Similar to the sarcotubules of the skeletal muscle, platelets house the dense tubular system (DTS) which is a closed-channel network of residual endoplasmic reticulum whose function is to sequester ionized calcium (White 1972).

Another characteristic feature of the platelet is the well-defined and highly specialized cytoskeleton which is crucial to maintain the disc shape of the resting platelet. Imaging of the platelet, like the erythrocyte has been done at high resolution and the three major cytoskeletal components of the resting platelet identified are (1) the spectrin-based membrane skeleton, (2) the action cytoskeleton and (3) the marginal microtubule coil (Hartwig 1991). Among the intracellular organelles like mitochondria, lysosomes, peroxisomes, are granules. A variety of biologically active molecules are stored in their granules. Two major storage granules in platelets are α- and dense granules. Alpha-granules are most abundant and contain proteins essential for platelet adhesion, (Blair & Flaumenhaft 2009) while the dense granules function to recruit additional platelets to sites of vascular injury. Dense granules store molecules that are secreted upon platelet activation. Contents of dense granules include substances such as catecholamine, serotonin, calcium, adenosine 5'-diphos-phate and adenosine 5'-triphosphate (Lesurtel et al. 2006).

2.3.2 Alterations of platelets in diabetes as caused by several mechanisms

Among factors contributing to the prothrombotic condition which characterise patients with diabetes mellitus, platelet hyperactivity plays a pivotal role. As

mentioned previously, multiple mechanisms are involved in platelet dysfunction in diabetics, and are categorised as follows: (a) hyperglycaemia, (b) insulin deficiency and resistance, (c) associated metabolic conditions and (d) other cellular abnormalities.

2.3.3 Hyperglycaemia

Hyperglycaemia which is an inherent finding in diabetes is associated with a hypercoagulable state since it induces platelet-vascular activation and post-prandial coagulation activation. There is a link between micro- and macrovascular complications and level of glucose control as tight control reduces its impact (Kluft 2002). A common finding in patients with diabetes is that their platelets exhibit increased expression of adhesion molecules (Ferreiro, Gómez-Hospital & Angiolillo 2010). Numerous biochemical abnormalities have been found that correlate with platelet hyper-reactivity. (i) non-enzymatic glycation of platelet membrane proteins that decreases membrane fluidity which may increase platelet adhesion, (ii) increased arachidonic acid metabolism which leads to, (iii) increased thromboxane A_2 production, (iv) disturbance in calcium homeostasis causing increased intracellular levels is linked to the reduction in membrane fluidity and altered magnesium homeostasis resulting in decreased intracellular magnesium levels which correlates to an increase in platelet hyper-aggregability and adhesiveness, (vii) diabetic platelets produce less nitric oxide and prostacyclin both important inhibitors that prevent platelets from attaching to endothelium, (vi) low antioxidant level in platelets is associated with increased aggregability, (vii) osmotic effect of glucose activates platelet GP IIb-IIIa and P-selectin expression which in turn is related to increased aggregability, (viii) increased generation of platelet dependent thrombin, (ix) active platelets in diabetic patients are rich in cytokines and chemokines which contribute to inflammation and atherogenesis, (x) increased platelet turnover results in increased reticulated platelets and this too contributes to platelet hyper-reactivity. It is not only factors directly linked to platelets that induces a prothrombotic state, other components in the plasma are contributing factors as well. In particular, hyperglycaemia increases concentrations of tissue factor and von Will brand factor, which increases platelet aggregability and inhibits fibrinolysis by increasing the levels of plasminogen activator inhibitor

(Colwell & Nesto 2003), (Ferreiro, Gómez-Hospital & Angiolillo JL, 2010) and (Balasubramaniam et al. 2011).

2.3.3.1 Insulin deficiency and resistance

It is a known fact that insulin resistance and hyperinsulinaemia is prevalent for a decade or two prior to the presentation of full blown diabetes. Upon manifestation of type 2 diabetes, progressive insulin deficiency is detected. Both insulin resistance and insulin deficiency results in platelet dysfunction (Schneider 2009). This is attributed to the presence of insulin receptors and insulin-like growth factor-1 which are expressed in platelets (Hajek & Joist 1992).

Insulin deficiency also a plays an important role in platelet dysfunction by different mechanism. Those postulated include increased intracellular calcium concentration leading to enhanced platelet degranulation and aggregation, and impaired response to nitric oxide and PGI_2, which enhances platelet reactivity (Ferreiro, Gómez-Hospital & Angiolillo D 2010).

2.3.3.1.1 Associated metabolic conditions

Other metabolic conditions that enhance platelet reactivity include obesity, dyslipidaemia and increased systemic inflammation. A number of factors contribute to platelet dysfunction in obesity, which is common among diabetic subjects, which include:

- Elevated platelet counts and mean platelet volume which is related to platelet reactivity;
- Increased leptin concentration which is associated with increased platelet aggregability;
- Higher calcium concentration in the cytosol that enhances platelet reactivity; and
- Increased oxidative stress.

The cholesterol parameters implicated in platelet activity is increased triglycerides and high density lipoproteins (HDL). The very-low-density lipoprotein in fact, impairs fibrinolysis as well. Endothelial dysfunction, a risk factor for

atherothrombotic events in diabetic subjects is associated with low HDL levels. The role of inflammation is described below in the discussion section.

2.3.3.2 Other cellular abnormalities

Patients with diabetes present with other platelet abnormalities that can enhance platelet adhesion and activation, such as impaired calcium metabolism, oxidative stress, upregulation of $P2Y_{12}$ signalling pathway and increased platelet turnover. (Calcium metabolism and oxidative stress are discussed below). The platelet adenosine diphosphate $P2Y_{12}$ receptor signalling pathway is upregulated in subjects with type 2 diabetes. The effect of this is inhibition of cAMP concentration, a diminished responsiveness to insulin which results in increased adhesion, aggregation and procoagulant activity (Ferreira et al. 2006). A special form of platelets, reticulated platelets are found in diabetic subjects. These are larger and more sensitive and thus hyper-reactive.

2.3.3.2.1 Structural abnormalities found in diabetic platelets

The work by Pretorius and colleagues that looked at the ultrastructure of platelets and fibrin networks in diabetic patients confirmed a changed platelet membrane ultrastructure. They found that the platelets themselves seemed shrunken and the membranes showed blebbing (Pretorius 2011a). There were also no open canalicular system channel pores. This blebbed morphology is typical of apoptosis as shown in Figure 2.3 (Pretorius 2011a).

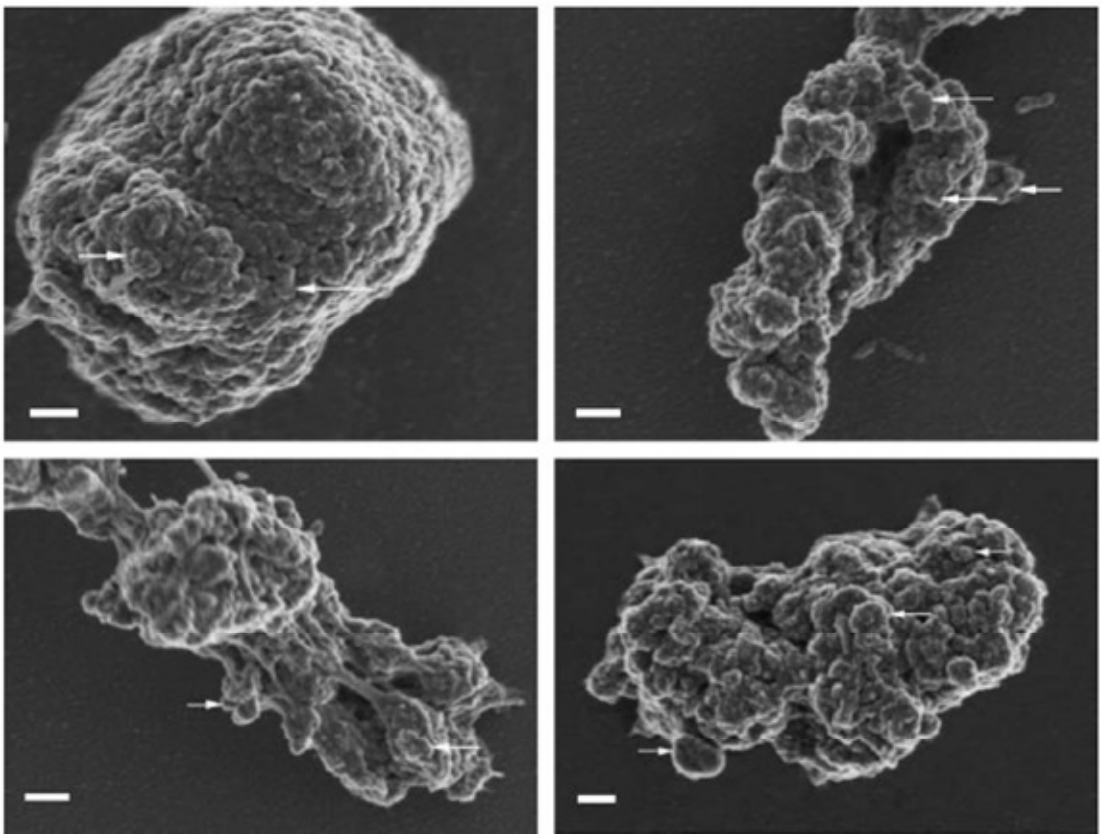


Figure 2.3: **Diabetic platelet, prepared as smear from platelet-rich plasma at 100 000 X magnification. White arrows representing blebbing. Taken from (Pretorius et al. 2011a). Scale = 200nm.**

2.3.4 Fibrin networks

There is an increased prevalence of atherothrombotic complications in subjects with diabetes. Prominent features contributing to premature atherosclerosis in this group include: increased platelet reactivity and activation of coagulation factors with associated fibrinolysis (Alzahrani & Ajjan 2010). During blood vessel injury, the normal physiological response is that fibrin is deposited at the atherosclerotic lesion. Thus, the structure of the deposited fibrin has become significant and is viewed as a probable risk factor for increased affinity for cardiovascular events in subjects with atherosclerosis (Fatah et al. 1996). Investigating ultrastructural morphological changes in activated platelets as well as that of fibrin networks is emerging as an important tool when studying different medical conditions. An etiologic factor postulated to be causing ultrastructural changes in fibrin networks is abnormalities in the coagulation process (Pretorius et al. 2007).

2.3.4.1 Physiology of clot formation

The final end product of the intricate coagulation cascade is the conversion of fibrinogen to fibrin as shown in Figure 2.4. One other critical step in this conversion, is the catalyzation of fibrin by the enzyme thrombin. Fibrin forms a loose netlike meshwork at the site of vessel injury as can be seen in Figure 2.4 (Sherwood 2004). The entrapped abundant erythrocytes give the clot a red appearance with the fibrin forming the foundation of the clot. The activated platelets are the stimulating agents initiating the ultimate conversion of fibrinogen to fibrin. Thrombin, in addition to its role in converting fibrinogen to fibrin, also activates factor XIII to stabilize the resultant fibrin mesh as indicated in Figure 2.4.

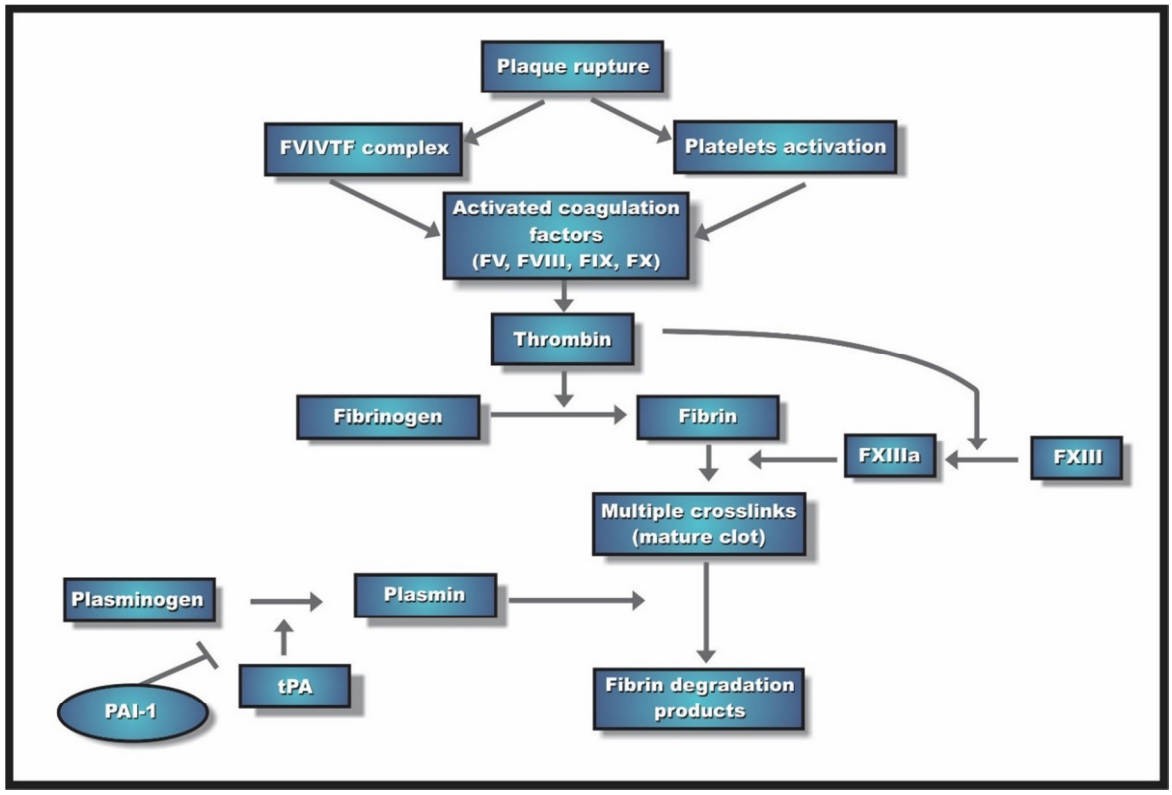


Figure 2.4: **The schematic representation summarizing the coagulation cascade and fibrin clot formation. Taken from (Alzahrani & Ajjan 2010). (tPA: tissue plasminogen activator, PAI-1: plasminogen activator inhibitor-1).**

2.3.4.2 Structure of fibrin network in various medical conditions

Among the cluster of risk factors for increased cardiovascular complications, fibrin network structure feature prominently. Fatah and co-workers examined compact clots formed ex vivo and showed that characteristics such as thinner fibres, increased branching and a tight network structure were more susceptible to cardiovascular events (Fatah et al. 1996). The structure of a fibrin clot as shown in

Figure 2.5, with features like fibre thickness and pore size mainly affects the effectiveness of fibrinolysis. The outcome on the fibrinolysis rate is greatly influenced by the fibrin network configuration rather than fibre thickness. Multiple factors which include, age, fibrinogen levels, genetic mutations in the fibrinogen genes and several environmental factors (smoking), influence fibrin architecture and resistance to lysis (Undas et al. 2009). Interestingly, fibrin clot properties have been shown to improve following specific pharmacotherapy for chronic diseases, as will be elaborated upon below.

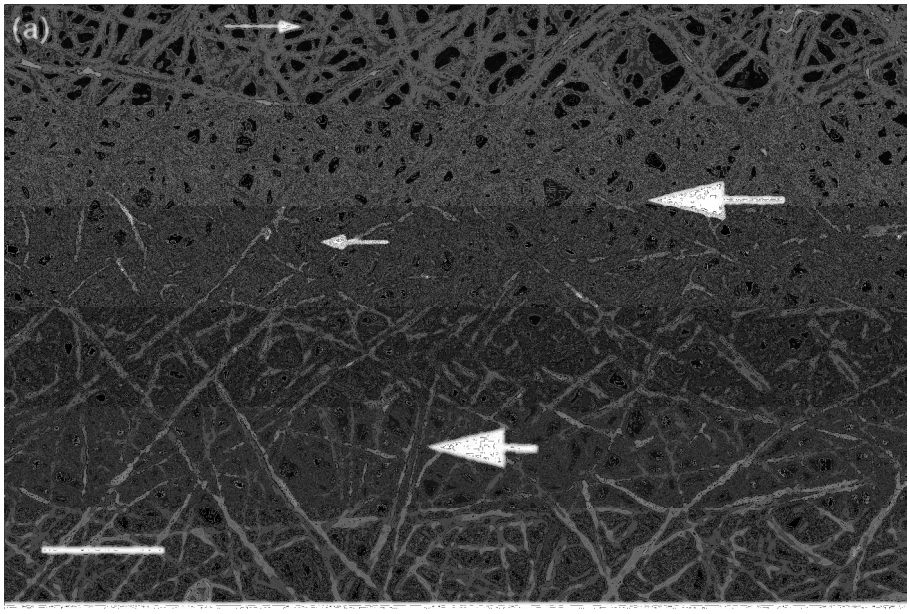


Figure 2.5: **Scanning electron microscopy of a fibrin network architecture, thick arrows representing major fibres and thin arrows the minor fibres, in a healthy control. Taken from (Pretorius et al 2011a). Scale = 1 µm.**

In subjects with chronic obstructive pulmonary disease, structure of the fibrin is altered and is associated with the formation of less permeable, more compact clots but less prone to lysis (Undas et al. 2009). Specific features used to describe a compact fibrin clot includes lowered susceptibility to lysis and lower permeability, typically found in subjects with acute coronary syndrome (Stępień et al. 2011). Depending on the severity of the atherosclerosis, subjects with advanced coronary artery disease have their altered clot structures affected by oxidative modification. On the other hand, young subjects with coronary artery disease, displayed a decreased fibrinolysis rate which is ascribed to higher stiffness, fibre thickness and density (Stępień et al. 2011). Research by Pretorius and colleagues 2014, investigated the ultrastructural changes in red blood cells and fibrin networks in

subjects with hemochromatosis and found profound morphological changes (Pretorius et al. 2014). In particular, the clot in subjects with hemochromatosis featured predominantly with red blood cells trapped within the clot which finally could result in a tighter clot.

2.3.4.3 The role of coagulation proteins in the coagulation cascade

The coagulation cascade entails both clot formation and fibrinolysis. Coagulation proteins play significant roles in both the processes. Diabetic subjects are known to have higher levels of circulating tissue factor (TF), FVII (factor VII), thrombin, fibrinogen, tPA (tissue plasminogen activator) and PAI-1 (plasminogen activator inhibitor-1) (Alzahrani & Ajjan 2010). TF initiates the thrombotic process with the ultimate production of thrombin which is crucial for the conversion of fibrinogen to fibrin. The increased TF levels in diabetes are under the control of both glucose and insulin. In fact, the two controlling factors tend to have an additive effect (Boden et al. 2007). Another mechanism implicated for the elevated TF levels is through the formation of advanced glycation end products and reactive oxygen species (Breitenstein, Camici & Tanner 2010). During the process of plaque rupture TF-FVII complex is formed as can be seen in figure 4. Together with the underlying platelet stimulation, this complex activates different coagulation factors with the ultimate generation of thrombin (Alzahrani & Ajjan 2010). Like TF, FVII is also elevated in subjects with diabetes and in those with the metabolic syndrome. Early work has shown that FVII coagulant activity has been associated with fatal cardiovascular events and more importantly, increased FVII coagulant activity is directly correlated with raised blood glucose (Alzahrani & Ajjan 2010).

In both type 1 and type 2 diabetes thrombin generation is enhanced (Boden et al. 2007). The hyperglycaemia found in diabetic subjects, is the culprit causing increased thrombin production and when the hyperglycaemia is controlled, thrombin production is reduced, proving the prothrombotic nature of hyperglycaemia (Undas et al. 2008). High concentration of thrombin results in altered clot structures as they are denser and less permeable making them more resistant to lysis (Boden et al. 2007). Fibrinogen, the precursor of fibrin is described as an independent risk factor for cardiovascular disease and is often used as a surrogate marker for cardio-vascular risk (Corrado et al. 2010). High

fibrinogen levels are known to have a predictive value in the setting of silent myocardial ischaemia especially in subjects with type 2 diabetes. Multiple mechanisms have been suggested to explain increased fibrinogen levels in diabetes: (a) it may be related to the associated low-grade inflammation, (b) insulin resistance, this is a crucial pathogenic mechanism in type 2 diabetes, which is associated with increased fibrinogen production in response to insulin and (c) fibrinogen levels are further increased in the presence of diabetic complications, highlighting its link to the pathogenesis of macro- and microvascular disease (Alzahrani & Ajjan 2010).

Clot dissolution is achieved by plasmin, with the precursor being plasminogen. The conversion of plasminogen to plasmin is finely controlled by tPA as seen in Figure 2.4. Furthermore, there is evidence that lowering glucose modulates PAI-1. In addition to hyper-glycaemia, hyperinsulinaemia also increases PAI-1 levels (Alzahrani & Ajjan 2010). Activity of tPA has been linked to diabetic complications. The recent work by Sahli and colleagues has shown increased tPA activity in type 2 diabetic subjects that present with peripheral vascular disease (Sahli et al. 2009). More research by Dunn and co-workers showed that clots derived from plasma purified fibrinogen in diabetic subjects had a more compact structure characterised by smaller pore size, increased fibrin thickness and number of branch points than that from healthy controls (Dunn, Ariëns & Grant 2005).

One interesting finding in the study by Undas and co-workers was that there appeared to be an improvement in the fibrin clot properties following a three-month duration of statin therapy in subjects with chronic obstructive pulmonary disease (Undas et al. 2008). Features suggestive of improvement included: more permeable clots, less compact and enhanced lysis. Similar findings were reported when glycaemic control was optimized. After glycaemic control with the use of insulin was obtained, there was a decrease in fibrinogen glycation, increased permeability and increased lysis of the clot (Pieters et al. 2008). Metformin, a common oral hypoglycaemic agent has beneficial effects on clot structure as well. The mechanism of metformin in reducing ischaemic heart disease risk is related to the modulation of early fibrin polymerisation and decreased FXIII cross-linking function (UK Prospective Diabetes Study (UKPDS) group 1998). Drugs used for

the treatment of hypertension and ischaemic heart disease such as angiotensin-converting enzyme inhibitors (ACEI) and angiotensin receptor blockers lower fibrinogen and PAI-1 levels thereby reducing the risk of cardiovascular events (Alzahrani & Ajjan 2010).

2.3.4.4 Description of fibrin networks in diabetes

Diabetic subjects have been shown to have altered fibrin network structures as illustrated in Figure 2.6, and this was first confirmed by Jörneskog and colleagues in 1996. Their findings indicated that plasma clots from Type 1 diabetes subjects have reduced permeability suggestive of a more compact structure which is independent of the presence of microvascular complications (Jörneskog et al. 1996). Using confocal microscopy techniques, Alzahrani and colleagues found that clots made from pooled plasma-purified fibrinogen in diabetic and insulin resistant subjects have a more compact fibrin network structure compared with controls, supporting earlier findings (Alzahrani & Ajjan 2010).

Mechanisms involved in changing the fibrin network architecture in diabetes causes both quantitative and qualitative changes. Hyperglycaemia and insulin resistance induce qualitative changes as a result of increased glycation and oxidation and quantitative changes associated with elevated levels of TF, thrombin, fibrinogen and PAI-1 as discussed above. The final end result is that the clot exhibits a denser structure and resistance to fibrinolysis (Alzahrani & Ajjan 2010). Clots formed at high fibrinogen concentrations show unique properties and includes: thin fibres, reduced pore size and increased tensile strength, also this clot is degraded at a lower rate by plasmin (Marchi-Cappelletti & Suáres-Nieto 2010).

The first intervention trial by Pieters et al, investigated the effect of glycaemic control on fibrin network structures of type 2 diabetic subjects using isolated fibrinogen. A variety of parameters were measured to highlight their findings and included, fibrinogen glycation, clot permeability, turbidity measurements, fibre diameter, visco-elastic properties, lysis rate and effect of fibrinogen glycation on FXIIIa cross-linking (Pieters et al. 2008). Results obtained were as follows:

- Fibrinogen glycation: higher level of fibrinogen glycation among the diabetic group with a significant decrease upon achieving glycaemic control.
- Clot permeability: this parameter reflects the clot structure, specifically the average pore size. This was increased in the diabetic subjects and a significant correlation between permeability and HbA1c was also proven.
- Turbidity measurements: analysis of this parameters results in turbidity curves which are used to characterize the kinetics of polymerization and clot structure. The slope of increase in turbidity, representative of lateral aggregation was higher in the diabetic group.
- Fibre diameter: this was measurement using scanning electron microscopy (SEM), and the median fibre diameter of the clots from the diabetics and controls did not differ.
- Viscoelastic properties: were similar between the controls and diabetics however, there was a lower proportion of inelastic component in the fibrin clots of the diabetic subjects.
- Lysis rate: the diabetic subjects had a lower lysis rate.
- Effect of fibrinogen glycation on FXIIIa cross-linking: No differences were detected among the diabetic and control groups.

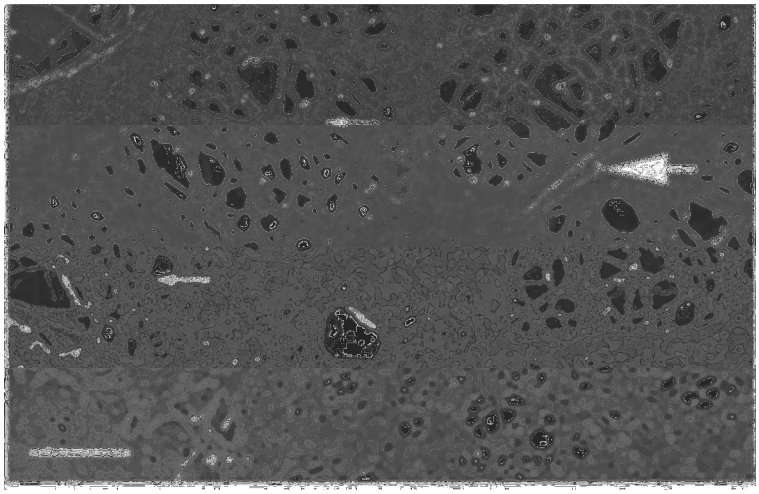


Figure 2.6: Scanning electron microscopy of a fibrin network in a diabetic subject. Thick white arrows represent major, thick fibres; thin white arrows represent netted fibrin masses. Taken from (Pretorius et al. 2011a). Scale = 1 µm.

2.4 Erythrocytes

The erythrocyte is a disc-shaped cell indented in the middle on both sides, with a flattened centre, a biconcave disc measuring 8 mm in diameter, with a thickness of

2 µm at the outer edges and 1 µm at the centre, devoid of organelles. Despite its relative simplistic form, its structural, biochemical and physiological properties continue to fascinate biophysicists, cell biologists and biochemists (Sherwood 2004) and (Shin et al. 2007). It is no surprise that just like platelets and fibrin networks, erythrocytes have been shown to play a role in coagulation as well. This is in striking contrast to their traditional physiological role of oxygen transport. In fact, emerging evidence suggests that erythrocytes enhance functional coagulation properties and platelet aggregation (Brown et al. 2014). Among the spectrum of haematological abnormalities in diabetes, erythrocyte aggregation and decreased deformability of erythrocytes feature strongly (Mahindrakar et al. 2007). More importantly, they are implicated in the pathogenesis of microvascular complications of diabetes (Desouky 2009).

2.4.1 Structural characteristics of erythrocyte membrane

The RBC membrane comprises of proteins, lipids and carbohydrates, arranged in a lipid bilayer and cytoskeleton. Phospholipids and cholesterol are the main components of the lipid bilayer. The phospholipids are asymmetrically dispersed in the bilayer. The outer half of the bilayer contains sphingomyelin, glycolipids and phosphatidylcholine while the inner half consists of phosphatidylinositols, phosphatidylserine and phosphatidylethanolamine. The cholesterol is distributed evenly in the bilayer and accounts for the flexibility and is the crucial component providing stability to the membrane as it undergoes considerable cellular deformation especially in the capillaries (Smith 1987) and (Mohandas & Chasis 1993). Proteins found on the membrane are classified into two groups: integral and peripheral as shown in Figure 2.7.

Integral proteins are the glycophorin and band 3 which are tightly bound to the membrane through hydrophobic interactions lipids in the bilayer. A filamentous network of proteins is anchored to the bilayer by the integral proteins. This network is made up of three principal components: spectrin, actin and protein 4.1, details of which are shown in Figure 2.7. The protein band 4.1 stabilizes the attachment of spectrin and actin. Holding the cytoskeleton and bilayer together is the protein ankyrin which anchors spectrin to band 3 and via band 4.1 to glycophorin as clearly defined in Figure 2.7. The non-linear spring action of spectrin is revealed

under the action of an AFM tip as the individual spectrin chain is stretched (Shin et al. 2007). The membrane proteins provide specific functions. The most abundant trans-membrane protein is band 3 and it is responsible for anion exchange at the level of the plasma membrane. Other proteins serve as channels facilitating movement of ions and the transport of glucose and other small molecules. The cytoskeletal proteins function is to ensure adequate structural integrity and to maintain the biconcave shape of the erythrocyte (Desouky 2009).

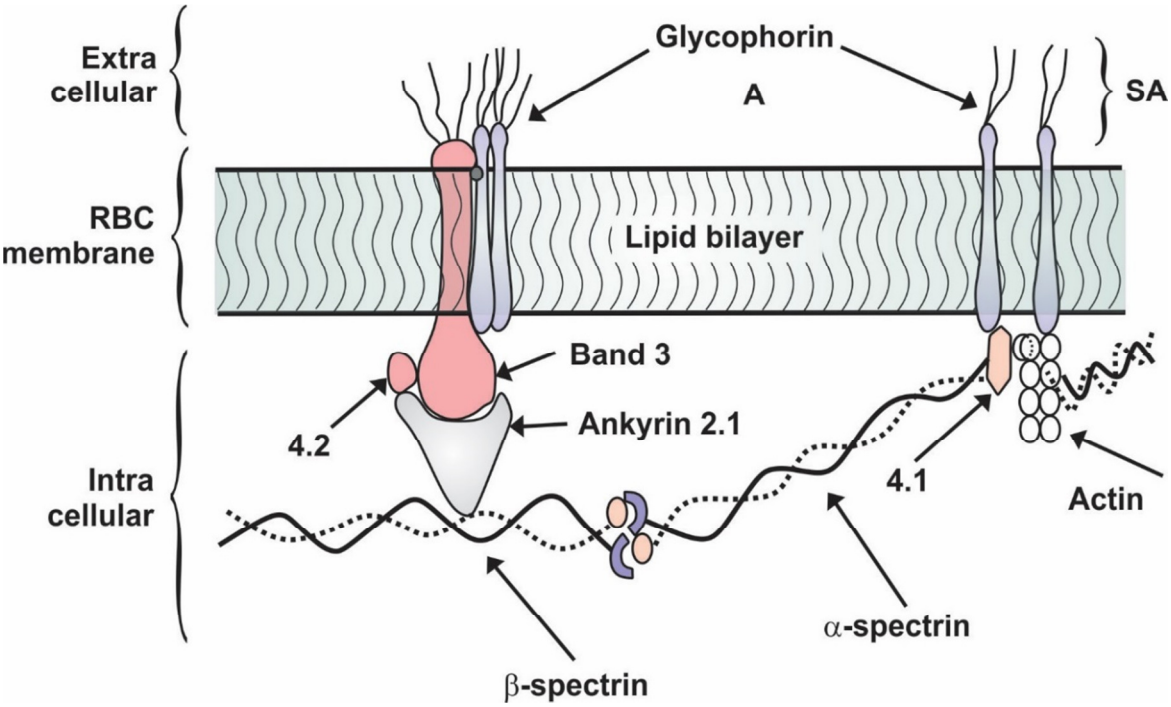


Figure 2.7: Schematic representation of RBC membrane showing protein orientation. Taken from (Desouky 2009). SA=sialic acid.

2.4.2 Major determinants of erythrocyte deformability

Diabetic subjects have their erythrocytes exposed continually to a hyperglycaemic environment and therefore are more prone to compositional changes, which has a direct effect on their flow properties (Singh & Shin 2009). 'Cellular deformability' is the term used to describe the red blood cells (RBC) ability to undergo deformation during flow (Mohandas & Chasis 1993). This characteristic of erythrocytes, the deformability improves blood flow in the microvessels and in large arteries at high shear rate. The major determinants of deformation response include cell geometry, cell shape and internal viscosity (i.e. mean cell haemoglobin concentration and components of the erythrocyte membrane) (Shin et al. 2007).

2.4.2.1 Red blood cell geometry

The dimensions of the erythrocyte are finely suited for its complex functions. The diameter of the cell in its undeformed state is about 8 µm with a membrane surface area of 135 μm^2 and mean cell volume of 94 μm^3. The disproportion in area and volume, with the area considerably greater allows the erythrocyte to deform uniaxially at constant surface area and cell volume under different physiological conditions (Chien 1987).

2.4.2.2 Rheological characteristics of intracellular fluid

The erythrocyte of the diabetic subject is more rigid and has a decreased deformability due to the interaction of haemoglobin with the membrane. In the absence of shear stress, when erythrocytes are placed in suspension they adhere face-to-face and form aggregates called rouleaux (Desouky 2009). At normal mean cellular haemoglobin concentration (MCHC), blood viscosity is low (7 mPas) and has exhibits no elastic properties. However as MCHC increases, viscosity increases (Chien 1987)

2.4.2.3 Rheological characteristics of erythrocyte membrane

The erythrocyte membrane has been described in detail above. Alterations in the membrane lipids results in a membrane that is rigid and non-deformable. This abnormality therefore, predisposes the cells to be disrupted as they circulate through microvessels (Caimi et al 1992).

2.4.3 Oxidative stress and the erythrocyte

Oxidative stress is a term referring to the presence of products called free radicals and reactive oxygen species (ROS). Even though their production is part of normal physiological processes, adverse events occur when their synthesis exceeds their removal. For this purpose, antioxidants are essential (Fang, Yang & Wu 2002). Mounting evidence has demonstrated that diabetes is an oxidative stress disorder and it is responsible for the production of reactive oxygen species which are contributory factors for initiation and progression of ageing, atherosclerosis and other complications in diabetes (Saha et al. 2005). The study by Adak et al, explored how hyperglycaemia induced oxidative stress modifies the erythrocyte membrane dynamic and electrokinetic properties in diabetic and diabetic

cardiovascular disease (CVD) subjects with reference to healthy control. Their results investigated potential mechanisms that might underlie this association of CVD with diabetes mellitus (Adak, Chowdury & Bhattacharyya 2008). The latter included:

- Enhanced lipid peroxidation in diabetes and diabetic CVD subjects compared to the erythrocytes from control subjects. (Lipid peroxidation is an index of membrane damage which promotes irreversible dysfunction of essential cellular components and ultimately triggers accidental cell death and necrosis (Kolwaltowski & Vercesi 1999).
- Osmotic fragility experiment reveals erythrocytes of diabetic CVD patients to be more fragile compared to diabetic and control subjects.
- Decreased erythrocyte membrane fluidity has been evidenced in diabetes and diabetic CVD, which reveals significant alteration in the dynamic properties of the hydrophobic core of the erythrocyte membrane in pathologic state.
- Decreased zeta potential (erythrocytes have a strong net negative charge called the zeta potential produced by the scialoglycoprotein coat such that approximately 18 nm is the shortest span between two cells).
- Significant decrease of ankyrin level in diabetic and diabetic CVD. (Ankyrin is an extrinsic protein of the red cell membrane which links cytoskeletal network to the membrane by forming a bridge between spectrin and transmembrane anion-channel protein, band 3.)

2.4.4 Diabetic induced changes in the erythrocytes

2.4.4.1 Alterations in erythrocyte structure

In diabetic subjects the erythrocyte membrane becomes rigid and non-deformable. A decrease in the cholesterol to phospholipid ratio is responsible for this abnormality. Not only is the cholesterol component of the membrane increased but there is a four-fold increase in the phospholipids concentration, which results in a significant decreased ratio (Baynes 1991). Cytoskeletal proteins, in particular, beta spectrin, ankyrin and protein 4.1 are heavily glycosylated (Schwartz et al. 1991). Disturbances in ionic balance is attributed to the lowered Na^+/K^+ - ATPase activity and this leads to complications such as increased serum and intra-erythrocyte sodium and serum potassium in diabetic subjects. The alterations in intracellular

ionic balance contributes to acceleration of cellular ageing (Gürbilek et al. 2004). This also results in an increase in the cell size and increased osmotic fragility which contributes to the development of microvascular complications (Kowluru et al. 1989). Elevated fibrinogen and glucagon is a common finding is uncontrolled diabetes (Singh & Shin 2009). Oxidative stress plays a role in causing increased membrane lipid peroxidation and this may lead to abnormalities in composition and function. Enhanced levels of malondialdehyde (an indicator of lipid peroxidation) (Rizvi et al. 2005) and reduced levels of glutathione and membrane – SH group are features of the diabetic erythrocyte (Srour et al. 2000).

2.4.4.2 Alterations in erythrocyte aggregation and deformability

The property of aggregation is guided by the composition of erythrocyte membrane and plasma proteins fibrinogen and globulin. When fibrinogen levels are increased and albumin is decreased, aggregation is enhanced. A mechanism that favours increased tendency of erythrocyte aggregation is the decreased ionic charge of the membrane. Microscopic examination of the erythrocyte aggregate reveals an increase in aggregate shape and size when compared to healthy controls. The parameter erythrocyte deformability unlike aggregation, is modified by the composition of the membrane, cytoplasmic contents and age of the erythrocytes (Singh & Shin 2009). It is the measure of the ability of the cells to deform under applied shear stress (Shin et al. 2007). Advanced technological measurements confirm that in diabetes, deformability is significantly decreased. This abnormality is attributed to the specific changes in the membrane structure. The consequence of altered deformability is the increase in blood viscosity which can lead to increase in shear stress on the endothelial wall (Singh & Shin 2009). Investigation of diabetic erythrocytes with cardiovascular complications are associated with lowered membrane fluidity when compared to healthy controls. The diffusion of protein and lipid molecules within the membrane is known as membrane fluidity and is dependent on the presence of saturated and polyunsaturated fatty acid (McMurchie & Raison 1979).

Diabetes, a multifactorial disease has a marked effect on the rheological and electrical properties (not discussed in this chapter) of the erythrocyte. The erythrocyte is commonly described as more rigid than normal with a reduced

deformability (Desouky 2009). However, diabetes also causes profound changes on the ultrastructure of erythrocytes, fibrin networks and platelets. Pretorius and co-workers showed a changed morphology in type 2 diabetic erythrocytes using the scanning electron microscope (SEM), which showed elongated cells forming extended projections that twist around spontaneously formed fibrin fibres as indicated in Figure 2.8A, Figure 2.8B. In addition, the membrane could also become

smooth as seen in Figure 2.8C. These findings were correlated with changes detected using atomic force microscopy (AFM). AFM was opted to determine the shape and membrane roughness. Like the SEM, the AFM highlighted that the erythrocytes were smaller with diminished height, diameter and concave depth. The utilization of AFM measurements as pointed out by Pretorius et al, was deliberate as it allowed the isolation of three specific spatial domains each representing a typical erythrocyte feature (Buys et al. 2013). Measurement of the surface roughness using AFM revealed deviations in the first order surface of the cell, the roughness of the second order was also decreased reflecting changes in the cytoskeletal matrix and the connections between band 3 and band 4 proteins and the matrix. Finally, a decrease in the roughness parameters in the third order surface is indicative of the superficial protein structure rearrangement (Buys et al. 2013). The findings of the second and third order findings of the AFM described above can be linked to structural abnormalities found in skeletal protein and lipids of the diabetic erythrocyte. Research has shown that several erythrocyte membrane proteins are heavily glycosylated, viz., the beta-spectrin, ankyrin and protein 4.2 and that spectrin is damaged via oxidative stress (Schwartz et al. 1991).

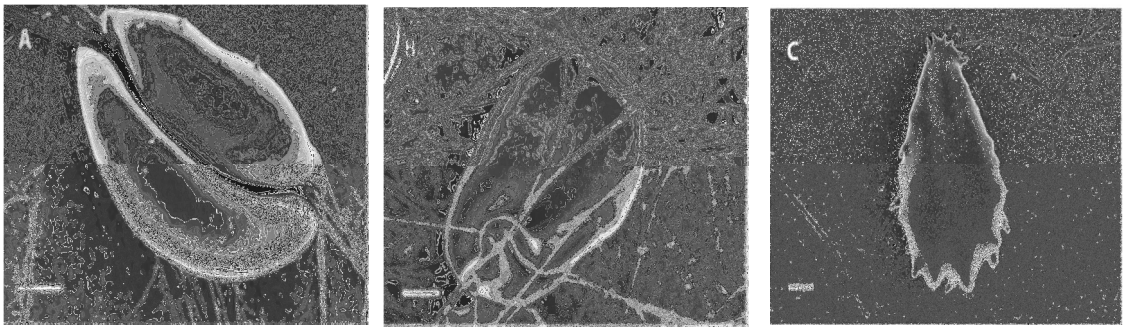


Figure 2.8: **Scanning electron microscopy of an erythrocyte in a diabetic subject: (A) RBC with very smooth membrane twisted around spontaneously formed fibrin fibres; (B) RBC showing lengthened ultrastructure; (C) RBC showing smooth membrane. Scale = 1 µm. Taken from (Buys et al. 2013).**

2.5 Discussion

Accelerated atherosclerosis is the main underlying factor contributing to the high risk of atherothrombotic events in patients with diabetes mellitus and athero-thrombotic complications are the main cause of mortality (Ferreiro, Gómez-Hospital & Angiolillo 2010) and (Alzahrani & Ajjan 2010). Like with many bodily systems, pathology is observed when the normal processes are exaggerated or uncontrolled. This applies to the processes of coagulation and thrombosis as well. In diabetes, in fact, the balance between prothrombotic and fibrinolytic factors is impaired and thus the scale is tipped towards a prothrombotic and hypofibrinolytic milieu, which in association with the vascular changes accompanying plaque formation and rupture, increases the prevalence of ischaemic events such as angina and myocardial infarction (Levi, van der Poll & Büller 2004). The morphology of platelets, fibrin networks and erythrocytes are thus essential role players in unravelling the pathogenesis of cardiovascular complications in diabetic subjects.

Among classical risk factors for atherosclerosis, diabetes features strongly, furthermore it is also associated with elevated levels of markers of subclinical inflammation. It is also known that inflammation plays a central role in the evolution of atherosclerosis, in particular, inflammation is an important feature of plaque initiation, progression and thrombosis (Gustavsson & Agardh 2004) and (Pradhan et al. 2002). The inflammatory response forms part of the body's normal protective mechanism when exposed to pathogens, infectious agents, mechanical factors and many more. One crucial component of the inflammatory response is activation of the coagulation system. Research suggests that coagulation also affects the inflammatory response. Evidence of this is highlighted during investigation of a thrombus on a ruptured atherosclerotic plaque as depicted in Figure 2.9, which shows that it is composed of a vast number of inflammatory cells, and which clinically presents as unstable angina or myocardial infarction (Levi, van der Poll & Büller 2004). This cross-talk between coagulation and inflammation occurs at multiple levels: at level of platelet activation, fibrin formation

and resolution including physiological anticoagulant pathways (Levi, van der Poll & Büller 2004).

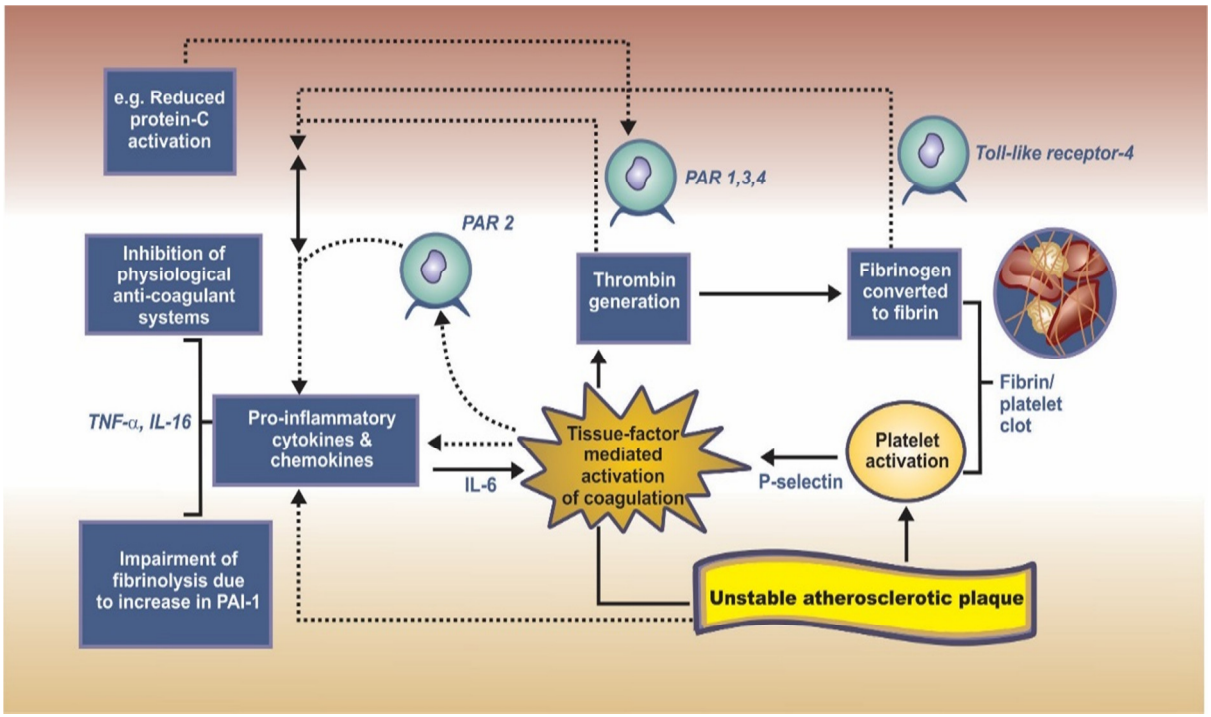


Figure 2.9: **The schematic representation of activation of coagulation and inflammation on rupture of atherosclerotic plaque. Taken from Levi van der Poll & Büller 2004. Coagulation pathways are indicated by straight arrows; inflammatory mechanism by dashed arrows.**

Evidence of coagulation and thrombosis contributing to pathology is highlighted by careful inspection of Figure 2.9. Upon rupture of the atherosclerotic plaque shown in yellow, both the processes of coagulation and inflammation are activated. Exposure of tissue factor-bearing inflammatory cells to blood results in thrombin generation and subsequent formation of fibrin from the conversion fibrinogen. Concurrently, activation of platelets is initiated occurs, stimulated both by thrombin and by exposure of collagen to blood. This promotes the binding of tissue factor, thrombin and other activated coagulation proteases to specific protease activated receptors (PARs) on inflammatory cells and induce release of pro-inflammatory cytokines. This binding is the most important mechanism by which coagulation proteases influence inflammation. Four types of PARs have been identified, PAR 1-4. Once pro-inflammatory cytokines are released, they will further modulate coagulation and fibrinolysis.

Insulin resistance is a common finding in subjects with type 2 diabetes as are the complications in the macrovascular and microvascular circulation. The insulin resistance is responsible for numerous alterations both at the metabolic and cellular levels. Included in the target tissues of insulin, in particular, the cellular systems that are affected is the endothelial cells, platelets, monocytes and erythrocytes (Vink AI, 2001). Insulin's action on platelets is to sensitize platelets to the inhibitory effects of prostacyclin and nitric oxide on aggregation and to diminish the proaggregatory properties of agonists such as prostaglandin E1, and E2. During platelet aggregation and activation, mechanisms like phospholipase C-induced hydrolysis of inositol phospholipids and opening of ion channels are activated. This results in various physiological responses by inducing changes in the phosphorylation state, activity of enzymes and structural properties of key platelet proteins (Vink et al. 2001).

The study by Pretorius and colleagues that examined the ultrastructure of fibrin networks and platelets, found unsuspected findings regarding platelets in diabetic subjects (Pretorius et al. 2011a). Barely no pseudopodia were seen, which normally develop spontaneously from platelets, as shown in Figure 2.3. The striking finding was the changed platelet membrane ultrastructure, which showed features of blebbing. The blebbed morphology was ascribed to the process of apoptosis. Wyllie et al, best defined apoptosis as a form of cell death characterized by cell shrinkage, nuclear condensation and membrane blebbing and fragmentation of the cell membrane into membrane-bound apoptotic bodies that are eliminated by phagocytosis (Wyllie, Kerr & Currie 1980). The finding of membrane blebbing is critical as it may cause an increase in microparticles in diabetes. Microparticles are membrane-coated vesicles that originate by budding from their parental cells upon activation or apoptosis (Burnier L, 2009). They also have the ability to activate the coagulation cascade with consequent thrombosis formation (Morel et al. 2010).

Subjects with diabetes have higher than normal tissue factor levels. More important is that tissue factor activity is controlled by both insulin and glucose. Another stimulating factor for the synthesis of tissue factor particularly in diabetes, is glycation end products and ROS. Also, the elevated production of thrombin in

diabetic subjects has a direct effect on the clot formation, structure and stability. The final product is thus a denser, less permeable clot which is more resistant to lysis. Linking the concept of diabetes as a prothrombotic state and inflammation is the elevated cytokine, IL-6 which stimulates the hepatocytes to produce more fibrinogen. Increased production of fibrinogen by hepatocytes is also a common finding in insulin resistance (Balasubramaniam et al. 2012). The findings by Pretorius et al, collaborated the association of inflammation and diabetes as the fibrin network profile of thin, minor fibres forms a distinct net over the major fibres as indicated in figure 6, which is the typical morphology seen in inflammatory conditions (Pretorius et al. 2011a).

Despite the absent mitochondria in the erythrocytes, they still depend on glucose as their energy source. However, in a hyperglycaemic environment, glycosylation of haemoglobin takes place which creates oxidative stress thereby making the cellular components of the erythrocyte more vulnerable (Vahalkar & Haldankar 2008). One of the functions of a membrane is to provide protection and this includes against oxidative damage as well. However, in diabetic subjects, lipid peroxidation causes structural damage to the membrane with a subsequent decrease in the cell deformability and fluidity. SEM and AFM findings in the study by Buys et al, confirmed the correlation between the cytoskeletal protein and lipid layer damage with the ultrastructural roughness of the erythrocyte membrane found with AFM (Buys et al. 2013). As mentioned previously, evidence points to glycosylation of cytoskeletal proteins and oxidative damage of spectrin (Shin et al. 2007). The adverse effects of glucose manifests in multiple ways: rearrangement of erythrocyte membranes, defects in haemoglobin oxygen binding activity, alterations of mechanical features of the membrane and general aspects of the cell as well (Desouky 2009).

2.6 Conclusion

Platelet dysfunction poses an increased risk for thrombotic vascular events. The significance of platelet abnormalities in the atherothrombotic process has been highlighted by the use of antiplatelet drugs that form part of the therapeutic regime in reducing cardiovascular risk (Colwell & Nesto 2003). The multifactorial aetiologies for platelet dysfunction is beyond the scope of this review, however,

diabetes and the associated hyperglycaemia cannot be ignored. Hyperglycaemia induces a hypercoagulable condition and contributes to micro- and macrovascular disease. Studies have shown that optimal control of both fasting and post-prandial glucose levels will reduce the impact (Kluft & Jespersen 2002). It remains to see if the latter will improve morphological findings of the platelet ultrastructure in diabetic subjects.

Suppression of fibrinolysis and increased fibrinogen are among the array of haemostatic abnormalities in type 2 diabetes. Increased fibrinogen adds to the burden of cardiovascular risk by increasing blood viscosity, increasing the size of the clot, tissue deposition is increased and stimulation of atherosclerosis and vascular thickening (Kluft & Jespersen 2002).

Erythrocytes too contribute to the high incidence of atherosclerotic diseases in diabetes partly due to the association of abnormalities of erythrocyte composition and rheological function with increased oxidative stress (Emam et al. 2008).

Clearly the abnormal ultrastructural findings of erythrocytes, platelets and fibrin network in diabetes should be considered as part of the clinical examination.

3. CHAPTER 3: SERUM INFLAMMATORY MARKER - FERRITIN, ITS CHEMISTRY AND LINK TO TYPE 2 DIABETES MELLITUS

3.1 Abstract

Iron deficiency anaemia is the most common nutritional anaemia worldwide. Yet, it is also known today that elevated body iron stores can lead to pathology. Increased iron stores have been associated with high blood pressure, dyslipidaemia, metabolic syndrome and increased risk of cardiovascular disease. Emerging evidence suggests that there is a strong link between type 2 diabetes mellitus and high body iron levels. The chemical structure of iron facilitates its role in the formation of reactive oxygen species. Superoxide has the ability of releasing iron from ferritin and the released iron can result in hydroxyl formation. The beta-cells are particularly susceptible to oxidative stress, which in turn culminates in pancreatic beta-cell damage resulting in decreased insulin secretion, insulin resistance and subsequently type 2 diabetes mellitus. Free radical species are key players in both myocardial ischaemic and reperfusion injuries. Part of the inflammatory response is elevated levels of fibrinogen. This influences the coagulation process, as in the presence of high fibrinogen, it causes abnormal fibrin fibre formation. These morphological abnormalities are best visualised by scanning electron microscopy. Abnormal ultrastructural features caused by high ferritin levels in the red blood cells and fibrin fibres are shown.

3.2 Introduction

In clinical practice serum ferritin forms part of routine laboratory testing and thus high levels of serum ferritin is becoming a common finding. Its incidence is likely to increase since both acute and chronic inflammation are associated with elevated ferritin levels (Camaschella & Poggiali 2009). Inflammation is the primary immune system response to get rid of pathogens in order to restore the cells to normal (Emmendoerffer et al. 2000). Current research points to chronic inflammation playing a role in various age-related diseases like diabetes, cardiovascular and autoimmune (Khansari, Shakiba & Mahmoudi 2009). In fact, increased levels of markers of systemic inflammation have been described in subjects with diabetes when compared to those without diabetes (Munter et al. 2004). Recent evidence is also suggesting that elevated levels of ferritin may assist to identify individuals at

high risk of type 2 diabetes. This is of particular interest since type 2 diabetes and its complications are becoming a global pandemic (Kunutsor et al. 2013). An outline of the chapter is depicted in Figure 3.1.

3.3 Chemical properties of iron

Trace elements even though found in minute amounts in the body; are considered to be essential to life. One such element is iron. In the earth's crust, iron is the fourth most abundant element and second most abundant metal (Bleackly et al. 2009). It plays a crucial role in a variety of cellular functions in metabolism, growth and differentiation, such as oxygen transport and storage, energy production, cell cycle and DNA synthesis (Theil 1987). The biological functions of iron are based on its chemical properties such as the ability to produce different complexes with organic ligands and its redox ability to exist in both Fe^{2+} and Fe^{3+} forms (Papanikolaou & Pantopoulus 2005).

The normal iron content in an adult man is 35 – 45 mg of iron per kilogram of body weight. Most of the body iron; almost 80% is incorporated into haemoglobin with 10-15% found in myoglobin. A 70 kg adult has about 3 mg of iron in the plasma, with all bound to transferrin (Emerit J Beaumont & Trivin 2001).

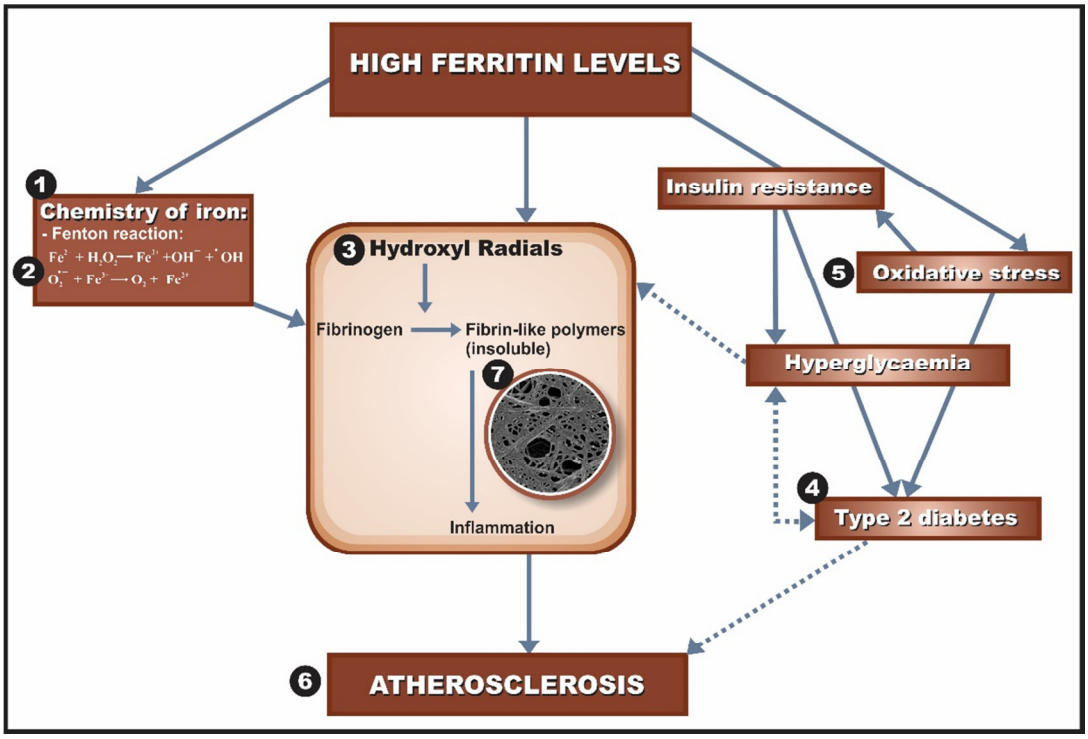


Figure 3.1: An outline of the chapter

The chemistry of iron (1) is briefly summarised with particular attention to the Fenton reaction (2). The end product of the Fenton reaction, the hydroxyl radical (3) and its effects on coagulation is described with detail on the morphology of the fibrin network (7). The multiple effects of elevated ferritin levels and its contribution to type 2 diabetes (4) and oxidative stress (5) is explored. Finally, the manner in which all factors contribute to the pathogenesis of atherosclerosis (6) is reviewed.

Iron absorption which takes place in the enterocytes lining the villi close to the gastroduodenal junction, is highly regulated since there is no physiologic pathway for active iron excretion (Heath & Fairweather-Tait 2002). The destination of the iron inside the enterocyte has 2 possible routes: it may be stored as ferritin or it may cross the basolateral membrane to reach the plasma transported by its transfer protein, transferrin (Emerit, Beaumont & Trivin 2001). Iron present in the circulation exists in two forms, transferrin bound iron and non-transferrin bound iron (NTBI) (Batey et al. 1980). Biochemically, NTBI is important as it has a significantly higher redox potential (Cotton, Thiry & Boeynaems 2000).

3.4 Toxicity of Iron

Under physiological conditions, free iron is found in one of two oxidation states, the soluble ferrous (Fe^{2+}) and insoluble ferric (Fe^{3+}) form (Bou-Abdallah 2010). Iron thus has the ability to accept and donate electrons readily; making it physiologically useful but biochemically hazardous as it can catalyse the conversion of hydrogen peroxide to free-radical ions that are potentially harmful (Emerit, Beaumont & Trivin 2001). A free radical is best described as any species that has the ability to exist independently and is reactive. Its reactivity is brought about by at least one unpaired electron in its outer atomic orbital. The unpaired electron is (denoted by $\bullet^-$) and examples are superoxide and hydroxyl radicals. An interesting characteristic of radicals is that they can react with each other to form nonradical derivatives like H_2O_2 that can cause more deleterious effects than the parent radicals (Halliwell 2005). The involvement of iron in an oxidation-reduction reaction with dioxygen results in the formation of superoxide radical $O_2^{\bullet -}$ which because of its ability to accept another electron and two protons produces hydrogen peroxide, (H_2O_2) as shown in the reaction below:

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

The formation of H_2O_2, now precipitates the formation of the reactive and harmful hydroxyl radical species via the Fenton reaction; whereby ferrous ions react with H_2O_2, whilst the superoxide radical reduces the ferric ion to ferrous ion (Wardman & Candeias 1996).

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

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

The hydroxyl radical, an end-product of the Fenton reaction, is a powerful reactive oxygen with the potential to damage DNA and cause lipid peroxidation and protein modification (Welch et al. 2002). To halt these reactions, the free, redox-active iron (Fe^{2+}) is sequestered in plasma in a safe redox-inactive form by ceruloplasmin (Gutteridge & Quinlan 1992). Ceruloplasmin is a copper-dependent metalloenzyme with unique plasma ferroxidase activity that maintains iron in the less chemically reactive oxidized state as Fe^{3+}. Another method to prevent the participation of iron in the Fenton chemistry reactions; is having it bound to transferrin as it is catalytically inactive. The site for the storage depots of iron are the hepatocytes and the reticuloendothelial macrophages. In healthy subjects, 30% of circulating transferrin binds to iron. In pathological conditions presenting with iron overload, transferrin binding sites are saturated by iron and forms redox-active, low-molecular-weight chelates. NTBI is eventually internalized into cells and tissues causing cell damage and tissue injury (Papanikolaou & Pantopoulus 2005).

3.5 Structural overview and functions of ferritin

There is a positive correlation between serum ferritin and body iron stores (Addison et al. 1972) and (Walters, Miller & Worwood1973). Ferritin is a key role player in iron homeostasis since it binds and sequesters intracellular iron. Normal levels in adults: men:12 to 300 ng/ml and women:10 to 150 ng/ml (Rajpathak et al. 2009). Ferritin synthesis occurs in all cells but predominately in hepatocytes and macrophages (Emerit, Beaumont & Trivin 2001). Serum ferritin is not only an indicator of iron stores and may reflect other mechanisms, particularly subclinical systemic inflammation related to insulin resistance and the risk of type 2 diabetes

(Witte et al. 1996). Ferritin not only forms part of the iron regulatory proteins, crucial in maintaining iron homeostasis, but ferritin is also a member of the protein family that facilitates the cellular defence against stress and inflammation (Pan & Jackson 2008).

Ferritin is a large protein (12 nm in diameter, 480 000 Da) comprising of a large cavity (256 nm^3) characterized by an assembly of 24 ferritin polypeptides folded into four-helix bundles bound to each other by hydrogen and salt bonds as shown in Figure 3.12. Iron is identified as a cofactor in the dioxygenases and as a substrate in ferritin (Theil 2000). Investigation of the ferritin molecule in most species illustrate a specific assembly of the subunits which are tightly packed leaving eight narrow hydrophilic channels around the 3-fold axes and six hydrophobic channels around the 4-fold axes as illustrated in Figure 3.23 (Harrison 1996) and (Chasteen 1998). The channels perform particular functions, the hydrophobic ones facilitate the diffusion of oxygen and hydrogen peroxide, while the hydrophilic channels allow the entry and exit of iron, in animal ferritin (Liu & Theil 2005) and (Theil 2000).

Ferritin is made up of a hollow sphere which comprises 24 subunits of 2 types, H and L subunits. Iron is stored within the lumen of the sphere and up to 4500 iron atoms can be stored in each ferritin multimer. It is the transport of iron through this multimer core that facilitates the oxidation of Fe^{2+} to Fe^{3+} (Torti & Torti 2002), (Carrondo 2003) and (Harrison 1977). Ferritin is an intracellular protein and large amounts are located in the cytoplasm with smaller amounts in the plasma. The regulation of ferritin expression is finely monitored by the metabolically active iron pool. This is facilitated by the presence of sequences on the ferritin gene structure that is capable of sensing the labile iron pool. It is the interaction between the iron regulatory proteins (IRP's) and iron responsive elements (IRE) localised in the 5′ untranslated region of the H and L ferritin mRNA that is crucial to the regulation of ferritin expression (Recalcati S, 2008) and (Koorts & Viljoen 2011). The IRE creates a binding site for the two IRP's, IRP-1 or IRP-2. The main regulatory factor of iron homeostasis is dependent upon the post-transcriptional control of ferritin and transferrin receptor mRNAs, as they are recognised by IRP-1 and IRP-2 which control their translation and stability respectively (Puntarulo 2005).

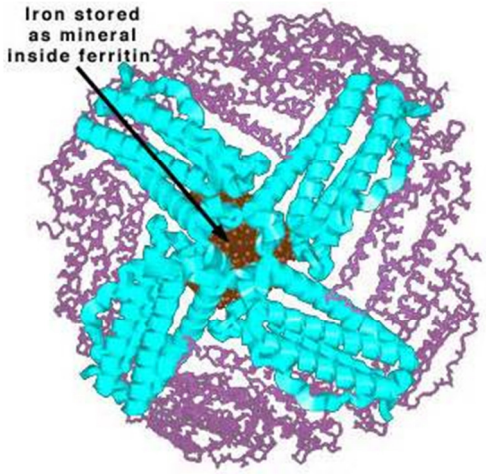


Figure 3.2: **This is a three-dimensional representation showing ferritin, the iron-storage protein in the body. Ferritin has a spherical shape, and iron (brown) is stored as a mineral inside the sphere. www.chemistry.wustl.edu.**

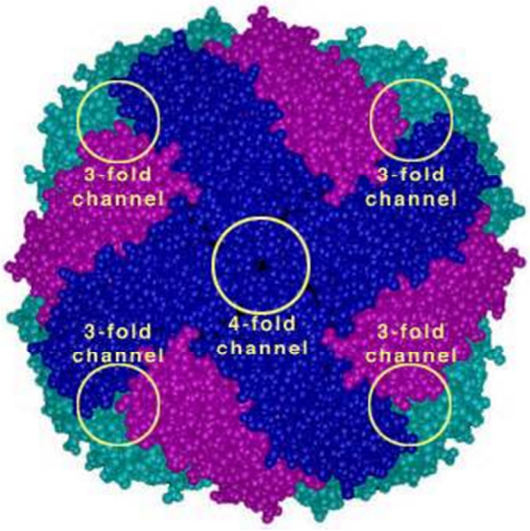


Figure 3.3: **This is a molecular model of ferritin in the CPK representation. CPK pictures represent the atoms as spheres, where the radius of each sphere is equal to the van der Waals radius of the atom. www.chemistry.wustl.edu.**

3.6 Oxidative stress

An imbalance between oxidant and antioxidant levels is instrumental in causing oxidative stress. Oxidative stress appears to be involved in over 100 diseases, (among which, diabetes mellitus, features prominently) but interestingly, more as a consequence of the pathology than as the aetiological factor (Halliwel 2005). Oxidative stress implies an increase in steady state concentration of reactive oxygen and nitrogen species. The highly reactive hydroxyl radical, an end-product of the Fenton reaction is responsible for the harmful effects to membranes, proteins and DNA, as described above. Even though the amount of iron within the

cell is finely regulated, accumulation of iron to toxic levels do occur and it is this iron excess that is the culprit causing oxidative stress (Puntarulo 2005). The body has mechanisms in place to counteract the damaging effects of free radicals in the form of antioxidants (cellular) such as superoxide dismutase, catalase, thioredoxin reductase, vitamin C, vitamin E, glutathione, glutathione-S-transferase and glutathione peroxidase (Halliwel 2005). As an example, one of the most important roles of glutathione is the protective function it offers to erythrocytes which are more prone to oxidative damage based on its transport function as the oxygen carrier in the body (Kennett & Kuchel 2006). More importantly, the level of antioxidant enzyme activity is linked to the susceptibility of tissues to oxidative stress and this is a crucial determinant related to the development of the complications in diabetes (Bisht & Sisodia 2010).

During the Fenton reaction, as detailed previously, hydroxyl radicals are produced in which hydrogen peroxide is an essential substrate. However, Lipinski and Pretorius have demonstrated that reactive free radicals can also be produced in the presence of ferric ions alone as indicated in the reaction $Fe^{3+} + HO^{-} \rightarrow Fe^{2+} + HO^{-}$ (Lipinski & Pretorius 2012). Hydroxyl radicals can convert soluble human fibrinogen into insoluble and plasmin-resistant polymer which are implicated in adding to chronic inflammation and making subjects more vulnerable to thrombosis, especially diabetic subjects (Lipinski & Pretorius 2012). The hyperglycaemia that is prevalent in diabetes, is commonly associated with an elevated level of free radicals. The hyperglycaemia induces binding of glucose to proteins, thereby glycating them with the final product being the formation of advanced glycation end products and many free radicals. (Keenoy et al. 2001) In diabetes particularly, the glycation of proteins is a significant process as it markedly increases the rate of free radical production which in turn will increase peroxidation of polyunsaturated fatty acids in the cell membrane, double to what is found in controls (Mullarkey et al. 1990). The glycation of proteins explains the clinical significance of the laboratory test, glycated haemoglobin (HBA_{1c}) which is elevated in subjects with poor glycaemic control. Since the lifespan of the erythrocyte is 120 days, the HBA_{1c} is an accurate measurement of glucose control of the past four months (Arese & Schwarzer 2003).

One of the mechanisms contributing to the increased risk of cardiovascular disease in diabetic subjects is the disparity between free radicals and antioxidants as this causes oxidative damage to biomolecules (Sies 1991). Evidence thereof is found in blood samples of diabetic subjects, particularly those with cardiovascular complications where high levels of lipid peroxidation products are found (Griesmacher et al. 1995), (Gallou et al. 1994). Iron induced oxidative stress in erythrocyte membranes of non-insulin dependent diabetics in Nigeria has been evaluated by Okunade and co-workers (Okunade GW, 1999). The results in their study highlighted the increased predisposition of erythrocyte membranes to lipid peroxidation and the formation of secondary breakdown products which are thiobarbituric acid reactive when compared to control subjects. In addition, the methods deployed to estimate non-heme iron and reaction thiol content of the erythrocyte membranes illustrated significant amounts of non-heme iron and reduced levels of thiols in these membranes (Okunade, Odunuga & Olorunsogo 1999). It is not only non-heme iron that is implicated, but iron atoms coming from intracellular ferritin can also auto-oxidize to form lipid-soluble iron-oxygen complexes, essential for the formation of reactive free radicals which can then lead to membrane abnormalities and subsequent tissue damage. This would support evidence that oxidative stress plays an important role in the pathogenesis of diabetes and its complex complications (Okunade, Odunuga & Olorunsogo 1999).

3.7 Cardiovascular risk

Despite the advancements made in reducing mortality due to cardiovascular disease, it remains a leading cause of mortality in developed countries. Modifications of traditional risk factors such as smoking, elevated cholesterol; high blood pressure and diabetes appear to be insufficient as they account for 50% of the incidence of cardiac disease. This may suggest that other less well-studied risk factors may play a role. Elevated iron is one such factor (Alpert 2004). Exploration of a pathological model for atherosclerosis reveals that through its free-radical damage caused by elevated iron, it triggers the inflammatory process, thereby promoting atherosclerosis (Alpert 2004). However, a moderate increase in iron load is implicated in accelerating thrombus formation especially following arterial injury, increasing vascular oxidative stress and altering vasoreactivity (Bester et al. 2014). Of greater importance is that vascular dysfunction caused by the chronic

iron-overload increased one's predisposition to developing an ischaemic cardio-vascular event (Bester et al. 2014).

The hypothesis that iron is a risk factor for cardiovascular disease was first postulated by J Sullivan in 1981 (Muñoz-Bravo et al. 2013). In 1992, evidence was presented that highlighted the connection between ferritin levels and the risk of cardiovascular disease in Finnish men (Salonen et al. 1992). In fact, Finnish men with an elevated but apparently normal ferritin had a two-fold risk of developing a myocardial infarct (Salonen et al. 1992). However, subsequent studies ranging from cross-sectional, case-control, retrospective cohort, prospective and clinical trials have not produced conclusive evidence to support the iron hypothesis (Muñoz-Bravo et al. 2013). Yet, through its role in free-radical formation, iron is implicated in the process of atherosclerosis (Pretorius & Lipinski 2013b).

A thrombus formed in the coronary artery on a ruptured atheromatosus plaque leads to the clinical problem of acute coronary syndrome (ACS). Of note, it that one of the main constituents of the thrombus is fibrin, which in turn, is a component of the atherosclerotic plaque (Undas et al. 2008). Investigation of the fibrin clot properties by Undas et al, demonstrated that the clots of subjects with ACS, were composed of dense networks and were more resistant to lysis (Undas et al. 2008). During the physiological process of coagulation, thrombin is a key component in converting fibrinogen to fibrin. As previously eluded to, ferric ions have the propensity to produce hydroxyl radicals in the absence of hydrogen peroxide. These hydroxyl radicals are powerful and can convert fibrinogen molecules into insoluble fibrin-like polymers without the essential action of thrombin (Lipinski & Pretorius 2012). Of significance is that such polymers are not only resistant to fibrinolytic dissolution but also to proteolytic digestion (Pretorius & Lipinski 2013b). The way in which these insoluble fibrin-like polymers contribute to the pathophysiology of cardiovascular disease is that the body views them as a foreign body with consequent recruitment of macrophages with the resultant being a continuous state of inflammation which is known to be associated with atherosclerosis (Ross 1999) and (Hansson 2005).

3.8 Link to Diabetes

Previous research using cross-sectional and case-control studies have confirmed a positive association between levels of ferritin and the risk of developing type 2 diabetes (Ford & Cogswell 1999), (Tuomainen et al. 1997) and (Perez de Nanclares et al. 2000). The most original evidence elucidating a link between iron and diabetes in humans was based on clinical observations of individuals with hereditary hemochromatosis (HH) and transfusional iron overload, both conditions are of pathologic iron overload (Buysschaert et al. 1997). The precise pathophysiology of diabetes in HH cannot be identified as evidence suggests both insulin deficiency (direct deposition of iron in pancreatic islets with β-cell failure) and insulin resistance can be implicated as causative factors (Hramiak, Finegood & Adams 1997), (Rajpathak et al. 2009). HH is commonly associated with markedly elevated levels of ferritin, typically in the range 1000 – 10 000 ng/ml. It is estimated that 25-60% of subjects with HH develop secondary type 2 diabetes (Rajpathak et al. 2009). The defect typically found in subjects with thalassemia is inadequate levels of haemoglobin. This needs to be corrected with repeated blood transfusions and this results in iron overload. Like with HH, the mechanism linking diabetes to iron overload in thalassemia is through insulin deficiency and insulin resistance (Merkel et al. 1988) and (Messina et al. 2002).

The underlying molecular mechanisms for the role of iron in the aetiology of type 2 diabetes is not fully defined. However, recent studies reveal a positive association between excess iron and risk of type 2 diabetes. More important is that it is the heme iron intake and body iron stores were positively associated with an increased risk of type 2 diabetes. This was confirmed through research by Bao and colleagues that performed a systemic review and meta-analysis after adjusting for known potential confounders. In fact, their meta-analysis of prospective studies established no significant association of other forms of increased iron intake such as, dietary intake of total iron, non-heme and supplemental iron intake with the risk of type 2 diabetes (Bao et al. 2012). Research close to home has also shown that even within the South African tribal populations, overload of dietary iron has been described. The culprit for this excess iron is the cooking utensil, the three-legged iron pots coupled to the acidic ingestion of cereal which enhances iron absorption. It has a similar clinical

presentation to HH with secondary type 2 diabetes appearing as a late manifestation (Walker & Segal 1999).

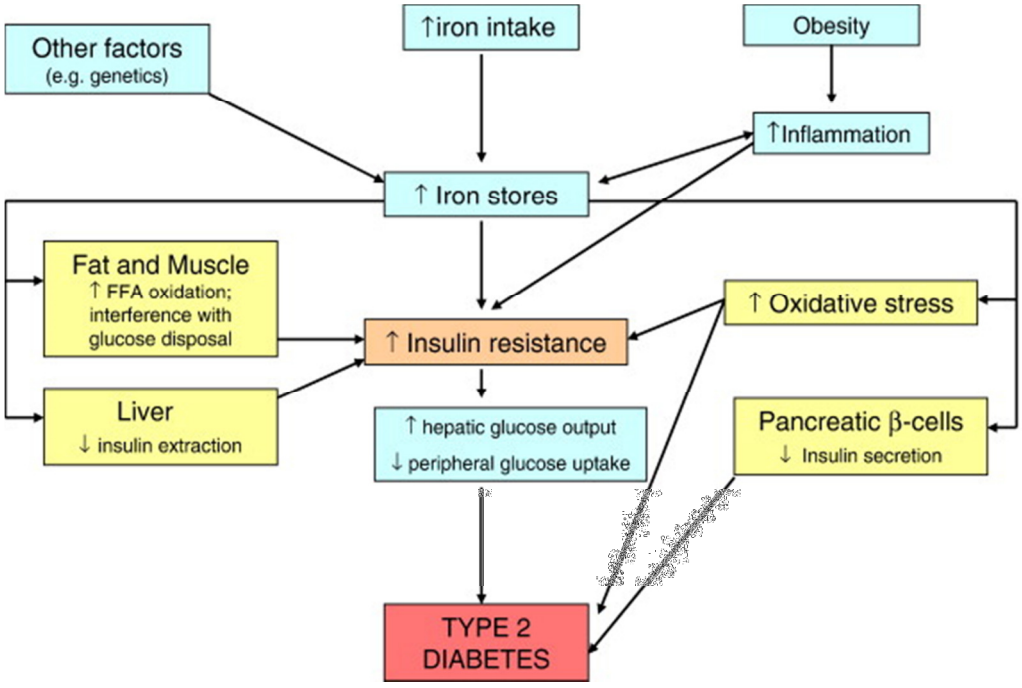


Figure 3.4: Potential mechanisms for the role of iron in aetiology of type 2 diabetes. Taken from (Rajpathak et al. 2009).

Multiple potential mechanisms linking iron and type 2 diabetes have been identified as highlighted in Figure 3.4. First of all, and as explained previously, iron is a powerful pro-oxidant and catalyst capable of reactions that lead to the formation of hydroxyl radicals with consequent oxidative stress. Oxidative stress in pancreas parallels to attack of the beta cells with impaired insulin synthesis and excretion (Evans et al. 2002). Increased oxidative stress, as shown in yellow in Figure 3.4 is one of the mechanisms implicated in increased risk of type 2 diabetes (Rajpathak et al. 2009). Secondly, when liver tissue is exposed to higher than normal iron levels, it causes impaired hepatic insulin extraction, thus glucose utilization in muscle tissue is diminished with a shift from glucose to fatty acid oxidation terminating in increased insulin resistance (Bao et al. 2012), (Jehn et al. 2007) and (Rajpathak et al. 2009). Thirdly, when iron stores are elevated, it interferes with insulin's function to suppress hepatic glucose production (Ferrannini 2000). It is clear that iron overload is responsible for both increased glucose production and decreased glucose utilization (Niederau et al. 1984). Not related to elevated oxidative stress, excessive iron by itself can be deposited in the

pancreatic β-cells which can lead to dysfunctional insulin secretion (Wilson et al. 2003).

Other factors influencing ferritin levels include genetic and environmental factors. It is thus possible that the association between ferritin and diabetes may not be suggestive of alterations in iron stores (Jehn et al. 2007). Ferritin cannot be viewed as a sensitive marker of body iron stores since it is an acute-phase protein and can be increased in the presence of inflammation and other conditions (Bao et al. 2012). It is known that increased systemic inflammation can influence iron metabolism. However, epidemiological studies show that inflammation does not seem to clarify the iron-diabetes association (Rajpathak et al. 2009). Iron status also has an impact on diabetic complications such as diabetic nephropathy and vascular dysfunction (Dymock et al. 1972) and (Swaminathan S, 2007). Epidemiologic, animal and human studies provide evidence that link iron to diabetic nephropathy, viz., research showing that in subjects with kidney disease there is an increased amount of iron; in those with a diagnosis of diabetic nephropathy, have a finding of increased urinary iron levels and studies that show limitation of the progression of nephropathy by treatment with iron-deficient diet or by the addition of iron chelators (Swaminathan et al. 2007).

Evidence pointing towards the possibility of iron status role in vascular diseases was highlighted in subjects with HH and transfusional iron overload in which there is an increased incidence of cardiac disease (Schafer et al. 1981). In addition, many other studies have demonstrated a direct link between increased iron intake, body iron stores and cardiovascular risk in the general population, whereas increased heme-iron intake has been coupled to increased cardiovascular events and increased body iron stores are associated with myocardial infarctions (Swaminathan et al. 2007). Pathophysiological mechanisms implicated in iron promoting vascular disease include increase in surface expression of adhesion molecules and monocyte adherence to the endothelium when NTBI is added to human endothelial cell culture cells (Kartikasari et al. 2004). Similar to subjects with nephropathy improving with iron chelators, endothelial dysfunction is improved in subjects who are high-frequency blood donors and those receiving iron-chelators as therapy (Swaminathan et al. 2007).

Table 3.1: **Ferritin concentrations for type 2 diabetic patients with and without CVD compared to established values for healthy controls. Also the percentage of individuals in each group with increased ferritin levels are shown. Values indicated are mean ± standard deviation.**

Variable	Diabetics without CVD (n=25)	Diabetics with CVD (n=25)
Ferritin concentration	136.36 ± 124.81	175.17 ± 175.44
Males (50%) **$(20 - 250 ng/mL)**	215.60 ± 151.75	178.65 ± 148.05
Females (50%) **$(10 -120 ng/mL)**	91.79 ± 82.20	169.63 ± 221.17
Increased Ferritin (%)	20.00	32.00

$ Control values as stipulated by the South African Ampath Laboratory services.

3.9 Ultrastructural findings in erythrocytes and fibrin caused by high serum ferritin.

Extensive research by Pretorius and her group, (Pretorius & Kell 2014) has demonstrated that the morphology of the red blood cells and fibrin is transformed in the presence of elevated iron, particularly coupled to serum ferritin levels. The red blood cells lose their characteristic spherical shape. A changed morphology is also noted in many inflammatory conditions, including diabetes mellitus as illustrated in Figure 3.5 (Pretorius & Kell 2014). In the section on iron toxicity above, it was described that increased iron levels cause oxidative stress. The blood cell most prone to this stress is the red blood cell and the pathology that occurs is eryptosis. Eryptosis can be described as a form of suicidal death of erythrocytes characterized by distinct findings of cell shrinkage, membrane blebbing, activation of proteases and phosphatidylserine exposure at the outer membrane leaflet. Any form of injury after several stressors to erythrocytes will trigger eryptosis and the exposed phosphatidylserine is the target of the macrophages that engulf and degrade the affected cells (Lang et al. 2006). Like diabetes, Alzheimer's disease (AD) is also associated with oxidative stress caused by increased iron levels with consequent hydroxyl radicals which are implicated in the pathology of neuronal damage in AD (Bester et al. 2013). Abnormal

erythrocyte membrane morphology was observed by Mohanty and co-workers, in their research which showed that 15% of erythrocytes in AD were elongated (Mohanty et al. 2010) as is also shown in Figure 3.5B. Research by Pretorius et al, also confirmed a changed morphology of the erythrocyte in diabetes, as shown in Figure 3.5A (Pretorius 2013).

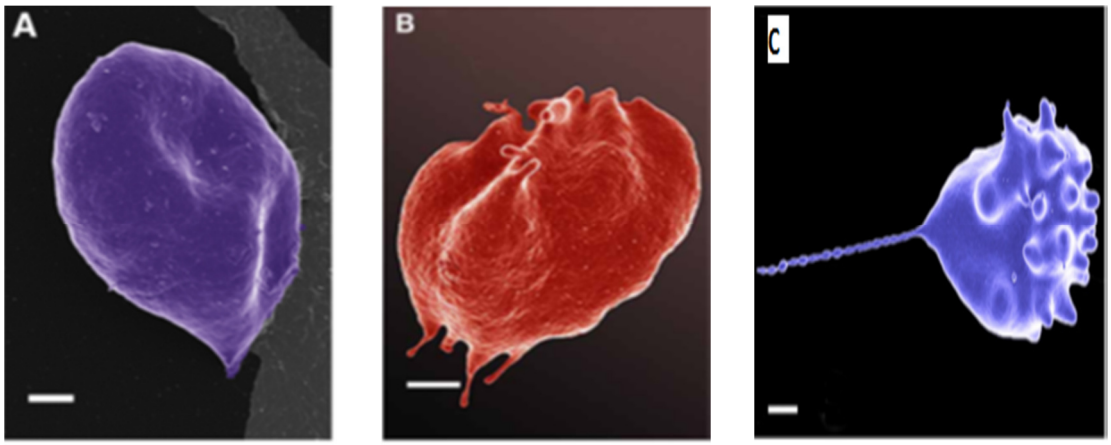


Figure 3.5: **SEM of red blood cell in (A) Diabetes, (B) Alzheimer and (C) Stroke. Taken from A: (Pretorius 2013) B: (Bester et al. 2013) C: (Pretorius & Lipinski 2013a).**
Scale = 1 µm.

Unique morphological findings are seen in the erythrocytes and fibrin fibres of subjects who have sustained thrombotic ischaemic strokes. These include, the presence of dense matted deposits (DMD) with the absence of characteristic fibrin fibres after mixing smears of blood with thrombin similar to findings seen with the addition of ferric chloride as seen in Figure 3.5B, while erythrocytes show formation of an extension from the erythrocyte toward the DMD as shown in Figure 3.5C (Pretorius & Lipinski 2013a).

Figure 3.6 shows six diabetic subjects from both groups, viz., those with and without CVD. However, common to all six subjects is the elevated ferritin levels. Preparation of the micrographs for scanning electron microscopy is described in chapter 6.

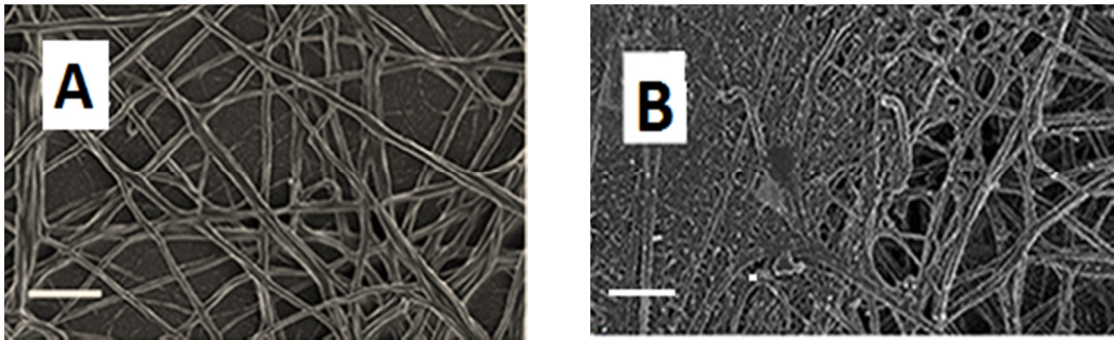


Figure 3.6: **(A) Platelet rich plasma smear with added thrombin in healthy individual. (B) Platelet rich plasma smear from HH with added thrombin (C282Y/H63D) (1166 µg/L^{-1}); Taken from (Bester et al. 2014). Scale = 1 µm.**

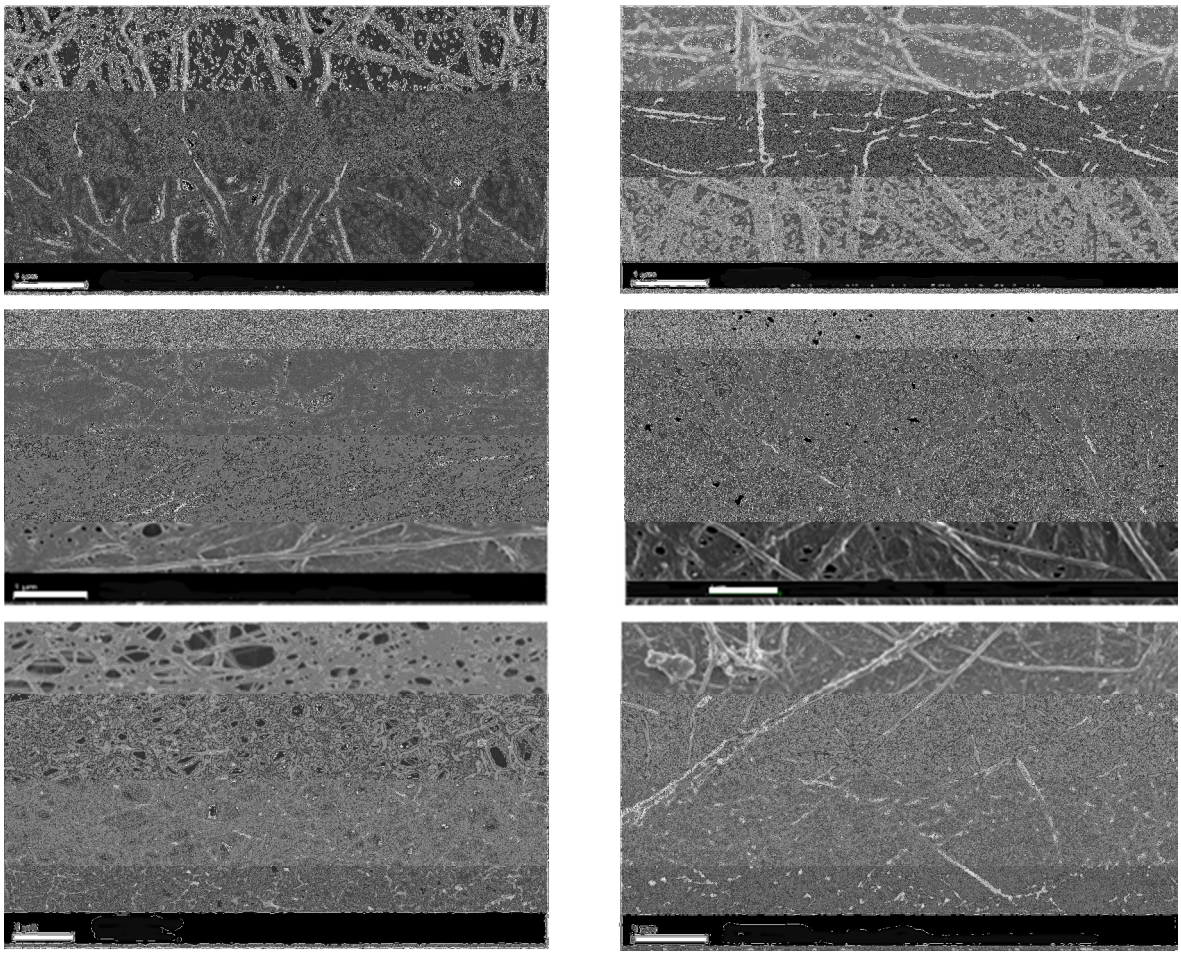


Figure 3.7: **Micrographs representing six diabetic subjects with elevated serum ferritin levels.**

3.10 Conclusion

Inflammation is no longer viewed as the simplistic definition of the body's immune system response to a stimulus. In fact, it is now recognised as a far more complex process with a healing, restoring and aggressive component. It is not only an acute process and can become chronic with pathophysiological changes such as

remodelling of the artery wall in atherosclerosis, remodelling of the bronchial wall in asthma and the debilitating destruction of the joints as seen in rheumatoid arthritis (Punchard, Whelan & Adcock 2004). A component of the inflammatory process includes the induction of various acute phase proteins such as highly sensitivity CRP and serum ferritin in the liver. Both these biomarkers are important key players in insulin resistance as well as atherosclerosis (Hotamisligil 2006).

Type 2 diabetes is a disease marked by chronic inflammation (Hotamisligil 2006) and ferritin increases with inflammation. (Simcox & McClain 2013). Thus, the question as raised by Simcox and colleagues that cannot be ignored is, whether high iron (whose best biomarker is high ferritin) causes diabetes or diabetes causes high ferritin (Simcox & McClain 2013). Evidence supporting the causal relationship of iron is provided in studies in which reversal of diabetes occurs with reducing iron levels (Simcox & McClain 2013). Similarly, treatment for subjects with HH include phlebotomy and/or iron chelation therapy such as desferoxamine. These modalities of therapy not only reduce ferritin levels but have an additional beneficial effect in that they also improve insulin sensitivity and glycaemic control (Rajpathak et al. 2009). These findings support the significant association between increased body iron stores and type 2 diabetes risk.

From a public health point of view, if we are able to confirm an increased risk of diabetes with moderately elevated ferritin levels, it would impact strongly, since subjects with high risk could be identified and targeted for more intensive screening and preventative interventions (Jehn et al. 2007). In this representative sample of government hospital, diabetic patients, a significant percentage seem to be burdened by high ferritin levels with remarkable effects on red blood cells and fibrin network morphology which impacts on their function and subsequently in the coagulation process and ultimately will lead to atherosclerosis. Screening and testing of iron and ferritin levels in the clinical setting should perhaps be strongly considered to be part of routine testing in our population, in particular diabetic patients.

4. CHAPTER 4: (FLOW CYTOMETRY) - PLATELETS IN DIABETES: ARE THEY ANGRY?

4.1 Abstract

4.1.1 Aim

The function of platelets has extended way beyond the horizon of haemostasis and thrombosis, and are recognised as active participants in vascular inflammation, as well as in prothrombotic complications of cardiovascular diseases. We describe and compare platelet function in diabetic patients (with and without cardiovascular manifestation) and healthy individuals using flow cytometry.

4.1.2 Materials and methods

Thirty subjects were recruited per group and informed consent was obtained from all participants. Diabetic patients were recruited from the diabetic clinic of the Steve Biko Academic Hospital (South Africa). Blood samples were drawn from all participants so that platelet specific antigens were analyzed in citrated whole blood. The platelet parameters used in the study were platelet identifiers (CD41 and CD42) and markers of platelet activation (CD62 and CD63).

4.1.3 Results

Results show that, compared to healthy individuals, both diabetic groups showed a significant difference in both platelet identifiers (CD41-PE, CD42b-PE) as well as markers indicating platelet activation (CD62P-PE and CD63-PE).

4.1.4 Conclusion

The flow cytometric data shows that the platelet surface receptors are statistically elevated. This is suggestive of enhanced platelet activation and it appears as if platelets are displaying ‘angry’ behaviour.

4.2 Introduction

Having diabetes as part of one’s chronic medical history accelerates your predisposition for cardiovascular disease (CVD), since diabetes causes a two to four-fold increase in the risk of coronary artery disease (CAD) (Feskens & Kromhout 1992) and (Grant 2007). In fact, diabetes worsens the early and late

outcomes in acute coronary syndromes (ACS) (Kjaergaard et al. 1999). Plaque rupture is an important pathophysiological mechanism of ACS. Emerging evidence shows that in addition to plaque rupture, the presence of multiple coronary plaques, persistent hyper-reactive platelets and vascular inflammation are principal risk factors for atherothrombosis as documented by the atherothrombosis expert column (Medscape cardiology 2003). It should be recognised that besides their role in haemostasis and thrombosis, platelets control a range of inflammatory responses and feature as key players in atherothrombosis (Wagner & Burger 2003) and (Gawaz, Langer & May 2005).

An indication of a connection between thrombosis and inflammation is highlighted at the molecular and cellular levels in the endothelium (Wagner & Burger 2003). Evidence for the latter is provided when, upon injury to the vascular tissue, platelets are recruited to adhere to the exposed subendothelial matrix and in doing so, facilitate additional platelet and leucocyte recruitment. In order to arrest bleeding, platelets provide the surface for the binding of leucocyte-derived microparticles, which holds tissue factor for a localized initiation of the coagulation cascade. Once platelets are activated they increase leucocyte activation and adhesion to the endothelial cells via deposition of chemokines on the endothelium. Leucocytes are thus now equipped to attach to vessel wall and to transmigrate into the subendothelial tissue and forming an atherosclerotic plaque (Wagner & Burger 2003) and (Gawaz, Langer & May 2005). Previous research by Trip and colleagues has provided evidence that ongoing platelet activation, so-called ‘angry platelets’ play a central role in ischaemic syndromes (Trip et al. 1990).

In subjects with type 2 diabetes mellitus hyperaggregation of platelets is reported (Hughes et al. 1983). In fact, a sub-threshold stimulus is needed to activate platelets which are constantly in activation despite the lack of a major plaque event and have thus been defined as ‘angry platelets’ (Viswanathan & Zaman 2013). This is significant as it has been postulated that circulating platelets in subjects with untreated type 2 diabetes mellitus are in a hyperactive state and are implicated as etiologic factors in thrombotic complications (Saboor, Moinuddin & Ilyas 2013) which are accelerated in diabetics (Grant 2007).

A great deal of progress has been made in the field of platelet function analysis, since the invention of the first in vivo test of platelet function in 1910, the so-called bleeding time (Lind 2002). Flow cytometry is a powerful technique, and its importance in research and clinical investigation has long been established (Marti, et al. 2001). Markers used in flow cytometry may give us valuable information regarding cellular activity and since 1989 it has been deemed an essential tool for the investigation of platelets (Ault et al. 1998). Flow cytometry thus provides a numerical technique, which is both objective and quantitative, to assess platelet function (Lazarus et al. 1995).

Platelets adhere to each other via adhesion receptors called glycoprotein receptors (Kasper & Harrison 2005). Several surface glycoproteins (GP) are found on the membrane of platelets and flow cytometry has been used to a great extent in the immunephenotyping of these entities. The study of platelet function, physiology and their interaction with other cells has been advanced by investigating the recognition of these surface glycoproteins by specific monoclonal antibodies (MoAbs). Not only can the glycoproteins on the surface of the platelet membrane be detected by MoAbs, but molecules transferred to the surface from the internal platelet granules can be identified (Lazarus et al. 1995). This is of particular interest in the study of platelet activation.

The application of a panel of MoAbs is preferred for flow cytometric analysis of platelets, since different flow cytometric probes reveals different characteristics of platelet function (Lazarus et al. 1995). CD42 is frequently used as platelets identifier, since it is only present on platelets and not on any other circulating blood cell (Coller et al. 1983) and (Montgomery, Kunicki & Taves 1983). Studies of extracorporeal circulation have mainly used CD62P and CD63 to investigate platelet activation (Gutensohn, Bartsch & Kuehnl 1979). Therefore, flow cytometry can be employed to establish the amount of activated and non-activated platelets (Lazarus et al. 1995).

How does flow cytometry actually work? The principle of flow cytometry is based on the science of fluorescence. Platelets are labelled with fluorescent monoclonal antibodies. The cell suspension is passed through a flow chamber equipped with a focused laser beam that activates the fluorphore. In order to identify the platelets,

fluorescence is measured. Since the intensity of the emitted light is directly proportional to the number of antibodies attached to the platelet receptors and or antigens, it allows the determination of the absolute number of antibody labelled cells (Michelson et al. 2007) (Saboor, Moinuddin & Ilyas 2013). It is a simple, reliable and cost effective technique with extensive utilization in the diagnosis of inherited and acquired coagulation disorders (Saboor, Moinuddin & Ilyas 2013).

Defined as important inflammatory markers, platelets are key players in atherosclerosis and CVD which are correlated with type 2 diabetes (Ghoshal & Bhattacharyya 2014). Platelet dysfunction can result in thrombotic or bleeding complications and in arterial thrombosis the level of platelet activity is enhanced and the expression of many platelet activation markers can be measured on the cell surface using flow cytometry. The aim of this chapter is thus to and to compare them to healthy individuals, so as to add to a better understanding of the pathophysiology of platelet activation. The following monoclonal antibodies: CD41 antigen (GPIIb-IIIa), CD42b (GPIb), CD62P (P-selectin), and CD63 (thrombospondin/GPIV) were used.

4.3 Materials and Methods

4.3.1 Participants

Thirty healthy individuals were used as controls. These individuals were non-smokers, who did not use any chronic medication and did not have a history of thrombotic disease. Sixty diabetic subjects were recruited from the Steve Biko Academic Hospital, diabetic clinic in Pretoria, South Africa. Inclusion criteria included: (a) subjects older than 18 years and willing to provide informed consent, (b) subjects with known diagnosis of diabetes, (c) for the cardiovascular group, history of previous myocardial infarction, peripheral arterial disease, stroke or coronary arterial bypass grafting. Exclusion criteria included: (a) subjects hemodynamically unstable and (b) subjected with documented life threatening disease (malignancy, HIV/AIDS). Two groups of thirty each were distinguished, with and without cardiovascular complications. Demographic information of the subjects is indicated in Table 4.1. 5 ml of blood was drawn into a citrate tube, from each participant. Ethical clearance was obtained for this study from the University

of Pretoria Human Ethics Committee. Informed consent was obtained from all participants.

4.3.2 Flow Cytometry

For each blood sample taken 4 tubes was prepared; each tube containing 1 ml sheath fluid and 20µl of blood. The various tubes were stained with 20µl of CD41-FITC (fluorescein isothiocyanate) and 20µl of one of the following probes: CD41-PE (phycoerythrin), CD42b-PE, CD62P-PE and CD63-PE (from Beckman Coulter). 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). The surface expression of platelet receptors was determined by flow cytometry using the different monoclonal antibodies as indicated in Table 4.1. Antibodies used in the study included CD41, CD42, CD62 and CD63.

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).

4.3.3 Statistical Analysis

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 groups. GraphPad Prism 5 was employed to perform one-way ANOVA for all statistical analysis, with a p-value of ≤ 0.05 considered significant. Post-hoc Dunnett's Multiple Comparison Test was performed to compare the two diabetic groups to the controls.

4.4 Results

Table 4.1 shows the demographic data of our study population. It provides information on the controls and the two diabetic groups. The diabetic group with

cardiovascular disease were slightly older when compared to the group without cardiovascular disease, (mean age of 61 years' vs 53 years respectively). It is notable to observe that both groups had uncontrolled glucose as evidenced by the HbA1c values. Detail of the chronic medication taken by the subjects are also shown. Anti-hypertensive agents included ace-inhibitors, calcium antagonists and β-blockers. A large proportion, more than 80% of diabetics with cardiovascular disease were taking statins.

Platelet-bound antibodies were detected either by fluorescein isothiocyanate (FITC) or by phycoerythrin (PE). As seen in Figure 4.1, the FL1 log (x-axis) is representative of CD41-FITC and the FL3 log (y-axis) represents CD62-PE and CD63-PE. The upper right quadrant F2 represents the population of platelets that are activated in Figure 4.1 and each graph is a representation of 2 subjects from each diabetic group and 2 controls. Results of activated platelets (as percentages) in the studied groups, inferred from Figure 4.1 is illustrated in Table 4.3 and Figure 4.3. When compared to controls both diabetic groups had significantly elevated activated platelets as measured with CD62 and CD63. The monoclonal antibodies CD41-PE and CD42b-PE MCFI, when compared to the controls were also increased, with a $p < 0.005$ as indicated in Figure 4.2 and Table 4.3. This was applicable to both diabetic groups.

CD62P-PE and CD63-PE MCFI were significantly decreased for both diabetic groups as shown in Figure 4.2 and Table 4.3. It should be noted that the platelet activation indicated CD63-PE showed the diabetic group with cardiovascular complication to have the largest percentage of activated platelets.

Table 4.1: Baseline demographic data and clinical characteristics of the study population

Variable	Controls (n=30)	Diabetics without CVD (n=30)	Diabetics with CVD (n=30)
Age, years	25 ± 9.64	53 ± 13.7	61 ± 9.4
Males, n (%)	6 (20)	11 (37)	20 (67)
Females, n (%)	24 (80)	19 (63)	10 (33)
Hypertension, n (%)		17 (57)	26 (87)
Diabetic treatment:			
Insulin, n (%)		5 (17)	3 (10)
Oral agents only, n (%)		16 (53)	12 (40)
Oral and insulin, n (%)		9 (30)	15 (50)
***HBA1c %**		9.0 ± 2.6	8.5 ± 1.7
Cardiovascular Complications:			
Previous MI, n (%)		n/a	19 (63)
PAD, n (%)		n/a	2 (7)
CABG, n (%)		n/a	9 (30)
Essential medication:			
ACEI, n (%)		10 (33)	20 (67)
Ca-antagonist, n (%)		8 (27)	3 (10)
B-blocker, n (%)		3 (10)	11 (37)
Nitrates, n (%)		1 (3)	14 (47)
Statins, n (%)		10 (33)	25 (83)
Disprin, n (%)		6 (20)	20 (67)
Warfarin, n (%)		2 (7)	5 (17)

Data expressed as mean +/- (SD) or n (%)

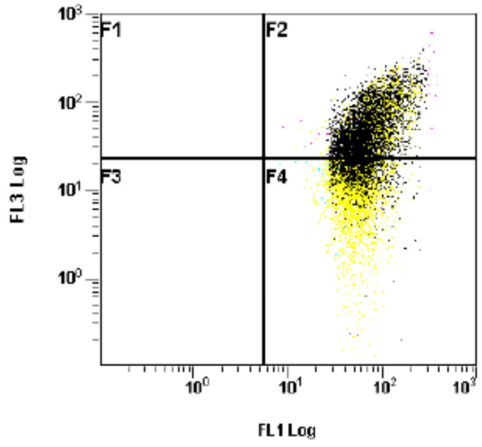
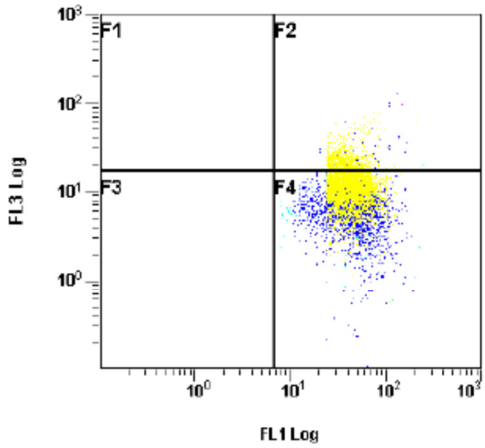
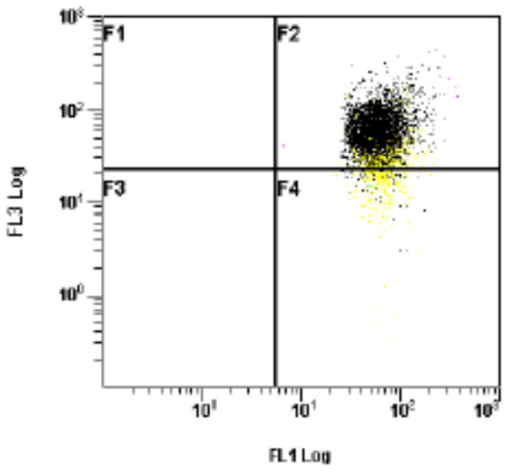
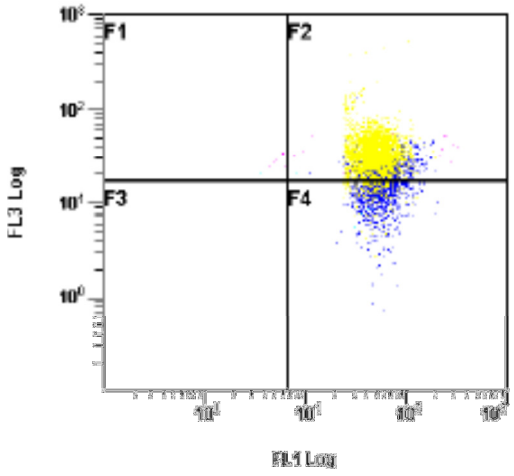
MI = Myocardial infarction, PAD = Peripheral arterial disease, CABG = coronary arterial bypass grafting, ACEI = Angiotensin converting enzyme inhibitor, Ca-antagonist = Calcium antagonist and HBA1c = haemoglobin A1c *not all 60 subjects had this test completed (results are available for 50% of the subjects).

n/a = not applicable

Table 4.2: Platelet parameters measured in this study

Cluster of differentiation	Glycoprotein (GP)	Function
CD41	GPIIb	Anti-CD41a reacts with platelet membrane GPIIb in the intact complex with GPIIIa but not with GPIIb or GPIIIa separately. Useful in the identification and enumeration of platelets.
CD42	GPIb	Anti-CD42b reacts with platelet membrane GPIb
CD62	P-Selectin	Anti-CD62 reacts with α-granule membrane protein which is expressed on the surface of activated platelet.
CD63	GPIV	Anti-CD63 reacts with lysosomal granule-membrane glycoprotein that is expressed on surface of activated platelet.

*In this study CD41-FITC (fluorescein isothiocyanate) and CD41, CD42b, CD62, CD63-PE (phycoerythrin) was used.

	CD62P-PE	CD63-PE
Controls		
Diabetes with CVD		

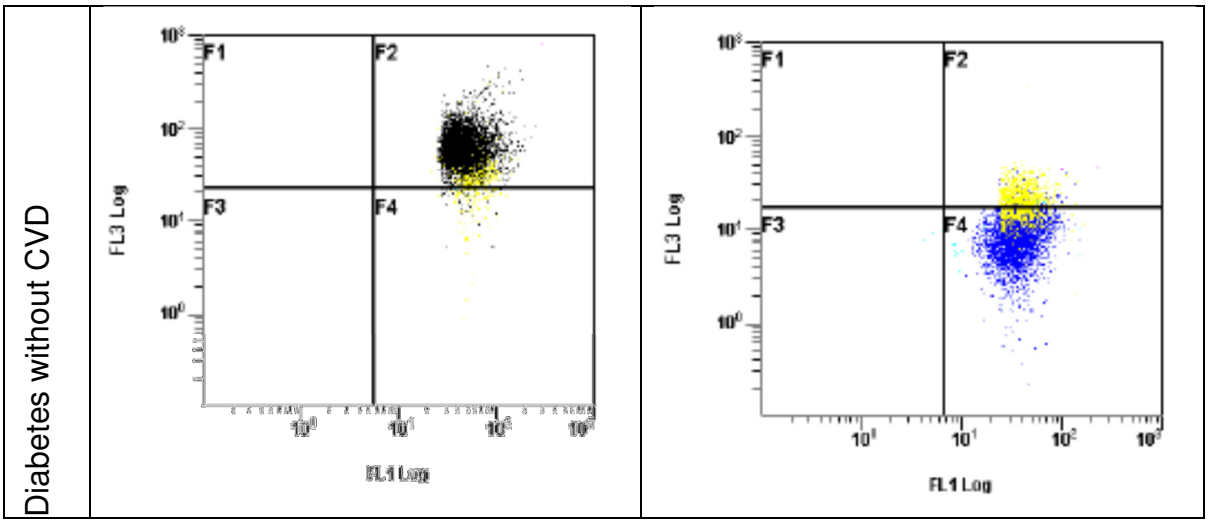


Figure 4.1: **Platelet activation probes of the control and two diabetic groups, the upper right quadrant F2 represents the population of platelets that are activated.**

Table 4.3: **Analysis of control and diabetic groups (with or without CVD), results presented as mean ± Standard Deviation (SD) of MCFI and percentage activated platelets. (n=10 000 platelets total analysed for each participant of each group).**

MoAb	Controls (n=30)	Diabetes without CVD[s] (n=30)	Diabetes with CVD (n=30)
CD41-PE	21.08±8.74	40.18±14.56*	47.81±24.49*
CD42b-PE	14.43±1.84	24.78±11.98*	24.09±7.27*
CD62P-PE X-mean	30.86±11.37	17.96±1.95*	17.79±2.266*
CD62P-PE % Activated Platelets	71.01±16.20	92.65±3.67*	92.11±4.31*
CD63-PE X-mean	16.40±3.32	13.05±3.68**	13.38±3.12**
CD63-PE % Activated Platelets	37.84±17.59	54.39±32.92**	61.24±29.73**

Statistically significant differences: p-value < 0.001 shown with *, p-value < 0.05 indicated with **.

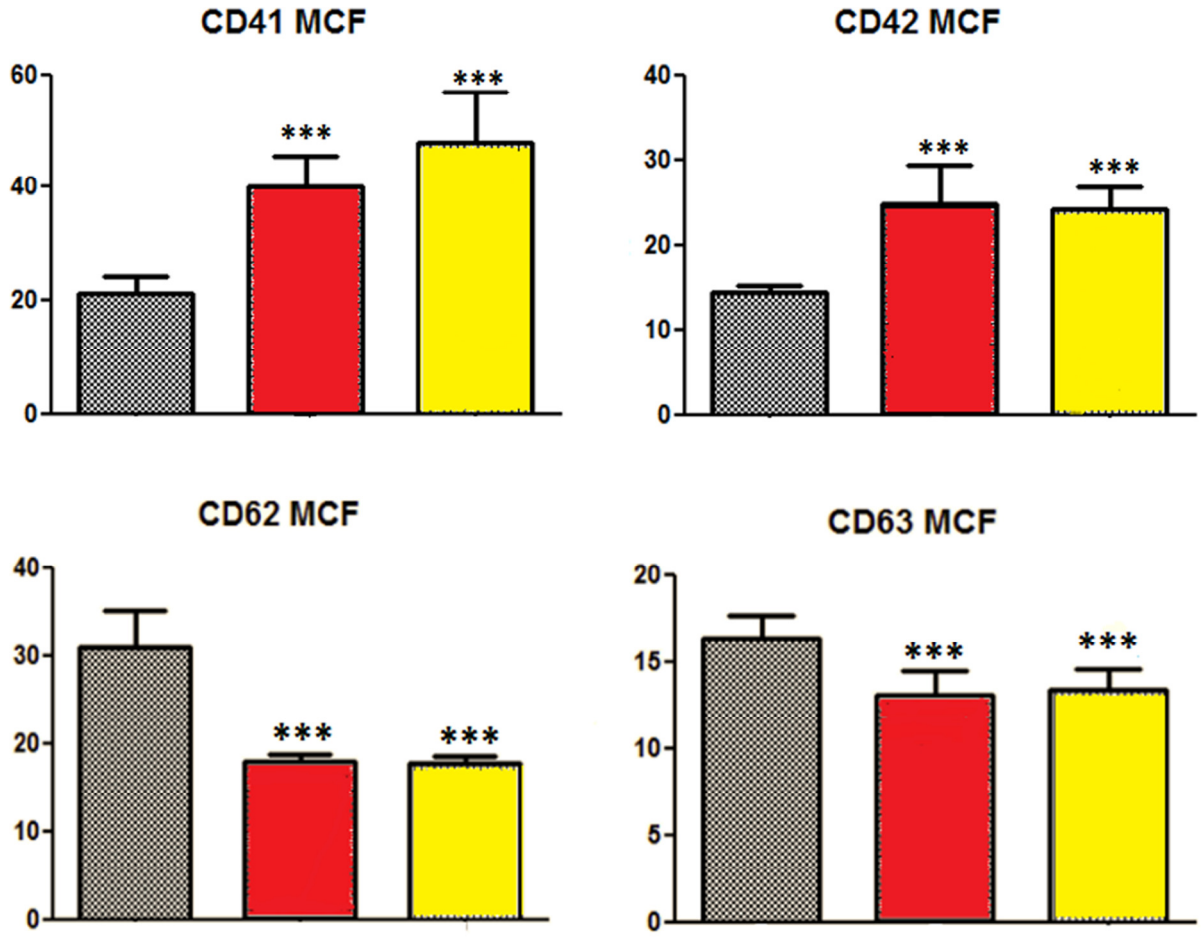


Figure 4.2: **Graphic representation of control and two diabetic groups showing CD41 MCF, CD42 MCF, CD62 MCF and CD63 MCF with standard deviations and asterisk indicating statistical significance.**

Control

Diabetic without cardiovascular disease

Diabetic with cardiovascular disease

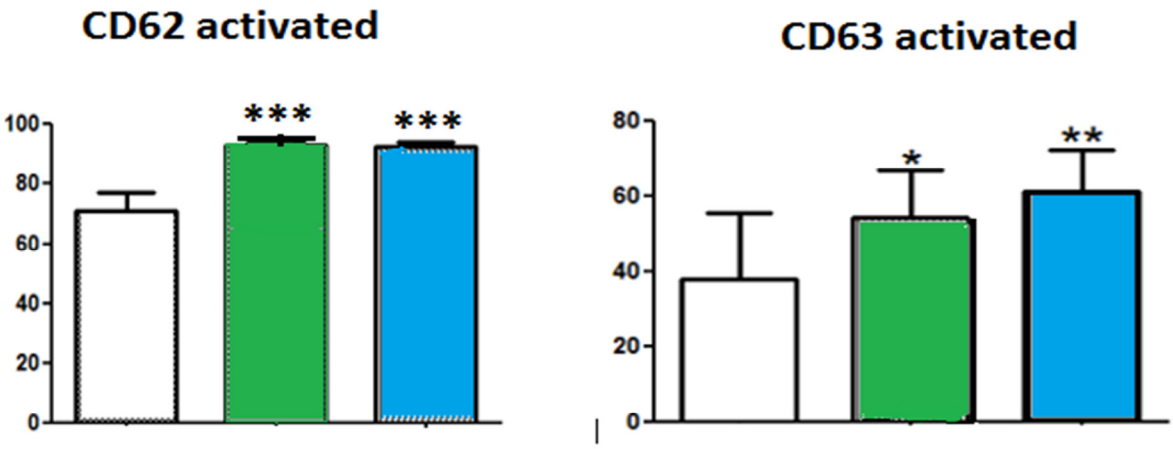


Figure 4.3: **Graphic representation of control and two diabetic groups showing percentage of activated platelets identified with CD62P-PE and CD63-PE with standard deviations and asterisk indicating statistical significance.**

Control

Diabetic without CVD

Diabetic with CVD

4.5 Discussion

Knowledge on the structure of the platelet plasma membrane is essential to unravel its role in thrombogenesis, atherogenesis and progression of athero-sclerotic lesions. The outermost layer comprises of glycoproteins which are key players in functions such as, adhesion and aggregation (Kamath, Blann & Lip 2001). Upon vascular injury, the platelet-endothelial contact is facilitated via adhesion molecules on the platelet surface, which belong to the families of selectin (P-selectin), integrin (GPIa, GPIc, GPIIa, GPIIb-IIIa) and immunoglobulin (Krejsek, Loudova & Kopecky 1997). During the process of adherence to the subendothelial matrix, platelets undergo a change in their shape from discoid to spherical with extrusion of the pseudopods (White 1974) as seen in Figure 4.4. Upon interaction between a platelet-activating agonist and its receptor, a rapid mobilization of

signalling molecules within the platelet is initiated which are sufficient to initiate and to mediate a complete shape change and aggregation responses (Willoughby, Holmes & Loscalzo 2002). In fact, flow cytometry identifies: (a) activated platelets by determining the change in shape of the activated platelets (Ruf & Patscheke 1995), (b) specific antigens on the membrane of activated platelets (P-selectin, GPIIb-IIIa) or platelet surface bound proteins (fibrinogen) (Lazarus et al. 1995) and (c) expressed procoagulant surface (Schmitz et al. 1998).

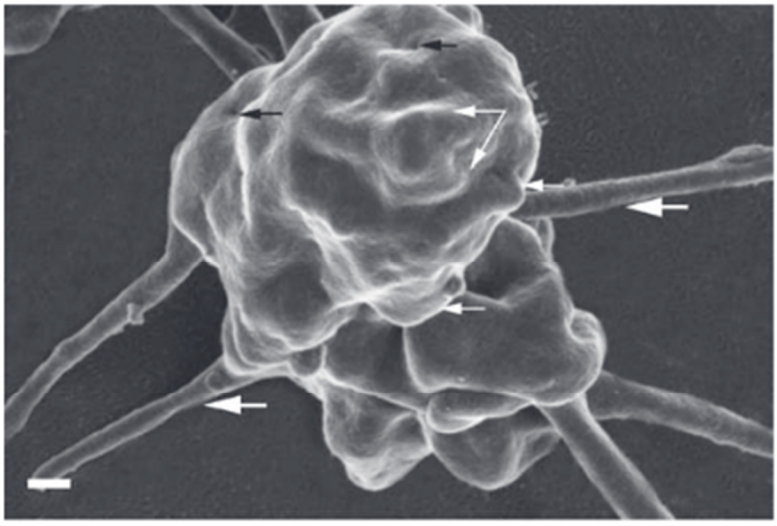


Figure 4.4: **Control platelet (prepared as smear from platelet-rich plasma), 40 000 x machine magnification. Thick, white arrows represent extended pseudopodia; thin, white arrow represents smooth platelet membrane; thin, black arrows represent open canalicular system. Scale 200 nm.**

CD41: This is a glycoprotein comprising 2 components, the first chain GPIIbα of 120 kDa and the second GPIIbβ of 23 kDa. Integrins are a large family of cell surface receptors. On platelets, β3 integrins are found, also called CD61 (GPIIIa), is a 110 kDa GP. 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 αIIbβ3 complex/integrin). CD41-CD61 complex (αIIbβ3) is the major protein (17% of the total platelet membrane protein) (Ni H 2003), making the CD41 antibody a useful tool to identify platelets. The function of platelet aggregation depends on fibrinogen binding to the CD41-CD61 complex. There is a low affinity for fibrinogen binding to the resting form of the CD41-CD61 complex on non-activated platelets. However, once the platelet is activated, the complex is stimulated to change its conformation after signals from

inside the cell are received and becomes a high-affinity receptor for fibrinogen (Zhang et al. 2007).

CD42: CD42a-d complex also known as GPIb-V-IX is the surface platelet receptor for von Willebrand factor (VWF) on endothelium. GPIbα (CD42b) and GPIbβ (CD42c) form main part of VWF (Lopez et al. 1998). The binding of GPIb to VWF after vascular injury has a dual effect: (i) facilitates initial platelet adhesion to the subendothelium and (ii) initiates signalling events with platelet that results in enhanced platelet activation, thrombosis and haemostasis (Lopez JA, 1998). CD42b also binds to thrombospondin-1, a GP abundant in arteriosclerotic plaques, leading to thrombus formation independent of VWF (Jurk et al. 2003). The CD42b moAb used in this study specifically binds to CD42b also known as GP Ib alpha chain (GPIbα).

CD62: The two flow cytometric parameters commonly used to quantify platelet activation are CD62 expression: % and MCFI, i.e. % CD62-positive platelets in the total population and mean fluorescence of CD62-positive cells expressed in arbitrary units of MCF. Thus, each of these parameters identify different specific aspect of platelet activation. The percentage positive depicts the proportion of activated cells in the total population without recording the activation level of individual cells; whilst the MCF positive indicates the mean epitope density of CD62 molecules on the platelet surface, reflecting the quality of activation of individual platelets, in contrast to the quantitative information provided by the percentage positive (Leytin et al. 2000). Also known as CD62P or P-selectin, this is one of the most widely researched type of activation-dependent MoAbs which is directed against the platelet granule membrane proteins (Schlossman et al. 1994). P-selectin is a component of the α-granule membrane of resting platelets and a unique feature is that it is only expressed on the platelet surface membrane after α-granule secretion (Stenberg et al. 1985), (Hsu-Lin S-C et al. 1984) and (McEver 1990). Many conditions are associated with increased expression of CD63 and CD62P such as acute coronary syndromes, acute cerebro-vascular ischaemia, peripheral vascular disease, diabetes mellitus and pre-eclampsia (Michelson et al. 2007).

Flow cytometric analysis found a significant increase in platelet activation regarding CD62 (P-selectin) for the diabetics group while the CD62 MCFI values decreased compared to the controls as shown in Table 3.1. 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 & Draper 2011). However, studies have shown an increase in both P-selectin on the surface of platelets and sP-selectin indicating that diabetes with or without cardiovascular complications are associated with chronic activation of platelets as P-selectin is being shed from activated platelets and as new P-selectin is being expressed on recently activated platelet (Inoue 2004). This study finding echoes results found by Véricel and colleagues whom also discovered hyperactive platelets in metabolically controlled diabetics without cardiovascular complications (Véricel et al. 2004). The diabetic subjects with cardiovascular disease, recruited in this study were those with ischaemic events many months and years prior to recruitment into this study. Our finding is in keeping with persistently hyper-activated platelets.

CD63: Also known as GP 53, it is as membrane protein of the platelet lysosomes. A unique feature of CD63 is that it is an antigen which only becomes accessible on the platelets' outer surface after platelet degranulation. It is known that the interaction between fibrinogen and fibrin to the platelet surface occurs during platelet activation in whole blood, their presence is thus viewed as a sign of activation (Klein B, 2002). 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. The study by Tschoepe and c0-workers showed that in diabetic subjects the expression of CD63- positive fraction was significantly elevated (6.1%) in comparison to controls. However, in subjects with angiopathy had a mean increase of 304% in CD63-positive fraction, implying that platelets becoming activated by vascular lesions. The trend observed in diabetics without vascular disease is suggestive of activated platelets contributing to the prothrombotic state in diabetes mellitus (Tschoepe et al. 1991).

Research on subjects with ischaemic stroke, show that CD62P and CD63 expression was higher on day 1 post stroke when compared to control groups. However, CD62P expression rapidly declined during follow-up whereas CD63 expression remained significantly increased until day 90. This persistent elevation in CD63 makes it a worthy marker to predict stroke recurrence (Marquardt et al. 2002). In our study CD63 percentage activated platelets were significantly increased compared to the healthy controls. The diabetic group with cardiovascular complication showed the greatest percentage of platelet activation (61.24% activation) while the diabetic group without cardiovascular complications showed a slightly lower activation percentage (54.39% activation), as shown in Table 4.3. It appears as if CVD may play a role in platelet hyperactivation with lysosomal involvement.

Increased platelet activation features in a variety of atherosclerotic diseases including CAD (Willoughby, Holmes & Loscalzo 2002), transplant vasculopathy (e.a. Fateh-Moghadam et al. 2000), and carotid artery disease (Fateh-Moghadam et al. 2005). Increased expression of platelet activation markers CD31, CD36, CD49b, CD62P and CD63 was confirmed by Eibl and co-workers when type 2 diabetics were compared with normal individuals (Eibl et al. 2004). In fact, increased expression of CD63 and CD62, enhanced platelet activation, and aggregation are viewed as one of the major causes of atherosclerosis and thrombosis in diabetes (Tschoepe et al. 1993). A feature that appears strongly in diabetics is that of platelet hyperaggregation. This is prevalent in both type 1 and type 2 diabetics (Hughes et al. 1983). From a pathophysiological view, this is significant as hyper-aggregated platelets have a tendency to block blood vessels (Ghoshal & Bhattacharyya 2014), contributing to atherothrombotic complications in diabetics.

4.6 Conclusion

Clinically, clues to defective platelets are highlighted when a patient complains of the following symptoms: unexplained or extensive bruising, epistaxis, gingival bleeding, heavy and prolonged bleeding at childbirth and bleeding from invasive procedures (e.g. dental extraction and tonsillectomy) (Sharathkumar & Shapiro

2008). However, using flow cytometry, the clinician is now enabled to diagnose inherited deficiencies of platelet surface glycoproteins, the diagnosis of storage pool disease and monitoring of treatment with GPIIb-IIIa receptor antagonists (Michelson 1996). With information, such as platelet hyper-reactivity and activated platelets provided by flow cytometry, in a variety of important conditions, the role of flow cytometry can no longer be ignored and may yet prove to be invaluable in the clinical setting for future practice of medicine. The use of flow cytometry in diabetic subjects confirms that platelets are in a hyper-reactive state and thus provides evidence that platelets in diabetes appear to exhibit ‘angry’ behaviour.

5. CHAPTER 5: (ELISA): TISSUE FACTOR - THE ROLE OF TISSUE FACTOR LIES BEYOND BLOOD COAGULATION

5.1 Abstract

5.1.1 Aim

Rupture of an atherosclerotic plaque is the definition for the term atherothrombosis. Known risk factors atherosclerosis includes high cholesterol, diabetes mellitus, smoking and hypertension, all of which increase tissue factor (TF) expression. High levels of TF have been recorded in atherosclerotic plaques. The aim of the chapter was to determine TF levels in both diabetic groups, those with cardiovascular disease and those without. A secondary objective was to elaborate on the coagulation-inflammation complex with the role of TF.

5.1.2 Materials and methods

Diabetic subjects were recruited from the diabetic clinic at Steve Biko Academic Hospital. Upon completion of the informed consent forms, blood was drawn from each subject and centrifuged on the day of recruitment and stored frozen. An ELISA, (IMUBIND® Tissue Factor ELISA assay, product no. 845) was used to measure TF levels.

5.1.3 Results

TF levels were remarkably elevated in both diabetic groups when compared to the control levels.

5.1.4 Conclusion

It remains to be seen if perhaps pharmacological control of plasma TF activity, in particular pathologic TF expression or augmentation of TFPI will lower thrombotic complications. Based on its physiology, the role of TF lies beyond blood coagulation.

5.2 Introduction

The classical view of the main role of tissue factor (TF) as the initiator of the coagulation cascade has to be reassessed, as it is also a mediator in the pathogenesis of cardiovascular disorders (Breitenstein, Camici & Tanner 2010)

and (Chu 2011). Atherosclerosis contributory risk factors such as smoking, hypertension, hyperlipidaemia and diabetes all increase TF expression (Tatsumi & Mackman 2015) and (Bode & Mackman 2015). The discovery of blood-borne TF has changed the opinion of labelling vessel-wall TF as the major determinant of thrombosis as emerging studies have recognised a hypercoagulable state associated with an increased circulating TF activity with the development of the concept of 'vulnerable blood' (Cimmino et al. 2011). In addition, it has been shown that this circulating pool of TF in blood that is associated with cells and microparticles is thrombogenic (Rauch & Nemerson 2000) and (Mackman 2004) is elevated in type 2 diabetes mellitus (Abdel-Hafiz et al. 2002) and (Sambola et al. 2003).

Under physiological conditions, the production/activation of prothrombotic and fibrinolytic factors are finely tuned and well balanced, so that haemostasis is appropriate, sufficient to protect from bleeding yet adequately suppressed to prevent pathological thrombosis. However, in diabetes, this fine balance is disturbed and tipped towards a prothrombotic/hypofibrinolytic phenotype which, in association with atheromatous vascular changes and platelet hyperactivity, poses an increased predisposition to cardiovascular ischaemic events (Alzahrani & Ajjan 2010).

Tissue factor, formerly known as thromboplastin, a 47 kDa transmembrane glycoprotein is the main activator of the coagulation cascade. It binds factor VIIa resulting in activation of factor IX and factor X, ultimately leading to fibrin formation. It is widely expressed in both vascular and non-vascular cells (Breitenstein, Camici & Tanner 2010). A source of high level of TF is the atherosclerotic plaque as macrophages and vascular smooth muscle cells express TF, as well as the presence of cell-derived TF-positive microvesicles. Hyperlipidaemia is another source of TF as it leads to the formation of oxidized low density lipoprotein (LDL) which in turn induces TF expression in circulating monocytes and the release of TF-positive microvesicles (Tatsumi & Mackman 2015), this is illustrated in Figure 5.1. The sources of TF that contribute to thrombosis after rupture of an atherosclerotic plaque is shown in Figure 5.1.

TF is the key initiator of inflammation-induced thrombin generation as indicated in Figure 5.2 (Levi, van der Poll & Büller 2004). There is ample evidence that inflammation and coagulation are intricately related processes that may considerably affect each other. Expression of TF (that acts as procoagulant stimulant) by inflammatory cells in the unstable plaque may initiate activation of a pathologic coagulation cascade and the resulting thrombin generation will both activate platelets and result in formation of platelet-fibrin thrombus (Levi, van der Poll & Büller 2004). Evidence of a link between inflammation and coagulation is demonstrated by the increase in TF expression and activity by different cell types. Whilst, in contrast, TF upregulation in turn can facilitate inflammation by enhancing intravascular fibrin deposition, formation of pro-inflammatory fragments of fibrin and by generating coagulation proteases (Cimmino et al. 2011). More importantly, based on the complexity of the biology of TF, it can also function as a signalling receptor (Cimmino et al. 2011) and (Chu 2011). The involvement of TF in wound repairs, embryonic development, angiogenesis, tumour metastasis, cell adhesion/migration, innate immunity and many pathological conditions demonstrates the diversity and complexity of TF (Chu 2011).

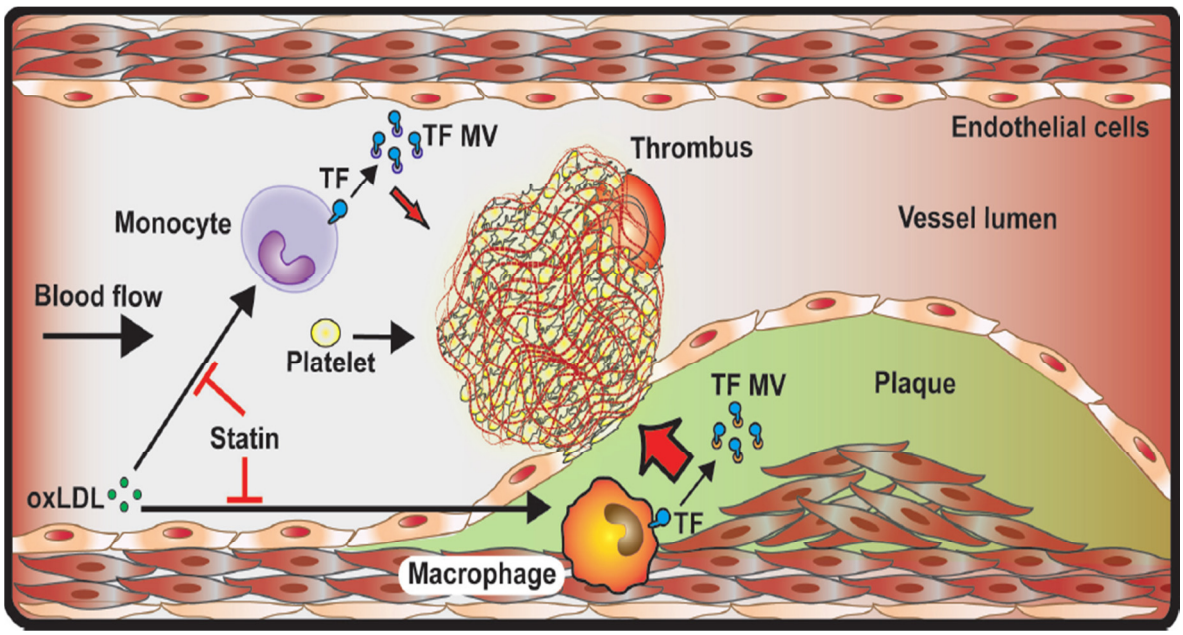


Figure 5.1: **Sources of TF contributing to thrombosis upon rupture of an atherosclerotic plaque. Taken from Tatsumi and Mackman (Tatsumi & Mackman 2015). TFMV=tissue factor microvesicles.**

As seen in Figure 5.2, exposure of TF-bearing inflammatory cells to blood plasma and cells results in thrombin generation, ultimately leading to fibrinogen conversion to fibrin. Simultaneously, exposure of TF-bearing inflammatory cells results in activation of platelets, both by thrombin and by exposure of collagen (and other subendothelial platelet-activating factors) to blood. Binding of TF, thrombin and other activated coagulation proteases to specific protease activated receptors (PARs) on inflammatory cells, may influence inflammation by inducing release of proinflammatory cytokines. These proinflammatory cytokines will subsequently further cause hypercoagulation and hypofibrinolysis. Coagulation pathways are indicated by straight arrows; inflammatory mechanisms by dashed arrows.

Moreover, tissue factor has been recognised to be involved in the pathogenesis of cardiovascular disease (Steffel, Lüscher & Tanner 2006). The work by El-Hagracy et al, demonstrated a significant positive correlation between tissue factor pathway inhibitor plasma level and factor VIIa among all study participants. In fact, cardiovascular complicated type 2 diabetes mellitus had significantly higher TF and tissue factor pathway inhibitor plasma levels compared to uncomplicated patients (El-Hagracy et al. 2010). The pathogenesis of type 2 diabetes involves beta cell death or failure and peripheral insulin resistance (Donath et al. 2005). Research by Edén and colleagues showed that TF signalling has a negative effect on beta cell function and promotes beta cell death in response to cytokines (Edén, Siegbahn & Mokhtari 2015).

The study by Vaidyula et al, that tested the hypothesis that hyperglycaemia and/or hyperinsulinaemia singly or combined may increase TF, highlighted the following results: in healthy volunteers, combined elevation of plasma insulin and glucose levels for 24 hours produced (a) a nine fold increase in circulating tissue factor procoagulant activity (TFPCA) associated with an increase in monocyte TF surface expression and mRNA and (b) changes in other components of blood coagulation as indicated in Figure 5.3 (Vaidyula et al. 2006). Figure 5.2, summarises the keys events that occur in normal subjects in response to high glucose and/or high insulin exposure (Vaidyula et al. 2006).

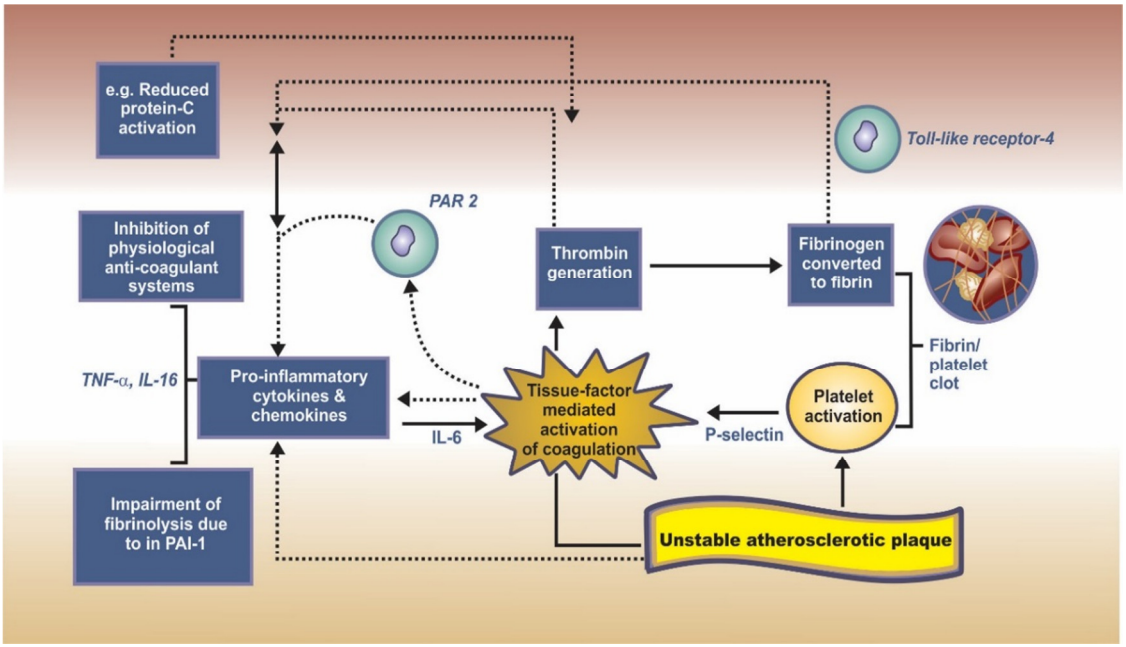


Figure 5.2: **The schematic representation of activation of coagulation and inflammation on rupture of atherosclerotic plaque. Adapted from (Levi, van der Poll & Büller 2004). Coagulation pathways are indicated by straight arrows → and inflammatory mechanisms by dashed arrows ⇢.**

Upon exposure to high glucose/insulin, there is an increase in TF protein and mRNA in monocytes and in circulating TF procoagulant activity. This results in a procoagulant state and the generation of thrombin. Thrombin activates platelets and promotes atherosclerosis. High glucose and/or high insulin are also associated with platelet activation and expression of CD40 ligand (CD40L), which induces tissue factor expression on monocytes and promotes endothelial cell inflammation and atherosclerosis. The decreases in plasma factor VII is considered to reflect increased binding to monocyte TF, which is upregulated (Vaidyula et al. 2006).

Activation of coagulation is regulated by 3 major anticoagulant pathways: anti-thrombin, the protein C system and tissue factor pathway inhibitor (TFPI) as shown in Figure 5.3 (Levi, van der Poll & Büller 2004). During inflammation-induced activation of coagulation, the function of all 3 pathways can be impaired. There is a differential pattern of expression of the various anticoagulant pathways in different vascular beds; for example, in the coronary circulation, TFPI is mostly expressed in microvessels, whereas the protein C system is more universally present (Levi, van der Poll & Büller 2004).

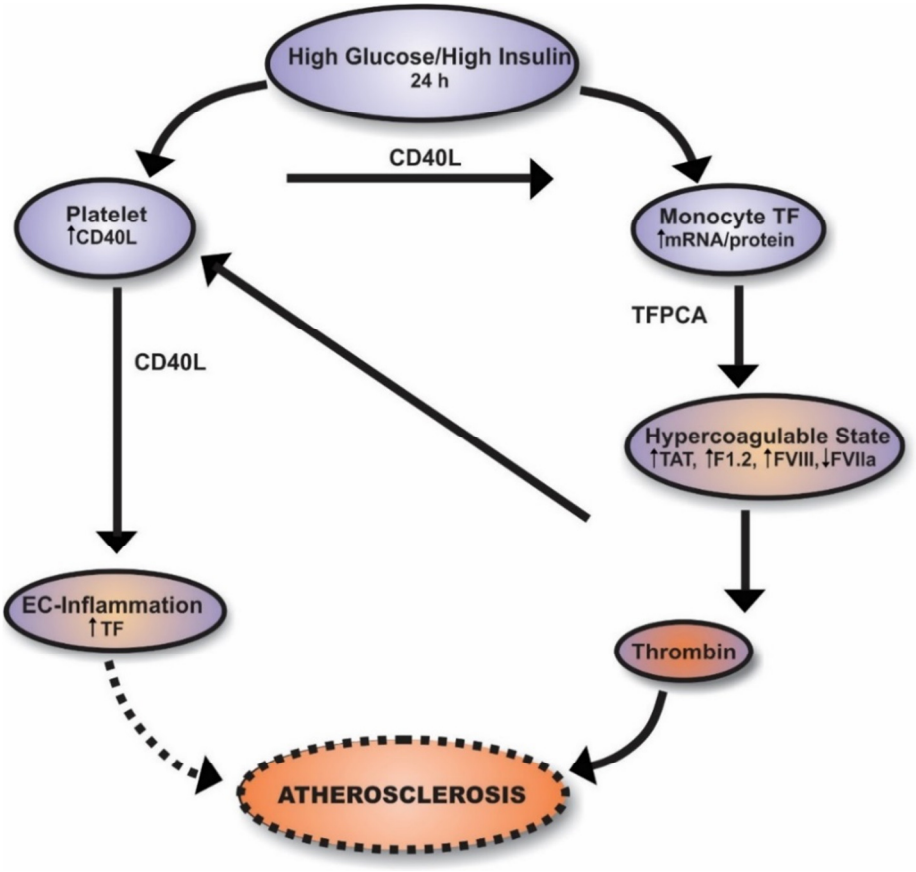


Figure 5.3: **Proposed scheme mechanisms explain changes in tissue factor procoagulant activity, factor VII, factor VIII, thrombin-antithrombin (TAT), prothrombin fragment (F 1.2) and CD40 ligand (CD40L) in normal subjects in response to hyperglycaemia. Taken from Vaidyula et al (Vaidyula et al. 2006). TFPCA = tissue factor procoagulant activity, EC = endothelial cell.**

Details of physiological anticoagulant pathways are highlighted in Figure 5.4. The top left hand corner of Figure 5.3 shows thrombomodulin which plays a central role as endothelial receptor and activator of protein C and TAFI (thrombin-activatable fibrinolysis inhibitor) respectively, and has direct effects on the endothelium. Activated protein C and TAFI have effects on coagulation/fibrinolysis as well on inflammatory processes. TFPI is the endothelial cell-associated inhibitor of tissue factor but also has direct effects on cytokine production in mononuclear cells. Antithrombin binds to endothelial glycosaminoglycan, which causes a more efficient inhibition of activated coagulation proteases and affects inflammation as well. Release of plasminogen activators from endothelial cells will affect local fibrinolysis; the complex of u-PA (urokinase-type plasminogen activator) and u-PAR plays an important in inflammatory cell recruitment and migration. Plus and

minus signs indicate stimulatory and inhibitory effects, respectively (Levi, van der Poll & Büller 2004).

This chapter aims to highlight the role of tissue factor in thrombosis by:

- exploring the physiology of tissue factor;
- review of the coagulation-inflammation-thrombosis circuit; and
- measuring levels of tissue factor (using ELISA) in 42 type 2 diabetic subjects divided into 2 groups, group 1 included 22 diabetic subjects with no cardiovascular complications and group 2 included 20 diabetic subjects with known cardiovascular complications.

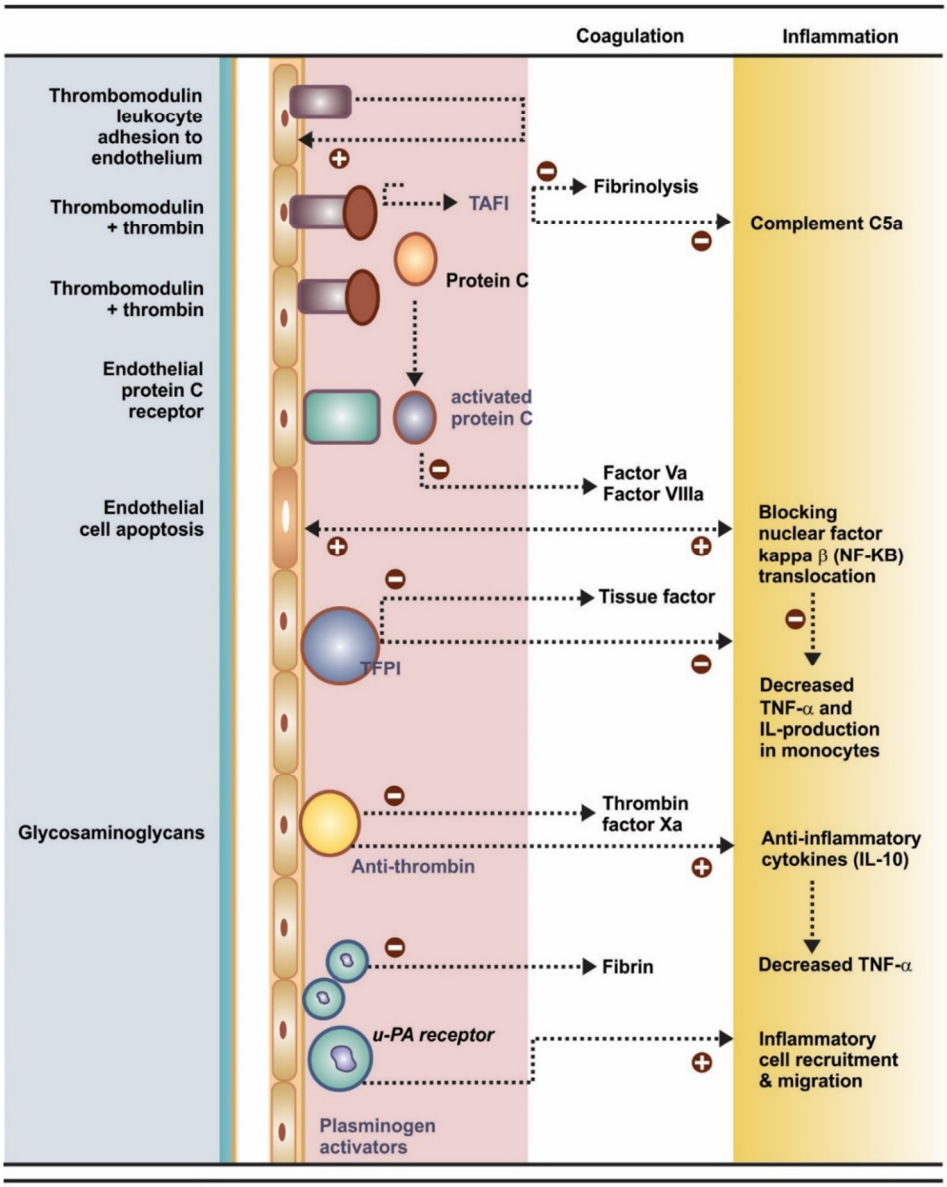


Figure 5.4: **The schematic representation of effects of physiological anticoagulant systems and fibrinolysis on coagulation and inflammation. TAFI=thrombin-**

activatable fibrinolysis inhibitor, TNF=tumour necrosis factor, IL=interleukin, u-PA=urokinase type plasminogen activator. Taken from (Levi, van der Poll & Büller 2004).

5.3 Materials and methods

5.3.1 Participants

42 diabetic subjects were recruited from the Steve Biko Academic Hospital, diabetic clinic in South Africa. Ethical clearance was obtained for this study from the University of Pretoria Human Ethics Committee. Informed consent was obtained from all participants. Inclusion criteria included: (a) subjects older than 18 years and willing to provide informed consent, (b) subjects with known diagnosis of diabetes, (c) for the cardiovascular group, history of previous myocardial infarction, peripheral arterial disease, stroke or coronary arterial bypass grafting. Exclusion criteria included: (a) subjects hemodynamically unstable and (b) subjected with documented life threatening disease (malignancy, HIV/AIDS). Two groups were distinguished, 20 diabetics with cardiovascular complications and 22 without cardiovascular complications.

5.3.2 TF Assay

The ELISA (enzyme-linked immunosorbent assay) was used for the quantitative determination of human TF by using a commercially available kit: IMUBIND Tissue Factor ELISA Kit no. 845 (American Diagnostica Inc., Stamford). The lower detection limit is approximately 10 pg/mL. This assay is designed in a way that it does not interfere with other coagulation factors and identifies TF-apo, TF and TF-VII complexes. The IMUBIND Kit uses a murine anti-human TF monoclonal antibody for antigen capture. This antibody recognizes and neutralizes human brain thromboplastin. Plasma samples incubate in micro-test wells precoated with capture antibody. Once captured the TF is detected using a biotinylated antibody fragment that specifically recognizes bound TF. The subsequent binding of the streptavidin conjugated horseradish peroxidase (HRP) completes the formation of the antibody - enzyme detection complex. TF levels are determined by measuring solution absorbance at 450 nm and comparing the values with those of a standard curve. The standard curve obtained for this assay is shown in Figure 5.5.

5.3.3 Sample Preparation for assay

5 ml of blood was drawn into a citrate tube, from each participant at time of recruitment. This was centrifuged at 3000 rpm for 10 min and stored frozen until time of analysis. To measure TF, blood plasma collected previously was used. The ELISA kit comprised of 96 wells. TF values were done in duplicate as well as standard values at 0, 50, 100, 200, 500 and 1000. On the day of the ELISA, the frozen samples of plasma were thawed at 37℃ for 15 minutes. The plasma was diluted 1:4 in sample buffer.

5.3.4 Assay Procedure

The assay procedure itself was performed over two days. On the first day the precoated micro-test strips were placed in the plate holder once removed from the foil pouch and this was resealed with the desiccant inside and stored in the refrigerator. 100 µL of TF standard was added to the micro-test wells, covered and incubated for three hours at room temperature. Measurements on 42 samples were performed in duplicate. The wells were then washed four times with wash buffer. On the second day, 100 µL of Detection Antibody was added to each well, covered with a lid and incubated for 1 hour at room temperature.

Thereafter, wells were washed 4 times with the 5 Wash Buffer. 12 µL of Enzyme Conjugate was added to 12 mL of Enzyme Conjugate Diluent. The next step was to add 100 µL of diluted enzyme conjugate to each well. They were covered with the lid and incubated for hour at room temperature. Thereafter wells had to be washed 4 times with Wash Buffer. After the hour, 100 µL of Substrate solution was added to each well, covered with the lid and incubated for 20 minutes at room temperature. The solution then turned into a blue colour. In order to stop the enzymatic reaction 50 µL of 0.5M H_2SO_4 was added. To ensure even distribution of the H_2SO_4 the sides of the strip-wells were tapped. The solution now turned a yellow colour. The absorbance's was read on a micro-test plate reader at a wavelength of 450 nm within 30 minutes. Final measurements were calculated by deducting the background average of the blanks from the standards and sample readings. A standard curve was drawn by plotting the mean absorbance for each TF standard against the corresponding concentration of TF as shown in Figure 5. The level of TF was expressed in pg/mL.

5.4 Statistical analysis

All statistical analyses were performed using the GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, https://www.graphpad.com. Mann-Whitney test was used to compare the two diabetic groups, with a p-value of ≤0.05 considered significant.

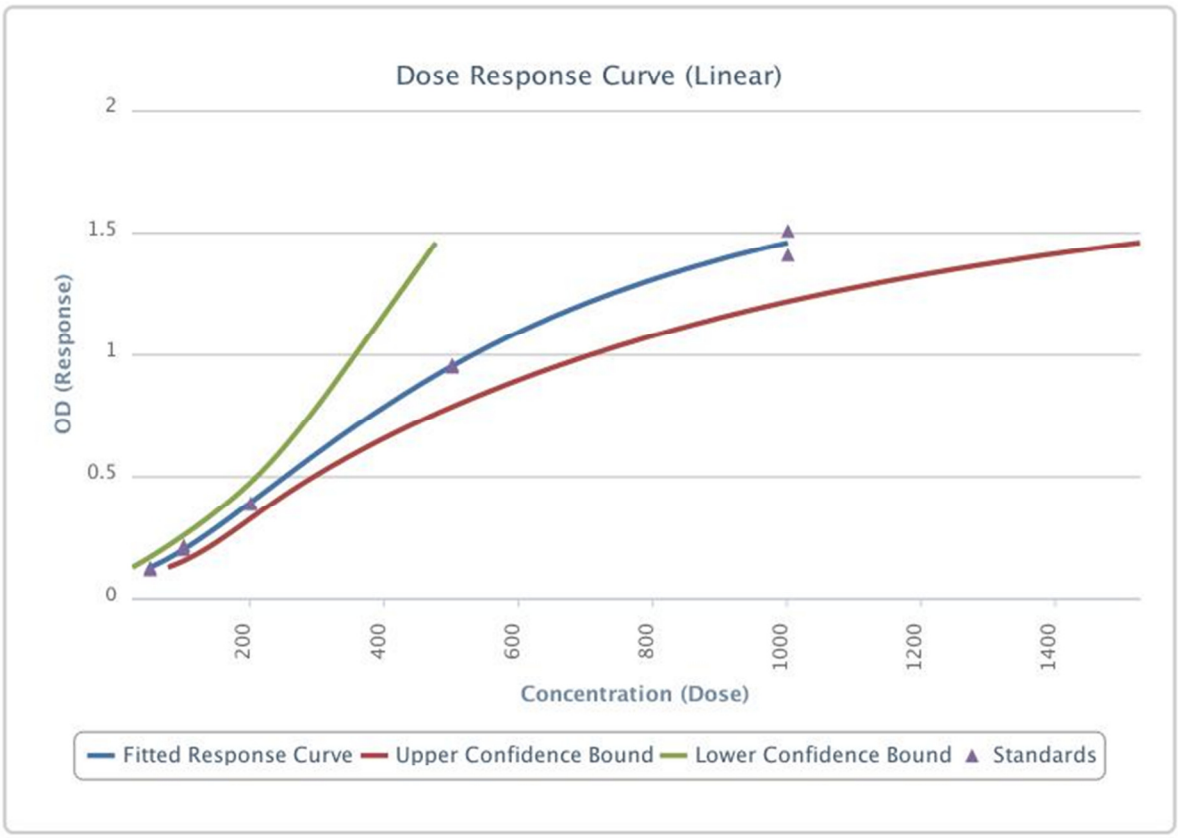


Figure 5.5: Standard curve for the IMUBIND tissue factor ELISA

5.5 Results

The ELISA kit used to calculate TF levels, has 96 wells. Of the total wells, 12 wells were utilized for the standard levels at 0, 50, 100, 200, 500 and 1000, leaving 84 wells for the diabetic subjects. Samples were done in duplicate, therefore analysis on 42 diabetic subjects could be performed. Characteristics of the study population is shown in Table 5.1.

Our results indicate that the concentration of TF was increased in both diabetic groups (with and without CVD) when compared to normal values, however there was no significant difference between diabetics without CVD and diabetics with

CVD. The TF values in both diabetic groups were almost two and a half times greater than the control value. Mann Whitney test showed no significant difference between the diabetic group with or without CVD.

Table 5.1: Baseline demographic data and clinical characteristics of the study population

Variable	Diabetics without CVD (n=22)	Diabetics with CVD (n=20)
Age, years	60 ± 8.7	52 ± 14.9
Males, n (%)	8 (36)	13 (65)
Females, n (%)	14 (63)	7 (33)
Diabetic treatment:		
Insulin, n (%)	4 (18)	3 (15)
Oral agents only, n (%)	18 (82)	16 (80)
Oral and insulin, n (%)	7 (32)	11 (55)
***HBA1c %**	9.0 ± 2.6	8.5 ± 1.7
Cardiovascular Complications:		
Previous MI, n (%)	n/a	9 (45)
PAD, n (%)	n/a	2 (10)
CABG, n (%)	n/a	9 (45)
Essential medication:		
ACEI, n (%)	7 (32)	12 (60)
Ca-antagonist, n (%)	4 (18)	1 (5)
B-blocker, n (%)	3 (14)	4 (20)
Nitrates, n (%)	1 (5)	11 (55)
Statins, n (%)	9 (41)	17 (85)
Disprin, n (%)	6 (27)	13 (65)
Warfarin, n (%)	1 (5)	3 (15)

Data expressed as mean ± (SD) or n (%)

MI = Myocardial infarction, PAD = Peripheral arterial disease, CABG = coronary arterial bypass grafting, ACEI = Angiotensin converting enzyme inhibitor and Ca-antagonist = Calcium antagonist. HBA1c = haemoglobin A1c *not all 42 subjects had this test completed (results are available for 50% of the subjects).

n/a = not applicable

Table 5.2: TF levels for type 2 diabetic patients with and without CVD compared to established values for healthy controls.

Controls	Diabetics without CVD (n=22)	Diabetics with CVD (n=20)
Tissue factor **$^{\$}$164.28** **$^{\$}$(117.39/183.85)**	452.60 ± 163.80	428.50 ± 120.60

All values are shown as medians with standard deviation for diabetic groups.

$^{\$}$Control value as recently calculated in study done by Ruszkowska-Ciastek, (Ruszkowska-Ciastek et al. 2015) with lower/upper quartile in parenthesis.

5.6 Discussion

According to the International Diabetic Federation, the number of people with diabetes worldwide is increasing and by 2030 this will have risen to 552 million (Whiting et al. 2011). Individuals with chronically elevated glucose and/or insulin levels, as is present in most patients with type 2 diabetes, have accelerated atherosclerosis and are prone to acute vascular events (Vaidyula et al. 2006). Evidence shows that diabetes has been considered to have a prothrombotic status. Characteristic findings in type 2 diabetes includes: increased coagulation, impaired fibrinolysis, endothelial dysfunction and platelet hyper-reactivity (Creager et al. 2003). Increased coagulation can be ascribed to the increased concentration of TF, FVIII, thrombin and fibrinogen (Alzahrani & Ajjan 2010) and (Ferreiro, Gómez-Hospital & Angiolillo 2010). Other mechanisms implicated for the prothrombotic milieu in diabetes is that of increased platelet activity (also proved in this study as showed in chapter 3) which increase thrombin expression (Martín-Timón et al. 2014). Diabetic subjects are also known to have elevated levels of plasminogen activator inhibitor type I (PAI-1) which contributes to the hypofibrinolysis (Olexa &, Olexová 2003) and (Fujii et al. 1998). Further evidence provided by Boden and colleagues showed that high levels of TF in poorly controlled type 2 diabetics which feature hyperglycaemia and hyperinsulinaemia contributes to the underlying low grade inflammation (Boden et al. 2007). Due to

the low grade inflammation, there is a rise in circulating levels of interleukin-6 (IL-6), fibrinogen and tissue factor expression in vascular cells (Martín-Timón I, 2014).

The finding in our study of elevated TF levels in both diabetic groups compares favourably with other studies where high TF levels are found in type 2 diabetic subjects (El-Hagracy et al. 2010), (Ruszkowska-Ciastek et al. 2015) and (Boden et al. 2007). In an environment of chronic hyperglycaemia, typical of type 2 diabetes, the binding of advanced glycated end products to their specific receptors creates an intravascular oxidative stress response, culminating in TF expression in vitro (Bierhaus et al. 1997). Furthermore, TF was discovered to be an independent factor related to microvascular diabetic complications (microalbuminuria, retinopathy and neuropathy) which is suggestive of endothelial dysfunction rather than procoagulant activity (Sommeijer et al. 2006). As seen in Figure 5.3, TFPI is mostly produced by vascular endothelium. Thus, increased levels of TFPI indicates endothelial dysfunction or altered binding of TFPI to the endothelium by glucosaminoglycans (Lindahl AK, 1992). In diabetic subjects, particularly those with nephropathy, elevated TFPI activity has been documented (Leurs et al. 1997). Physiologically, TFPI is the key factor in the initial phase of the coagulation pathway mediated by TF, controlling in turn, the production of thrombin which is so crucial in the pathophysiology of atherothrombosis (Opstad et al. 2010). More importantly, altered TF/TFPI ratio has been associated with the development of atherosclerosis, acute coronary syndrome, disseminated intravascular coagulation, sepsis or thrombotic complications related to malignancies (Ardissino et al. 1997) and (Creasy & Reinhart 2001).

Of note is the high incidence of dyslipidaemia and hypertensives among the participants, especially higher in the diabetic group with CVD. 85% of subjects in the CVD group were on statins compared to the 41% in the group without CVD. More subjects were on anti-hypertensive agents in the CVD group. There is an increased tendency for thrombosis in the presence of dyslipidaemia as cholesterol parameters alters TF and TFPI expression in atheromatous plaque (Zawadzki et al. 2007). In the study by El-Hagracy, the diabetic dyslipidaemic and hypertensive diabetic patients had significantly higher TF and TFPI plasma levels when compared to the non-dyslipidaemic patients (El-Hagracy et al. 2010).

Circulating TF is biologically active in converting factor X to Xa (Giesen et al. 1999). However, TF-bearing microparticles are very procoagulant and have been recognised to transfer TF to platelets which further propagates thrombus growth (Rauch & Nemerson 2000) and (Giesen et al. 1999). TF is by itself proinflammatory (Bokarewa, Morrissey & Tarkowski 2002) and in turn stimulates thrombosis and platelet release of CD40L as indicated in Figure 5.3, thereby perpetuating a vicious cycle of inflammation and thrombosis (Libby & Simon 2001). Activated platelets have also been implicated to play a role in decrypting and enhancing TF activity (Osterud & Bjorklid 2006). In our diabetic subjects, platelet hyperactivation was confirmed in both diabetic groups as evidenced by increased CD62P and CD63 percentage activated platelets in chapter 3. This could be contributing factor to the high levels of TF levels obtained in our study.

5.7 Conclusion

Patients with diabetes presenting with acute coronary syndrome have a higher risk of cardiovascular complications and recurrent ischaemic events when compared to non-diabetic patients. Multiple mechanisms have been implicated amongst which abnormalities in coagulation and fibrinolysis feature prominently (Balasubramaniam et al. 2011). Despite being on currently recommended dual antiplatelet therapy, diabetes still poses an increased atherothrombotic risk (Storey 2010). It is thus evident that more effective therapies are warranted for this group of high risk patients. Statins, cholesterol-lowering agents are not only indicated to lower cholesterol levels but have been shown to possess anti-inflammatory properties and to inhibit prenylation of intracellular signalling proteins (Monetti et al. 2007), (Tuomisto et al. 2008) and (Owens & Mackman 2014). Of significance it that statins reduce TF expression within atherosclerotic plaques in animal models (Bea et al. 2003), (Monetti et al. 2007) and (Aikawa et al. 2001).

It remains to be seen if perhaps pharmacological control of plasma TF activity, in particular pathologic TF expression or augmentation of TFPI will lower thrombotic complications.

6. CHAPTER 6: (SCANNING ELECTRON MICROSCOPY) - BASIC MEDICAL SCIENCES: A FUNDAMENTAL DIAGNOSTIC TOOL IN CLINICAL MEDICINE

6.1 Abstract

6.1.1 Aim

Apart from conventional modifiable risk factors such as smoking, hypertension and elevated cholesterol contributing to atherosclerosis, in diabetes mellitus, other factors gaining significance are rheological properties, endogenous fibrinolysis and impaired platelet activity. The aim of the chapter is thus to identify ultrastructural factors contributing to the pathogenesis of atherosclerosis and thrombosis in diabetic patients using scanning electron microscopy (SEM).

6.1.2 Materials and method

Patient's recruitment was completed at the Steve Biko Academic Hospital's diabetic clinic. 30 diabetics with cardiovascular disease (CVD) and 30 without, meeting inclusion and exclusion criteria were enrolled to participate. Bloods were drawn upon completion of the informed consent form. Blood was prepared for SEM so that the ultrastructure of erythrocytes, platelets and fibrin fibres could be investigated. In addition, axial ratios of erythrocytes and fibrin fibre diameters were calculated.

6.1.3 Results

Prominent morphological changes were identified in the erythrocytes, platelets and fibrin fibres. Erythrocytes in the diabetic patients showed changed shape, membrane abnormalities and presence of microparticles. Comparison of the axial ratios of the erythrocytes between the diabetic group with CVD and without, showed a significant (p-value <0.0001) increase in the diabetic group with CVD. Morphological alterations in the diabetic platelets were also distinct as extensive formation of pseudopia, features of spreading and blebbing dominated in almost all the platelets. The physiological abnormality of hypofibrinolysis was morphological confirmed as almost all diabetic subjects showed remarkable differences when compared to healthy platelets. The architecture of the fibrin network resembled that of thickened masses of fibres, described as dense-matted

deposits. Statistical analysis of the fibrin fibre diameters showed a significant (p-value <0.0001) increase the diabetic group with CVD.

6.1.4 Conclusion

Analysis of ultrastructural findings in the diabetic erythrocyte, platelet and fibrin fibres reveal significant findings which may add to a better understanding of the pathogenesis of atherosclerosis and thrombosis. It thus becomes imperative that microscopy analysis be considered as part of the clinical examination, especially in patients with diabetes mellitus.

6.2 Introduction

The idea of an electron microscope was born way back in 1926, when Busch examined the trajectories of charged particles in electric and magnetic fields. A scanning electron microscope (SEM) is a type of electron microscope with unique features as it produces images of a sample by scanning it with a focused beam of electrons. Literature review on the history of scanning electron microscopy developments show that success was achieved in 1963 when Pease and Nixon integrated all the improvements in one instrument, the SEM V with three magnetic lenses and an Everhart-Thornley detector (ETD) (Bogner et al. 2005). Eventually in 1965, the first commercial SEM was developed (Breton 1999).

An important principle of the SEM is that it can produce very high-resolution images of a sample surface, revealing details less than 1 nm in size. Other advantages offered by SEM include: (a) most of the specimen surface is concurrently in focus irrespective of the surface roughness; (b) much higher magnifications (up to 1,000,000x) can be attained compared to optical magnification; (c) additional information on the specimen's, crystalline structure, chemical composition and electrical behaviour is also provided (Vernon-Parry 2000). These added benefits make SEM the best suited technique to investigate the ultrastructural findings in diabetic erythrocytes, platelets and fibrin fibres.

The fact that the erythrocyte is devoid of organelles, the plasma membrane becomes an interesting source of research of age related changes in structure and function (Emam et al. 2008). Among features that are implicated in increased blood viscosity (McMillan 1976), in diabetics, especially those with complications

(Schut et al. 1992), is a decrease in erythrocyte deformability (McMillan, Utterback & La Puma 1978) and changes in erythrocyte membrane fluidity (Bryszewska & Leyko 1983) and (Juhan-Vague et al. 1986). The diffusion of protein and lipid molecules within the membrane is known as membrane fluidity. Thus, changes in membrane fluidity of type 2 diabetics may have an effect on the dynamic properties of erythrocyte membrane as membrane fluidity is a critical prerequisite for functioning of biological membranes (Emam et al. 2008). Previous research (Pretorius 2012), has shown that changes in membrane fluidity are structurally visible, making SEM the best technique to assess this functional aspect of diabetic erythrocytes.

Contributing to the prothrombotic milieu of diabetes mellitus is platelet hyper-reactivity (Ferreiro, Gómez-Hospital & Angiolillo 2010). Characteristics unique to platelets allowing them to be suited for one of their primary functions of responding to blood vessel injury include: changing shape, secreting granule contents and aggregating to form a clot (Blair & Flaumenhaft 2009). In this chapter, SEM will provide evidence on shape change of platelets. Like with erythrocytes, among the array of mechanisms postulated for platelet hyper-reactivity is decreased membrane fluidity (Sobol & Watala 2000).

The final participant involved in coagulation is the fibrin networks. Of particular interest in diabetes mellitus is the associated fibrinolysis, a culprit contributing to premature atherosclerosis in this group (Alzahrani & Ajjan 2010). During blood vessel injury, the normal physiological response is that fibrin is deposited at the atherosclerotic lesion. Thus the structure of deposited fibrin has become significant and is viewed as a probable risk factor for increased proneness for cardiovascular events in subjects with atherosclerosis. Fibrin networks do not all appear the same as they vary in structure from tight networks formed with thin, highly branched fibres to looser networks comprised of larger fibres (Fatah et al. 1996). It is therefore reasonable to consider structure of fibrin networks of diabetic patients as a serious contributing factor to increased CVD risk (Pieters et al. 2008).

Significant ultrastructural abnormalities are found in diabetic erythrocytes, platelets and fibrin networks. In addition, previous research (Pretorius 2012), has shown

that changes in membrane fluidity are structurally visible, making SEM the best technique to assess this functional aspect of cells involved in the coagulation process.

6.3 Materials and Methods

6.3.1 Subjects

60 diabetic subjects were recruited from the Steve Biko Academic Hospital, diabetic clinic in South Africa. Inclusion criteria included: (a) subjects older than 18 years and willing to provide informed consent, (b) subjects with known diagnosis of diabetes, (c) for the cardiovascular group, history of previous myocardial infarction, peripheral arterial disease, stroke or coronary arterial bypass grafting. Exclusion criteria included: (a) subjects hemodynamically unstable and (b) subjected with documented life threatening disease (malignancy, HIV/AIDS). Two groups were distinguished, 30 diabetics with cardiovascular disease and 30 without cardio-vascular disease. Upon completion of informed consent, 5 ml of blood was drawn into a citrate tube, from each participant which was used for microscopy analysis. Ethical clearance was obtained for this study from the University of Pretoria's Human Ethics Committee.

6.3.2 Sample preparation for SEM of red blood cells, platelets and fibrin fibres

From the citrate tube 10 µL of the whole blood was used to make a smear on a small (10 mm in diameter) glass coverslip to investigate RBC morphology. For the preparation of the platelets and fibrin fibres, after the whole blood smears were made, the blood was centrifuged at 65 rpm for eight minutes to obtain platelet rich plasma (PRP). 10 µL of the PRP was placed on a glass coverslip to study platelets. For fibrin fiber formation, thrombin was added to PRP to make an extensive fibrin fiber network. All the coverslips were incubated for five minutes at 37℃ and thereafter washed in a 50% phosphate buffer solution (PBS): 50% dd H_2O solution for 20 minutes, on a shaker to separate the red blood cells from plasma proteins. The washed samples were then fixated for 30 minutes in a fixative comprising of 5mL PBS, 1mL gluteraldehyde and 4mL ddH2O. In order to remove the fixative samples were washed three times. The cover slips were then fixated a second time in 1% osmium tetroxide (OsO_4) for 15 minutes. This second

fixation step was followed by three more washes. The samples were then serially dehydrated in 30%; 50%; 70%; 90% and 100% ethanol. Each dehydration step lasted three minutes and 100% ethanol dehydration was performed three times. The samples were then dried using undiluted hexamethyldisilazane (HMDS, 161.39 g/mol), after which the cover slips were mounted and coated with carbon. Viewing of the samples were done using a Zeiss ULTRA plus FEG-SEM with InLens capabilities. Micrographs were taken at 1 kV. All preparation and analysis of samples were done at the Microscopy and Microanalysis Unit of the University of Pretoria.

6.3.3 Axial ratio determination of erythrocyte shape and fibrin fibre diameters

In order to determine axial ratios, 20 red blood cells from each subject were measured. The longest axis referred to as the major axis was measured, after which a perpendicular line was drawn in the centre to establish the minor axis length. The axial ratio for each cell was obtained by dividing the major axis length by the minor axis length; a value of 1 represents a perfect circle. Fibre diameters were measured from micrographs at 40 000 x magnification. The thickness of 50 different fibres were measured per micrograph.

Axial ratios and fibre diameters were captured using ImageJ (ImageJ is a public domain, Java-based image processing programme developed at the National Institutes of Health: http://rsbweb.nih.gov/ij/). Box plots and other statistics were calculated using Microsoft Excel, together with the add-in template downloadable from http://www.vertex42.com/. In descriptive statistics, a box plot is a convenient way of graphically depicting groups of numerical data through their quartiles.

6.3.4 Statistical analysis

The data for axial ratios and fibrin fibre diameters are expressed as means ± standard deviations. All statistical analyses were performed using the GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, https://www.graphpad.com/. Mann-Whitney test was used to compare the two diabetic groups, with a p-value of ≤0.05 considered significant.

6.4 Results

6.4.1 SEM of RBC's

Demographics of the subjects recruited in the study have been described previously in chapter 2. Ultrastructural analysis of the healthy red blood cells (RBC's) is shown in Figure 6.1. RBC changes observed in diabetic subjects are shown in Figure 6.2 (diabetics without CVD) and in Figure 6.3 (diabetics with CVD). The RBC of a healthy individual is concave and disc shaped. However, in both diabetic groups RBC's structure is compromised and appear irregularly formed as some are elongated or folded. In addition, the RBC membrane forms extended projections, as clearly visible in Figure 6.2 and Figure 6.3. Figure 6.2(C) shows a RBC twisted spontaneously around fibrin fibres. High magnification SEM analyses was adopted to examine RBC structure and membrane surface. Inspection of the micrographs in Figure 6.5 and Figure 6.6 show highly deformed RBC membrane. The abnormalities appear to be of a more severe degree in the diabetic group with CVD.

6.4.2 Axial ratios

Axial ratios of RBC's in healthy individuals are close to 1, suggestive of their discoid shape. Both the diabetic groups have axial ratios greater than 1, inferring that they have an elongated or larger shape. Statistical analysis of the axial ratios when using the Mann Whitney test showed a significant increase in axial ratios of the diabetic group with CVD when compared to the diabetic group without (p-value <0.0001). Detail of the measurements of the RBC's are shown in Table 6.1.

Table 6.1: Axial ratios of RBC's in both diabetic groups

	Diabetes without CVD	Diabetes with CVD	p-value
Mean	1.238	1.183	< 0.0001
Standard deviation	0.22	0.17	

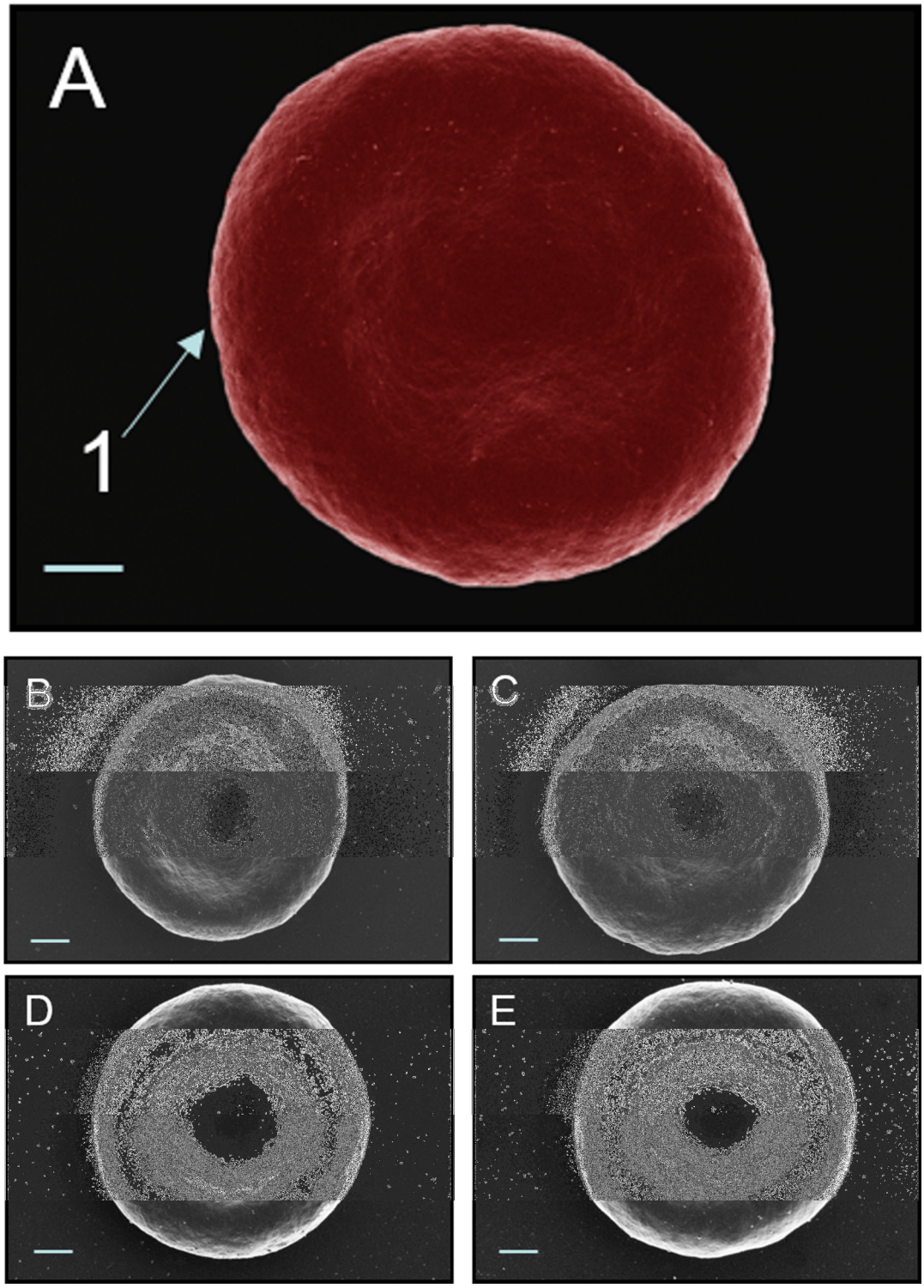


Figure 6.1: RBC of healthy individuals showing typical discoid shape.

Label 1 in A indicating discoid shape and smooth but slightly granular appearance of membrane. A-E typical RBC from healthy individuals (Scale = 1 µm).

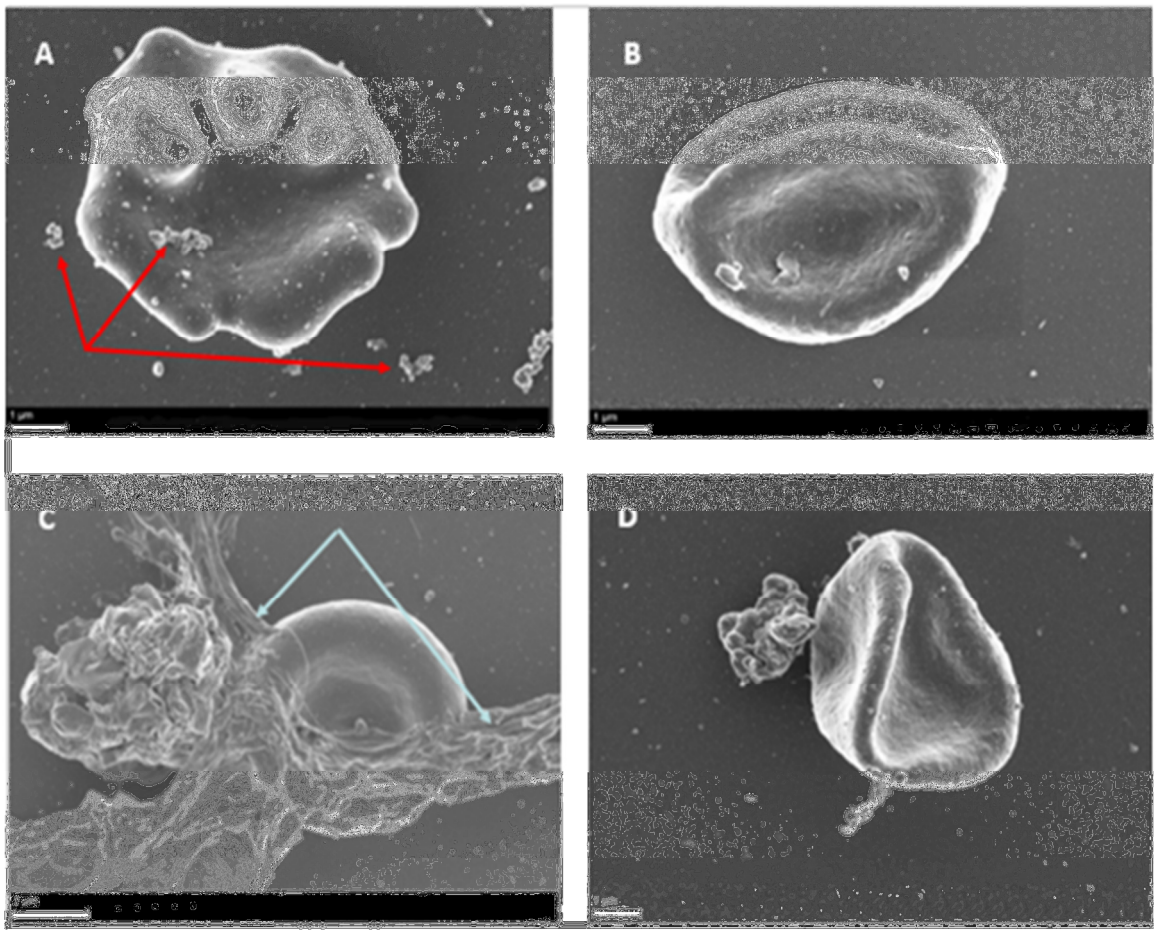


Figure 6.2: Micrographs A-D are those of diabetic subjects without CVD.

Red arrows in A indicate the presence of microparticles, in C arrows show fibrin fibres wrapping around RBC. B and D represent folded RBC shape. Micrograph A also shows the projections of the RBC membrane. (Scale = 1 µm)

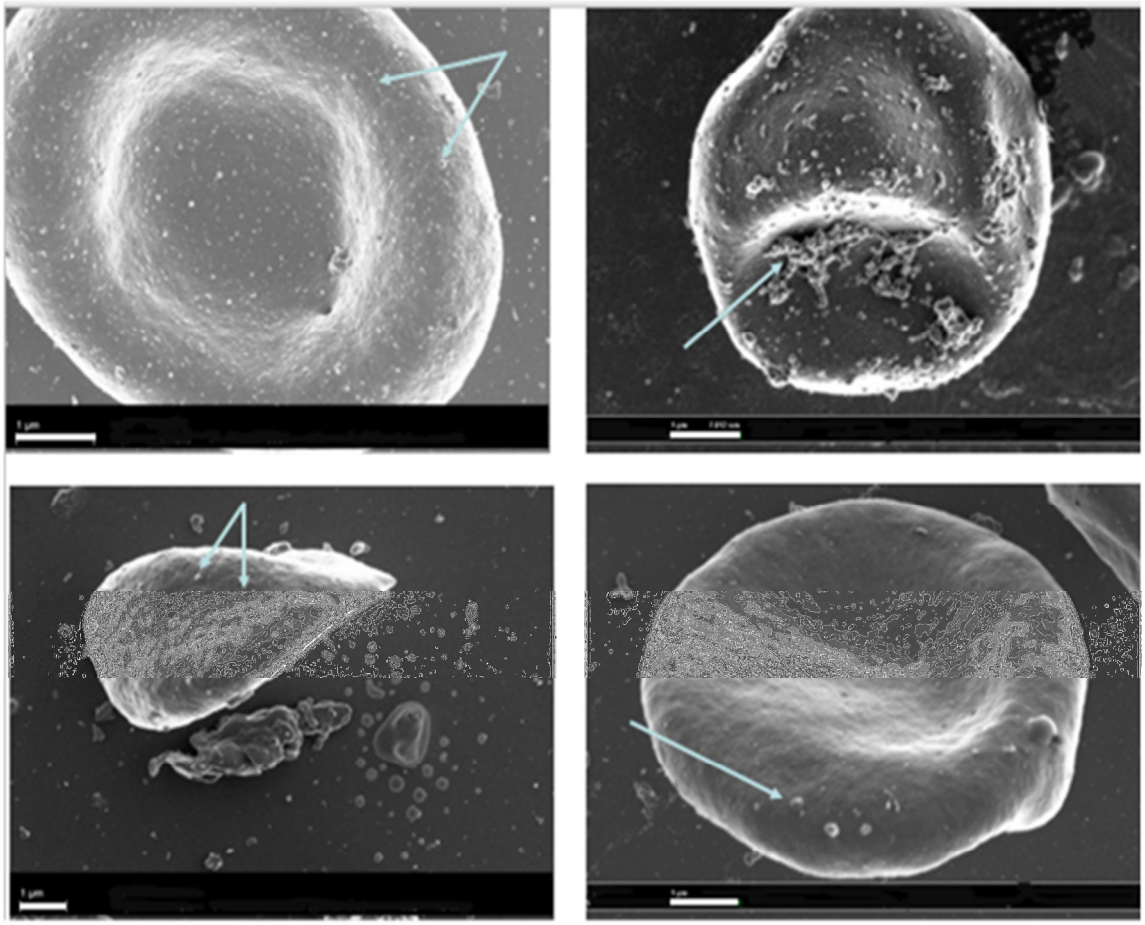


Figure 6.3: Micrographs are those of 4 diabetic subjects with CVD.

Blue arrows indicate presence of microparticles which are visible in all 4 diabetics. RBC are clearly seen as deformed as they lose their discoid shape. Elongated and pointed RBC's are some to the deformities that can be seen. Blue arrows are indicative of the microparticles present of the RBC membrane surface.

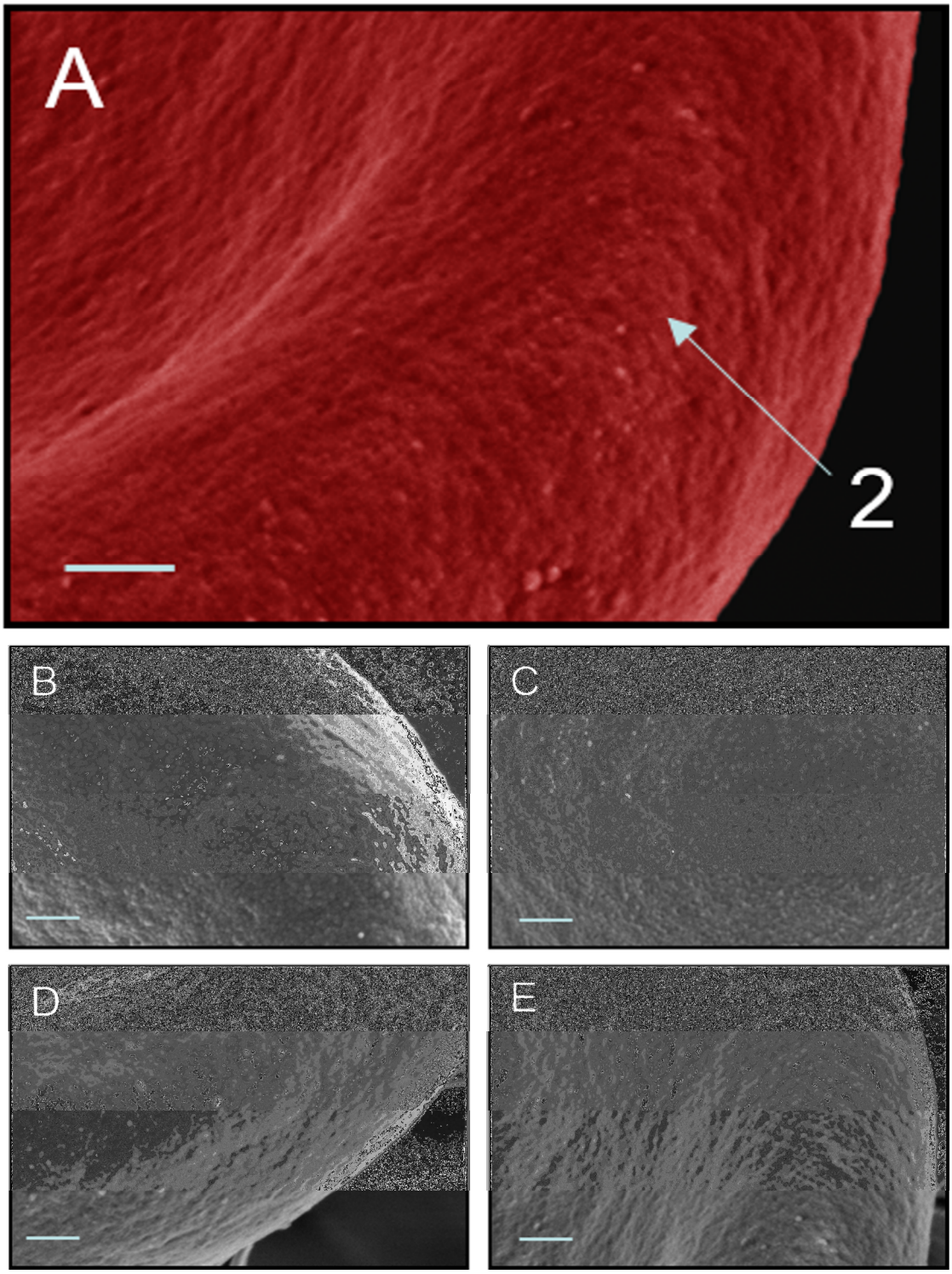


Figure 6.4: **High magnification of RBC membrane showing structure.**

Label 2: Slightly globular erythrocyte membrane. **A-E:** Erythrocyte membranes from healthy individuals. (Scale = 200 nm).

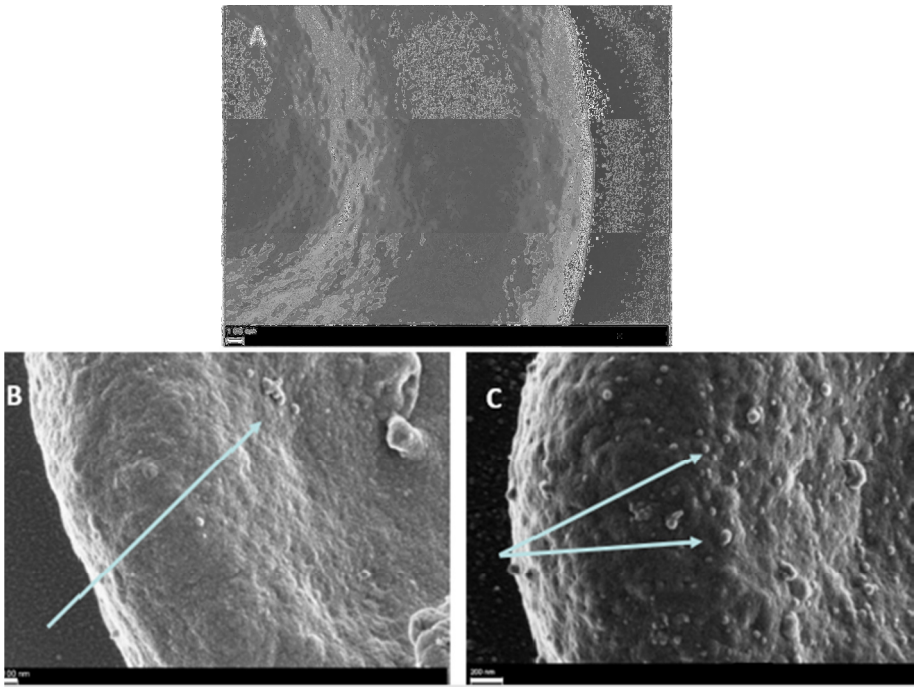


Figure 6.5: High magnification of RBC membrane of 2 diabetic subjects without CVD.

Micrograph A is that of a healthy RBC. Blue arrows indicate presence of microparticles which are pathological.

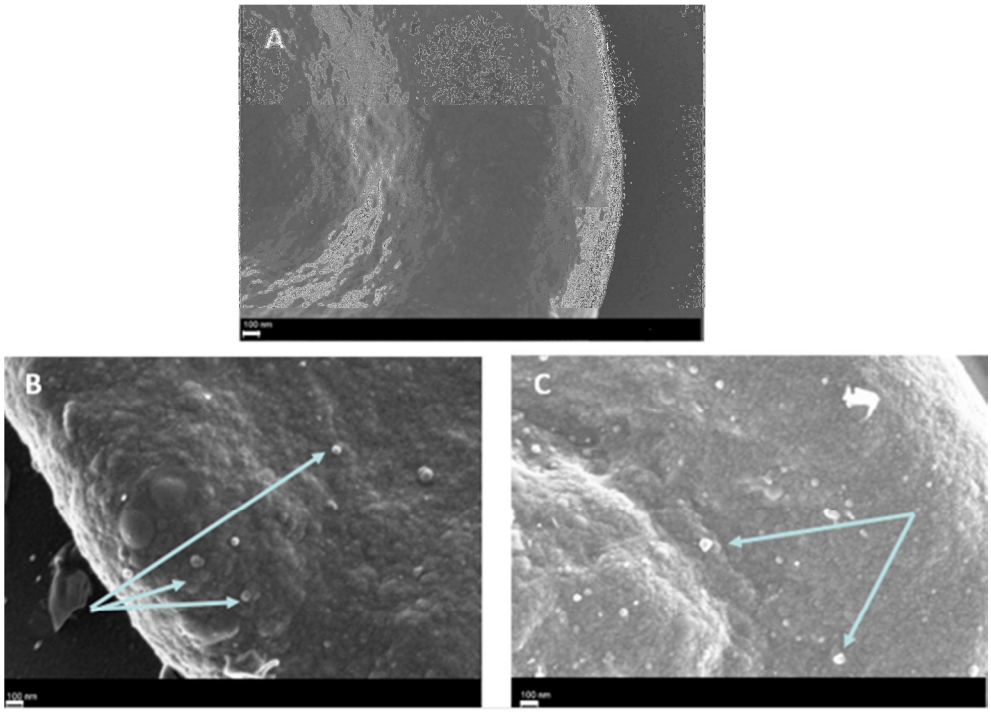


Figure 6.6: High magnification of RBC membrane of diabetic subjects with CVD.

Blue arrows indicate the presence of microparticles. A represents a healthy RBC at x100 000 manchine magnification. Both micrograph B and C, representing 2 diabetic patients, show a globular and rough membrane surface.

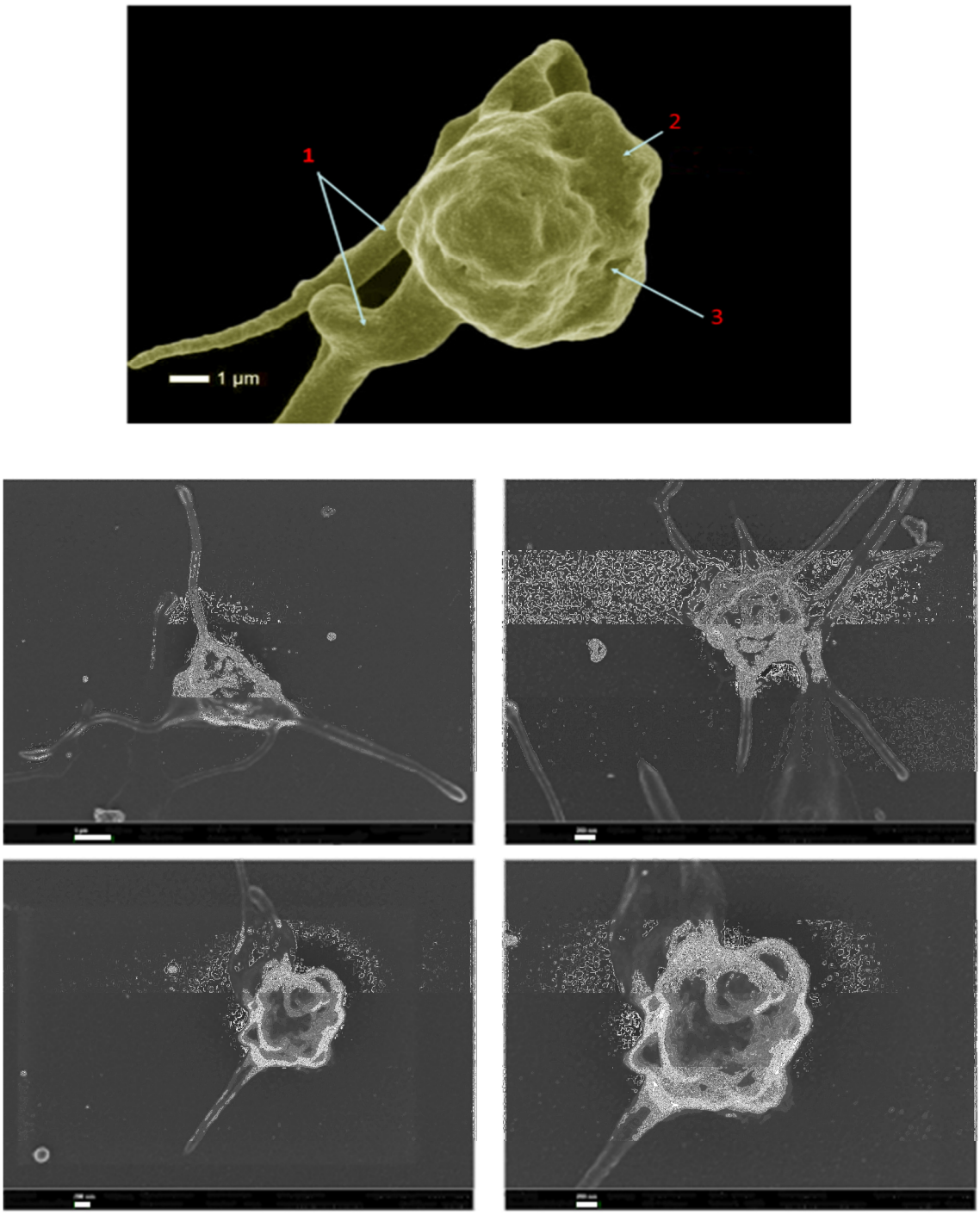


Figure 6.7: **Colour plate of a healthy platelet. Label 1 indicates pseudopodia, label 2 shows the smooth membrane and label 3 is the OCS (open canalicular system). Other micrographs are representative of healthy platelets.**

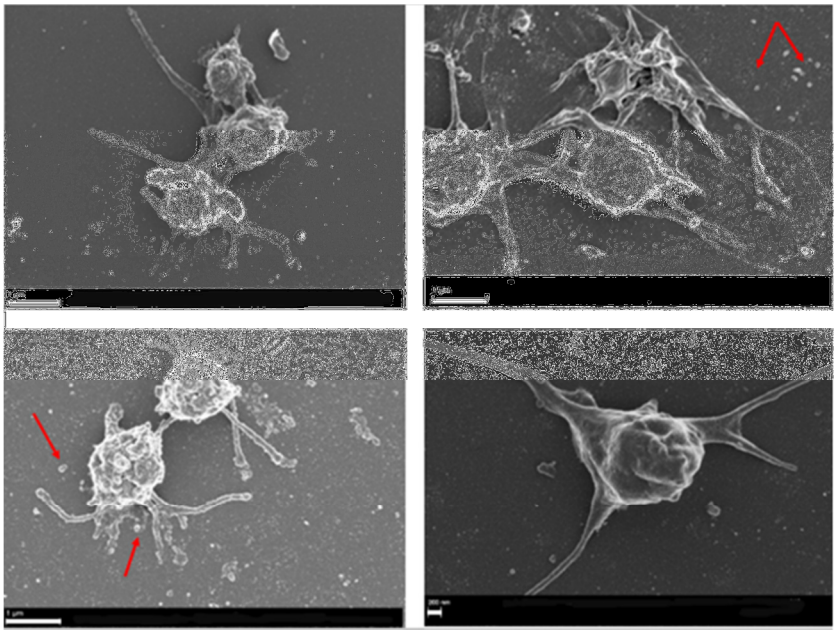


Figure 6.8: Micrographs represent platelets of 4 diabetic subjects without CVD.

Features of spreading and blebbing are seen in all the micrographs. Another important feature clearly visible is the presence of microparticles indicated by the red arrows. All of these features are absent in the healthy platelet.

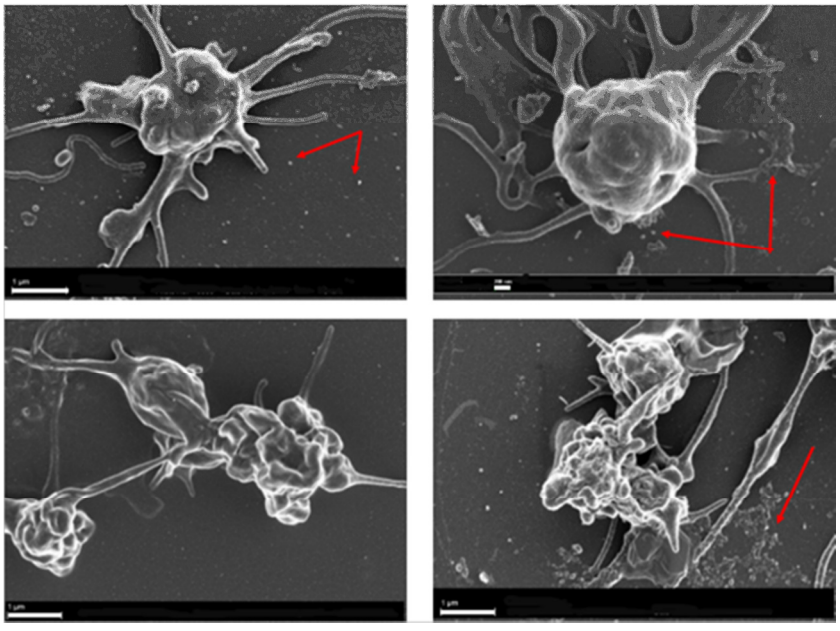


Figure 6.9: Micrographs represent platelets of 4 diabetic subject with CVD.

Compared to healthy platelets, diabetic platelets have lost their normal disc shape. There is also very prominent pseudopia formation in all 4 micrographs with features of spreading. Microparticle formation, indicated by the red arrows is another feature that is predominating in this group of diabetic patients.

6.4.3 SEM of platelets

Healthy platelets, typically show slight contact activation, with minimal pseudopodia formation as evidenced in Figure 6.7. However, during inflammation, platelets form numerous pseudopodia as is shown in Figure 6.8 and Figure 6.9, as well as spreading and extensive clumping as is visible in Figure 6.9, which is the hallmark of over-, or hyperactivation. This hyperactivation is seen in platelets from individuals with diabetes with and without CVD. However, diabetic patients with CVD is characterised further by an increased presence of hyperactivation and microparticles formation as is visible in Figure 6.9. Hyperactivated platelets in the diabetic group with CVD was confirmed by flow cytometry studies as is detailed in chapter 2.

6.4.4 SEM of fibrin fibres

Inspection of the fibrin fibres in the healthy subjects depicted in Figure 6.10 clearly shows individual thick and thin fibres. In comparison, the fibrin fibres in the diabetic subjects is indicative of thickened masses of fibres with a netted morphology as shown in Figure 6.11 and Figure 6.12, giving the appearance of dense-matted deposits, much more prominent in diabetic group with CVD.

6.4.5 Fibrin fibre diameter analysis

Table 6.2 provides detail on measurements obtained by measuring the diameters of 50 fibres per subject. There is a clear difference between the two diabetic groups, with an increase in diameter size in the diabetic with CVD. Statistical analysis of the difference in diameter size between the two groups was significant as the Mann Whitney test revealed a $p<0.0001$.

Table 6.2: Calculations of fibrin fibres in diabetic subjects with and without CVD

	Diabetes without CVD	Diabetes with CVD	p-value
Mean	113.0	123.0	< 0.0001
Standard deviation	41.71	43.90	

Values are in nm

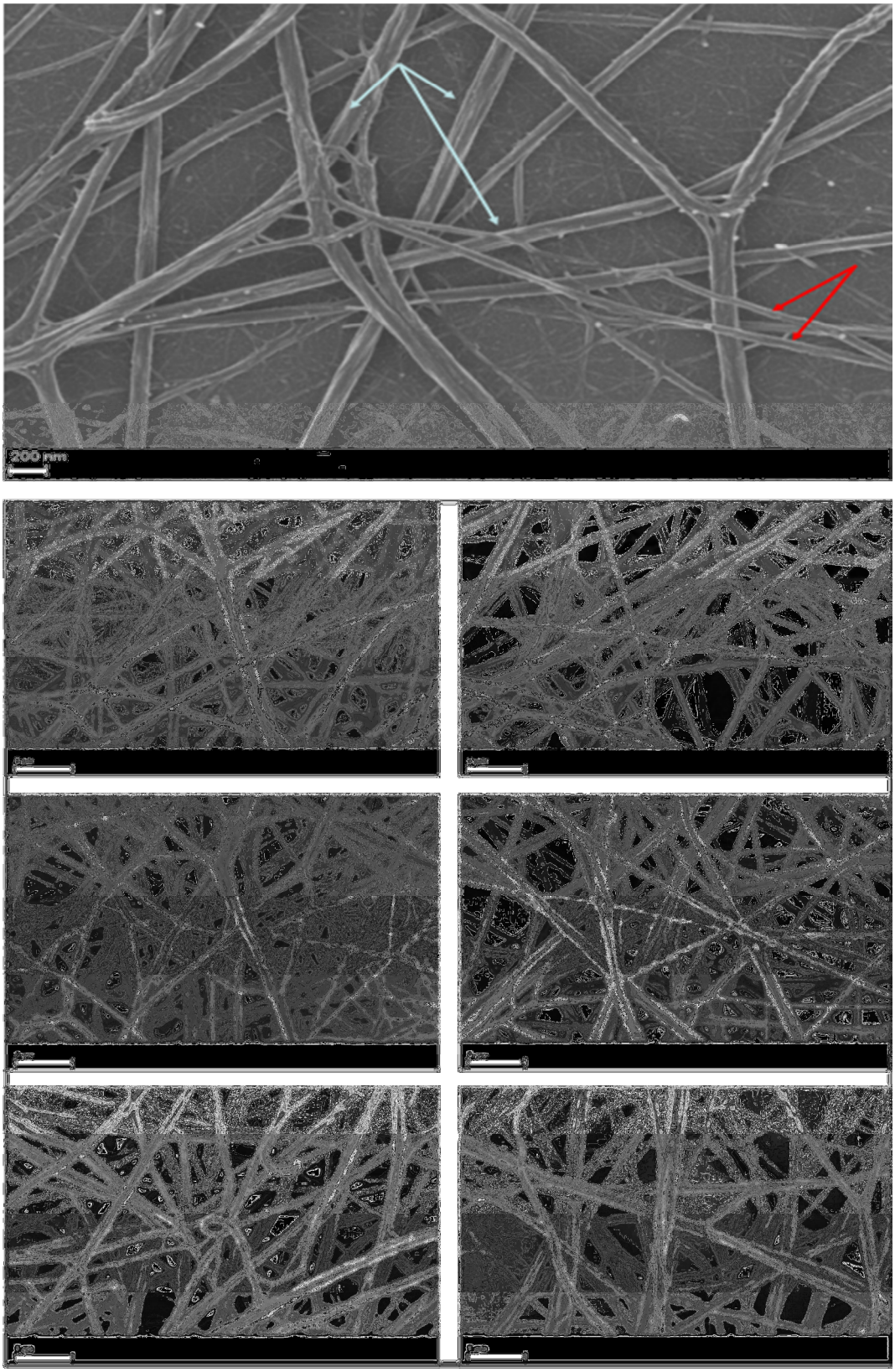


Figure 6.10: **Micrographs above are a representation of an extensive fibrin network of a healthy subject. Red arrows indicate thin fibres and blue arrows represents thick fibres. All other micrographs are taken from healthy subjects as well.**

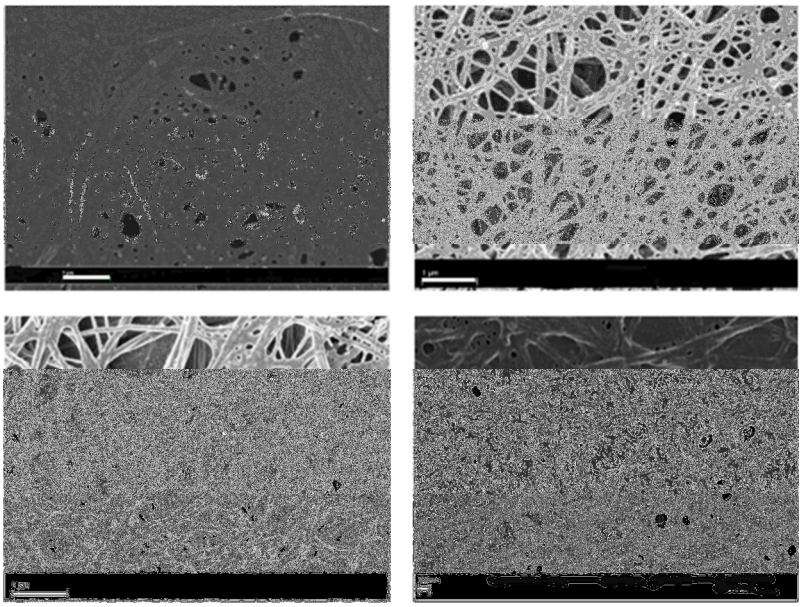


Figure 6.11: Above four micrographs represent fibrin fibres of 4 diabetic subjects without CVD.

In comparison to the healthy fibrin fibres where individual thick and thin fibres are easily distinguished, a pattern of thickened masses of fibres is a prominent feature in all 4 diabetic patients. This pattern is referred to as dense-matted deposits.

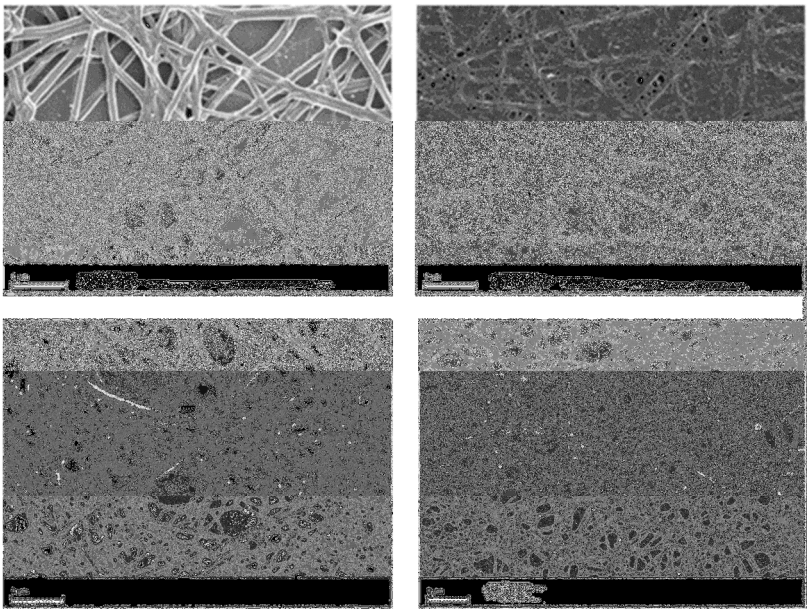


Figure 6.12: Above four micrographs represent fibrin fibres of 4 diabetic subjects with CVD.

Some individual fibres, both thick and thin can be identified, however, the predominant feature is that of thickened masses of fibres with a net-like morphology, giving the appearance of dense-matted deposits.

6.5 Discussion

6.5.1 Ultrastructural alterations in RBC

Alterations in erythrocyte structure and erythrocyte aggregation and deformability are features that characterise diabetic-induced changes in erythrocytes (Singh M, 2009). The erythrocyte cytoskeletal proteins, in particular, beta spectrin, ankyrin and protein 4.1 are heavily glycosylated (Schwartz et al. 1991). Disturbances in ionic balance is another finding common in diabetes. This is attributed to the lowered Na^+/K^+- ATPase activity which leads to complications such as increased serum and intra-erythrocyte sodium and serum potassium. This also results in an increase in the cell size as confirmed by the SEM axial ratios and increased osmotic fragility which contributes to the development of microvascular complications (Kowluru et al. 1989). One of the functions of the erythrocyte membrane is to provide protection and this includes against oxidative damage as well. However, in diabetic subjects, lipid peroxidation causes structural damage to the membrane with a subsequent decrease in the cell deformability and fluidity (Buys et al. 2013). Technological advances confirm that in diabetes, deformability is significantly decreased and is attributed largely to the specific change in the membrane structure. Evidence of this structural changes was confirmed in this study as illustrated in the high magnification micrographs of RBC as visualised in Figure 6.5 and Figure 6.6.The consequence of altered deformability is the increase in blood viscosity which can lead to increase in sheer stress on the endothelial wall adding to the burden of accelerated atherosclerosis in diabetes mellitus (Singh & Shin 2009). Therefore, changes in membrane fluidity are structurally visible and translate into a more globular and bulbous appearance of the membrane surface.

Another participant in the process of atherothrombosis in diabetes is microparticles. Microparticles are clearly visible in the SEM figures of the RBC's and those of the platelets. Microparticles are membrane-coated vesicles that emerge by budding from their parental cells upon activation or apoptosis (Burnier et al. 2009). They retain some functions of their cells of origin, which can include platelets, endothelial cells and various leucocytes. Microparticles have the ability to activate the coagulation cascade with consequent thrombosis formation (Morel et al. 2010). Review of the RBC membrane as seen in Figure 6.5 and Figure 6.6,

show the presence of microparticles. Cell-derived microparticles support coagulation and inflammation and they may be involved in accelerated atherosclerosis in diabetic patients (Morel et al. 2006).

6.5.2 Ultrastructural alterations in platelets

Previous investigations by our research team on the ultrastructure of diabetic platelets confirmed a changed platelet membrane ultrastructure (Pretorius et al. 2011a). In this research study, platelets seemed shrunken and the membranes showed blebbing. Barley no pseudopodia were seen which develop spontaneously from platelets. This blebbed morphology is typical of apoptosis as shown in Figure 6.8 and Figure 6.9. Due to the blebbed morphology, suggestive of apoptosis, the integrity and surface of the platelet membrane is impaired which translates into functional impairment. The finding of membrane blebbing is critical as it may cause an increase in microparticles as indicated by red arrows in Figure 6.8 and Figure 6.9. The stimulus for change in platelet shape is during platelet adhesion to the subendothelial surfaces. Platelet changes shape during the process of adhesion to the subendothelial surfaces it empties its granule contents and aggregates (Vink et al. 2001). The change is platelet shape is facilitated by the formation of pseudopods when intracellular calcium concentration exceeds a threshold (Smith, DeWitt & Garavito 2000). Change in platelet membrane fluidity in diabetes has been ascribed to glycation of membrane proteins which is grossly impaired in diabetes. A mechanism of decreased membrane fluidity is an increased cholesterol-phospholipid molar ration in platelet membranes. The impact of reduced membrane fluidity is associated with hypersensitivity to thrombin in particular to intact platelets of diabetic patients (Winocour, Watala & Kinglough-Rathbone 1992) and (Winocour et al. 1992).

6.5.3 Ultrastructural alterations in fibrin fibres

Jörneskog and colleagues were the first to confirm an altered fibrin network structure in diabetic subjects (Jörneskog et al. 1996). Their findings indicated that plasma clots from type 1 diabetes subjects have reduced permeability indicative of a more compact structure which is independent of the presence of microvascular complications. A likely and significant mechanism implicated in altering fibrin networks in diabetic subjects is the non-enzymatic glycation of fibrinogen in the

presence of uncontrolled blood glucose levels (Pieters et al. 2008) and (Ruszkowska-Ciastek et al. 2015). The subjects recruited in this study were mostly those with poorly controlled glucose. Fibrin clots with thinner fibres, more branches and smaller intrinsic pores fit the profile of a denser and resistant to lysis, both features adding to increased thrombotic risk (Collet et al. 2000). Figure 6.11 and Figure 6.12 show fibrin fibres in diabetics with CVD and without CVD and the appearance is that of thickened masses with a netted morphology, creating a dense-matted deposit/net. This is in blatant contrast to healthy fibrin fibres, Figure 6.11, where individual fibres are clearly discernable. The architecture of a dense-matted net is suggestive of systemic inflammatory profile (Pretorius et al. 2011a) and may contribute to the hypofibrinolysis which is a frequent and significant feature of diabetes mellitus.

6.6 Conclusion

The research findings highlighted in this chapter exposes significant ultrastructural findings. The combination of changed erythrocyte morphology, blebbed platelets with microparticles and dense-matted deposits creates a thrombotic risk cluster which underpins the development of cardiovascular disease and complications. It is clear that ultrastructural findings, especially in diabetic subjects are significant and may add to a better understanding of the pathogenesis of atherosclerosis and thrombosis. As we advocate history taking and measurement of vital signs compulsory tasks, prior to starting a clinical examination, the time has come to include the basic medical sciences, in particular microscopic analysis an obligatory component of clinical medicine.

7. CHAPTER 7: EARTHING: A POSSIBLE NEW MODALITY OF TREATMENT TO IMPROVE MORPHOLOGICAL ABNORMALITIES IN TYPE 2 DIABETIC ERYTHROCYTES, PLATELETS AND FIBRIN NETWORKS

7.1 Abstract

7.1.1 Aim

Non-pharmacologic measures to delay or prevent the onset of type 2 diabetes mellitus includes, a healthy diet, regular physical activity and maintaining a normal body weight. Interventions that do not really require medical expertise. Similarly, earthing is a natural, safe procedure whereby contact with the ground allows your body to naturally receive and become charged with free electrons. The aim of the chapter was to investigate the effect of earthing (two-hour session) on the morphology of erythrocytes, platelets and fibrin fibres.

7.1.2 Materials and methods

60 diabetic subjects were recruited from the diabetic clinic in South Africa. Two groups were distinguished, 30 diabetics with cardiovascular disease (CVD) and 30 without CVD. Grounding system comprised of 4 ECG type patches: one on each palm (middle of palm) and one on each sole (just below the ball of the foot). Patches were connected to a ground cord that attached to a dedicated ground stainless steel rod thirty centimetres long driven into the earth outdoors (through open window), with soil made moist. Bloods were drawn at baseline (0 min) and at 120 min, just prior to cessation of the earthing session (two-hour). Tests done included blood withdrawal for flow cytometry analysis, SEM (of erythrocytes, platelets and fibrin fibres) and measurement of axial ratios of erythrocytes and fibrin fibre diameters.

7.1.3 Results

Comparison of the axial ratio at 0 min and at 120 min showed a slight increase in the both diabetic groups at 120 min. Analysis of the fibrin fibre diameter revealed a slight increase in diabetics without CVD and a slight decrease in CVD group at 120 min. All the changes were statistically not significant. Review of SEM micrographs at 0 min conforms to changes typically found in diabetes. Findings at 120 min, on the other hand showed that earthing seems to have impacted largely

on the morphology as the erythrocytes (to a lesser extent), the platelets (almost all subjects) and the fibrin fibres (almost all subjects) abnormalities were improved as at 120 min, morphology resembled that of the healthy/control erythrocyte, platelet and fibrin fibre.

7.1.4 Conclusion

It appears as if earthing as an adjunct modality of treatment could still offer new hope for diabetes. The visual differences in morphology between the 0 min and 120 min can clearly not be ignored. Other benefits of earthing offered to diabetics include: reduction of inflammation, electrical stability, improvement of the autonomic nervous system, optimisation of blood glucose levels and enhancement of erythrocyte's zeta potential. Long-term effects of earthing on morphology will be needed in the future to assess its impact on morbidity and mortality in diabetes.

7.2 Introduction

It is known that the earth maintains a negative electrical potential on its surface. Earthing or grounding is defined as placing one's bare feet on the ground, whether it be dirt, grass, sand or concrete (Anisimov, Mareev & Bakastov 1999). What happens during earthing? Briefly, the theory is that your body is filled with negative-charged free electrons which are abundantly present on the surface of the earth. When your body comes into contact with the earth, your body immediately equalises to the same electric energy level or potential as the earth (Ober, Sinatra & Zucker 2010). Emerging evidence suggests that the earth's negative potential can create a stable internal bioelectrical environment for the normal functioning of all body systems (Oschman 2007). This means that the earth's electrons are conducted to the human body (free electrons are conducted onto the skin surface and into the body via the mucous membranes of the digestive and respiratory system), bringing it to the same electrical potential as the earth, when in direct contact with the ground, either through walking, sitting or lying down (Callahan & Kornberg 1993) and (Feynman, Leighton & Sands 1963).

Ongoing scientific research is suggesting that people feel significantly better when they connect with these ever-present energy fields as documented by the Earthing Institute (http://www.earthinginstitute.net/index.php/faq). The term "earthing" in this

thesis, refers to connecting a person to the ground with a wire and conductive patches, whilst it has to be differentiated from “grounding” which implies to protect the body from high electrical voltages as is practiced in electrical power and electrostatic discharge industries. It is suggested that the free electrons absorbed from the earth, may have a protective effect as they cross the inflammatory barrier, neutralizing the free radicals accumulating at the site of inflammation (Oschman 2008). It is known that electrons from antioxidant molecules neutralize reactive oxygen (ROS) species, or as commonly called, free radicals, which are involved in the body’s immune and inflammatory responses. However, it is assumed that the influx of free electrons absorbed into the body through direct contact with the earth likely neutralizes ROS and thereby decreases acute and chronic inflammation (Oschman 2007). Another significant source of ROS is diabetes as it is also described as an oxidative stress disorder. In diabetes mellitus, this is particularly imperative as the generation of ROS contributes to the initiation and progression of aging, atherosclerosis and other complications in diabetes (Saha et al. 2005).

The discovery of inflammation as a central event in the pathogenesis of diabetes mellitus was made more than a century ago when Ebstein observed a reduction of glucosuria in diabetic subjects taking an anti-inflammatory drug (Ebstein 1876). Type 2 diabetes is usually associated with elevated inflammatory markers and acute phase proteins such as fibrinogen, C-reactive protein, interleukin-6, plasminogen activator inhibitor-1, sialic acid and white cell count (Pradhan et al. 2001). Of greater interest is the emerging research showing that changes in erythrocyte membrane structures, details of which are shown in Figure 7.1, also contribute to the pathophysiology of the disease process (Saha et al. 2005). A structurally and functional cytoskeleton are features that are crucial in regulating cell function which is dependent upon the cell membrane structure (Adak, Chowdhury & Bhattacharyya 2008). Like with most cell membrane structures, the erythrocyte is composed of proteins, lipids and carbohydrates as can be viewed in Figure 7.1. The erythrocyte membrane will be discussed in further detail in the chapter. In addition, the research by Adak and colleagues reveals that oxidative stress in diabetic subjects including those with cardiovascular manifestations, is attributed to the hyperglycaemia which modifies the erythrocyte membrane

dynamic and electrokinetic properties when compared to healthy controls (Adak, Chowdhury & Bhattacharyya 2008).

A key event in atherosclerosis is a maladaptive inflammatory response to the sub-endothelial lipoproteins. A critical aspect of this response is failure of inflammation resolution. Understanding the principles of inflammation resolution is important in deciphering the complex process of atherosclerosis progression (Tabas 2010). Accelerated atherosclerosis is the main underlying factor contributing to the high risk of atherothrombotic events in patients with diabetes mellitus and athero-thrombotic complications are the main cause of mortality (Alzahrani & Ajjan 2010) and (Ferreiro, Gómez-Hospital & Angiolillo 2010). Despite being on currently recommended dual antiplatelet therapy, diabetes still poses an increased atherothrombotic risk (Storey 2010). It is thus evident that more effective therapies are warranted for this group of high risk patients.

The aim of this chapter is thus to highlight the significance of earthing as a possible new treatment strategy to reduce the mortality rate in diabetic subjects as a two-hour session of earthing improves morphological and physiological abnormalities in cells involved in the coagulation process. The morphological and physiological abnormalities were evaluated using scanning electron microscopy (SEM) and flow cytometry respectively.

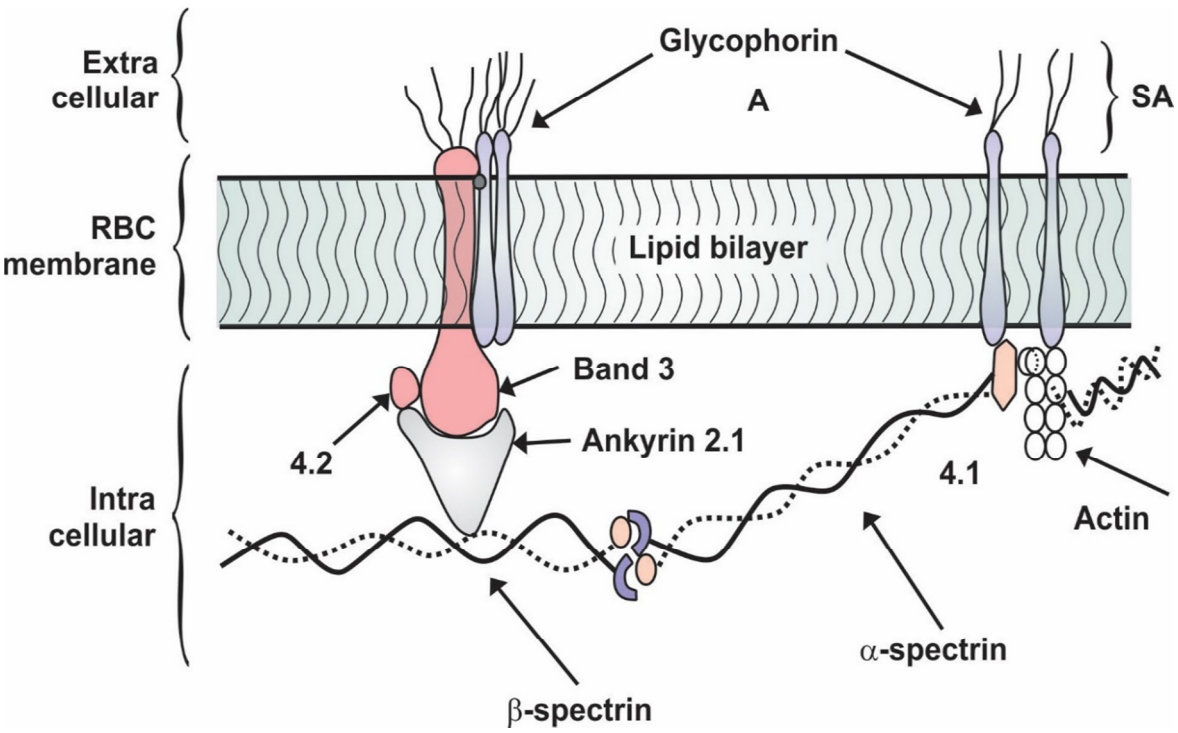


Figure 7.1: Schematic representation of RBC membrane showing red blood cell integral protein orientation. Taken from (Desouky OS, 2009). SA = Sialic acid.

7.3 Materials and methods

7.3.1 Subjects

60 diabetic subjects were recruited from the Steve Biko Academic Hospital, diabetic clinic in South Africa. Inclusion criteria included: (a) subjects older than 18 years and willing to provide informed consent, (b) subjects with known diagnosis of diabetes, (c) for the cardiovascular group, history of previous myocardial infarction, peripheral arterial disease, stroke or coronary arterial bypass grafting. Exclusion criteria included: (a) subjects hemodynamically unstable and (b) subjected with documented life threatening disease (malignancy, HIV/AIDS). Two groups were distinguished, 30 diabetics with cardiovascular disease and 30 without cardio-vascular disease. Ethical clearance was obtained for this study from the University of Pretoria's Human Ethics Committee. The study and informed consent was conducted in accordance with the Declaration of Helsinki and Good Clinical.

7.3.2 Practice guidelines

Upon completion of informed consent, blood samples were drawn at baseline (0 min). Bloods were drawn in a citrate tube (which was clearly marked as 0 min and 120 min) for SEM analysis and flow cytometry. Thereafter, participants were made comfortable on a bed, in a quiet environment, where a two-hour session of earthing was performed. The University of Pretoria Clinical Trial Unit was utilized to perform the clinical aspect of research. Blood samples were drawn at baseline, prior to the commencement of earthing, and at the end of the two-hour session (120 min) just before the grounding was terminated.

7.3.2.1 Earthing/Grounding system

Prior to commencing collection of any data, subjects were made comfortable on a bed and conductive adhesive patches were placed on the sole of each foot and palm of both hands. Patches were connected to a ground cord that attached to a dedicated ground stainless steel rod thirty centimetres long driven into the earth outdoors (through open window), with soil made moist. Grounding system comprised of 4 ECG type patches: one on each palm (middle of palm) and one on each sole (just below the ball of the foot).

7.3.2.2 Sample preparation for SEM of red blood cells, platelets and fibrin fibres

This has been described in detail in Chapter 5.

7.3.2.3 Axial ratio determination of erythrocyte shape and fibrin fibre diameters

To determine if differences in axial ratios of the RBC's and fibrin fibre diameters were present as a result of earthing. Measurements were done on RBC's and fibrin fibres drawn at baseline (0 min) and 120 min. The method employed has been described in Chapter 5.

7.3.2.4 Flow cytometry

The laboratory method and materials used to perform flow cytometry is explained in detail in Chapter 3. Bloods drawn at baseline included an additional citrate tube for flow cytometry analysis and this was done at 120 min as well.

7.3.3 Statistical analysis

For each participant the MCFI (flow cytometry) 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 groups. GraphPad Prism 5 was employed to perform one-way ANOVA for all statistical analysis, with a p-value of ≤0.005 considered significant. Post-hoc Dunnett's Multiple Comparison Test was performed to compare the two diabetic groups to the controls.

The data for axial ratios and fibrin fibre diameters are expressed as means ± standard deviations. All statistical analyses were performed using the GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, http://www.graphpad.com. Mann-Whitney test was used to compare the two diabetic groups, with a p-value of ≤ 0.05 considered significant.

7.4 Results

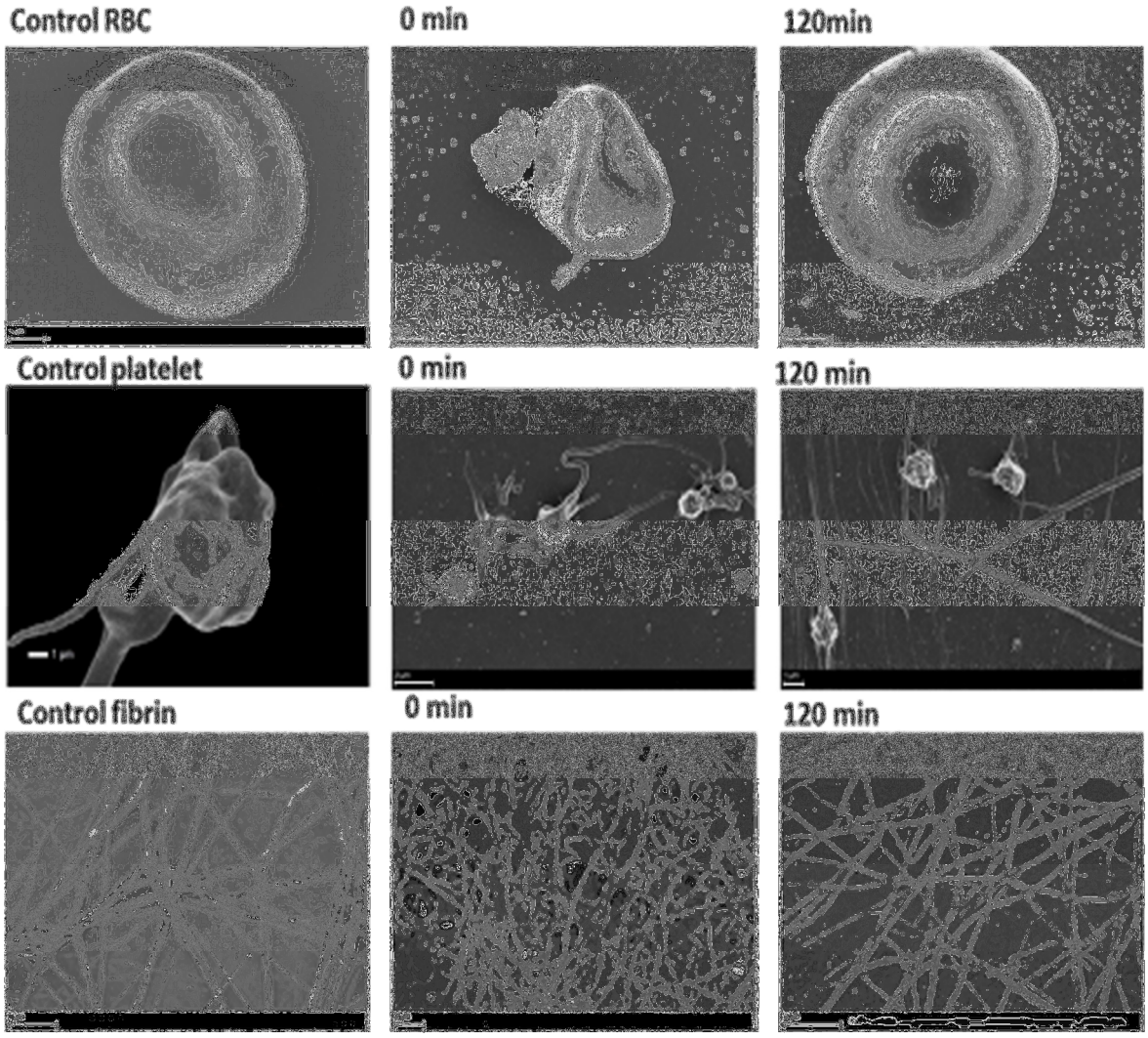
The demographics of the study participants is shown in Chapter 3, Table 3.1. Both diabetic groups were characterised by poor glucose control as revealed by the high HbA1c values. The effects of grounding on the ultrastructure of red blood cells (RBC's), platelets and fibrin fibres in the two diabetic groups is illustrated in Figure 7.2 (diabetics without CVD) and Figure 7.3 (diabetics with CVD). Ultrastructural abnormalities induced by diabetes on RBC's, platelets and fibrin fibres have been elaborated upon in all the preceding chapters of this thesis. Upon inspection of the ultrastructural abnormalities despite the noticeable changes in diabetic RBC's, platelets and fibrin networks, an interesting observation is apparent in the 120 min micrographs as the RBC's, platelets and fibrin networks appear to have more similarities to the morphology of the healthy ones.

Analysis of Table 7.1 shows that the RBC axial ratio in both diabetic groups increased after the two-hour earthing session. However, this change was not statistically significant when applying the Mann Whitney test. Similar findings were observed when the fibrin fibre diameters were analysed as indicated in Table 7.2. In the diabetic group without CVD, a slight increase in diameter was observed after the two-hour earthing session. However, in the group with CVD, a minimal decrease was noted. Both these changes were not significant as revealed by Mann Whitney statistical test.

Table 7.3 is a summary of flow cytometry analysis performed in the 2 diabetic groups. Results obtained at baseline (0 min) were compared with results after a two-hour session of earthing (120 min). Baseline (0 min) results show that, compared to healthy individuals, both diabetic groups showed a significant difference in both platelet identifiers (CD41-PE, CD42b-PE) as well as markers indicating platelet activation (CD62P-PE and CD63-PE). Review of the results shows minimal changes (both increases and decreases) in CD41, CD42, CD62, CD63 as well as platelet activation values when comparing 0 min parameters to 120 min. All the changes detected were statistically not significant.

Figure 7.4 and Figure 7.5 illustrates a detailed view (100 000 x magnification) of the RBC membrane at baseline and at 120 min. Remarkable changes are

observed when comparing baseline morphology to changes at 120 min. Details of the morphological differences are compared in Table 7.4. Changes in properties such as smoothness, roughness, globular texture and presence of microparticles are reported upon in Table 7.4. Pronounced changes in the erythrocytes, platelets and fibrin fibres are observed when comparing the 0 min and 120 min SEM

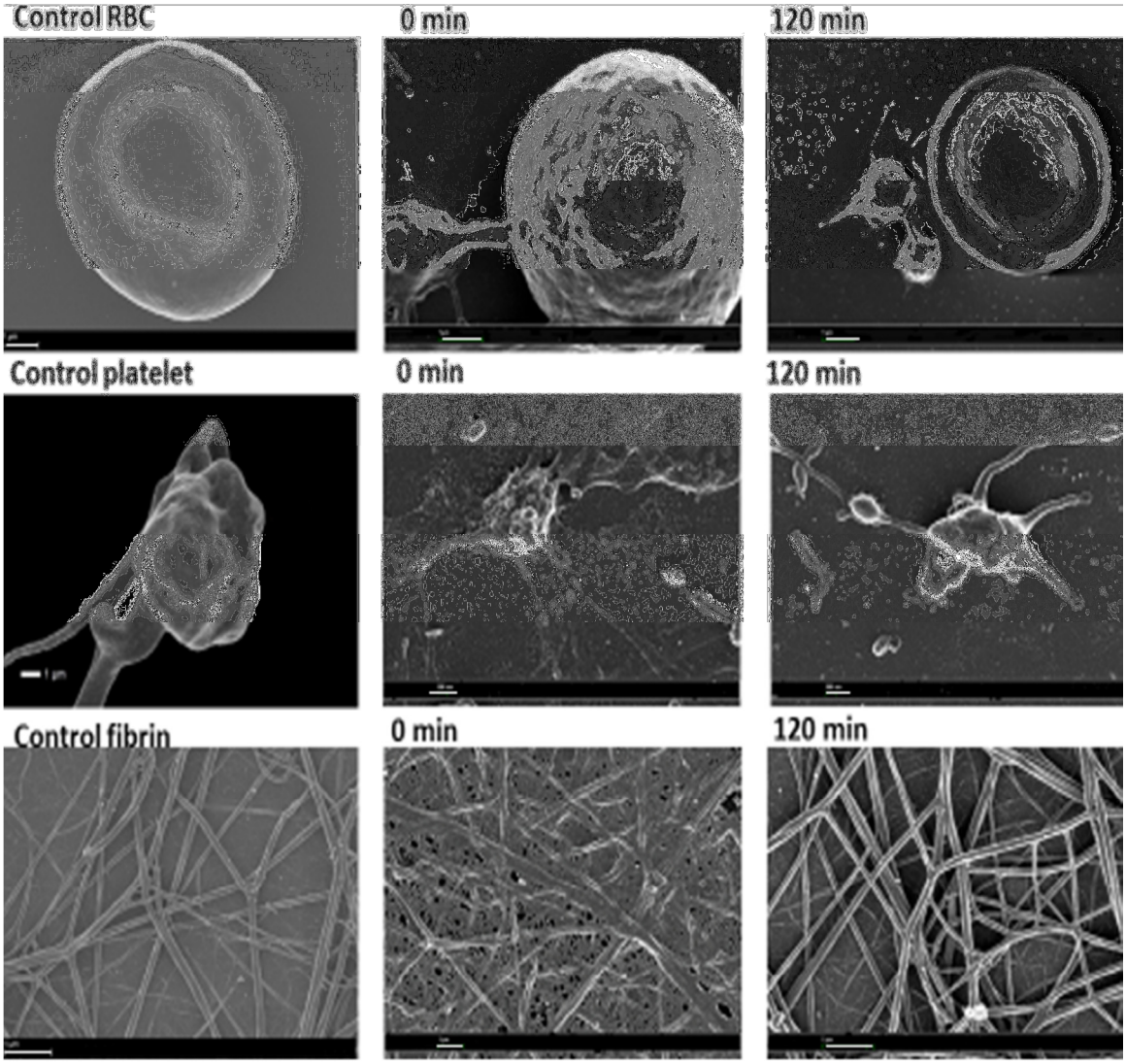


micrographs. This applies to both diabetic groups. Ultrastructural abnormalities present at 0 min are in keeping with changes prevalent in diabetes as described in chapter 6. Improvement in morphology had also appeared to be of a greater extent in the diabetic with CVD group.

Figure 7.2: Summary of SEM findings of diabetics without CVD at baseline (0 min) and post-grounding session (120 min).

Control RBC, platelet and fibrin morphology is described in chapter 6. Prominent changes are observed when comparing micrographs at 0 min to that at 120 min. At 0 min, the RBC is folded and at 120 min regains it biconcave disc shape. The

platelet morphology at 0 min is indicative of a granular surface membrane and features of hyperactivation. There appears to be less platelet activation at 120 min.



The fibrin fibre network at 0 min is in keeping with dense-matted deposits, prevalent in many other inflammatory conditions. Individual thick and thin fibres are difficult to discern. At 120 min, the fibrin network architecture resembles that of the control.

Figure 7.3: Summary of SEM findings of diabetics with CVD at baseline (0 min) and post-earthing session (120 min).

Prominent changes are observed when comparing micrographs at 0 min to that at 120 min. The membrane properties of the RBC at 0 min suggests increased roughness with a globular texture of the membrane. However, at 120 min the RBC shape is that of biconcave disc and a smoother membrane texture. The platelets in this group of diabetics, clearly show signs of diffuse spreading and apoptosis. At

120 min, the spreading can no longer be visualised and pseudopia are clearly seen. The net-like appearance of the fibrin fibres at 0 min conforms to the architecture of dense-matted deposits. At 120 min, both thick and thin fibres are easily discernible and takes the exact appearance of that of healthy fibrin fibres.

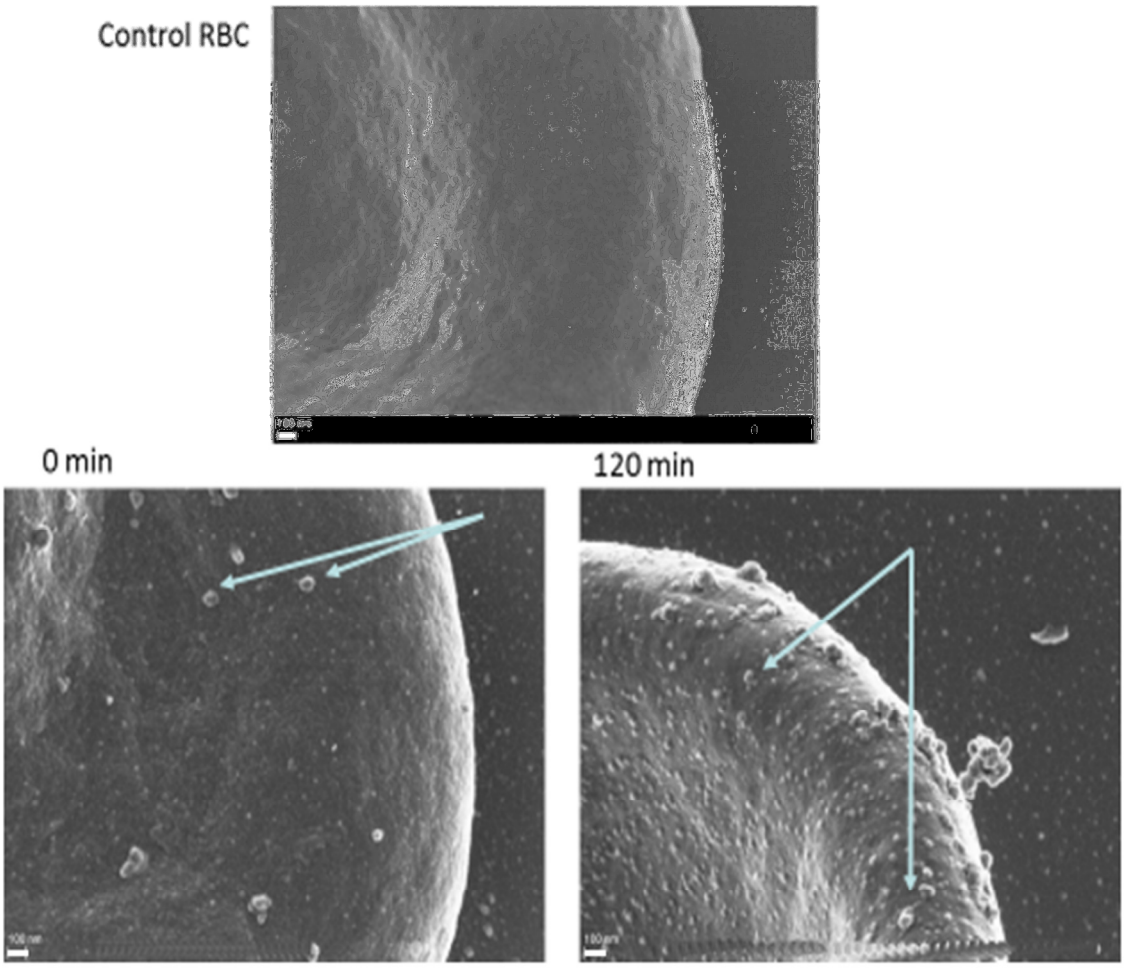


Figure 7.4: High machine magnification SEM of RBC at 100 000 x of a diabetic subject without CVD.

Inspection of the RBC membrane shows a rough appearance with the presence of microparticles (indicated by the arrows) at both 0 min and 120 min. There are some smooth areas of the RBC membrane visible at 120 min. Compared to the control RBC, the diabetic membrane has a particular globular appearance at 0 min.

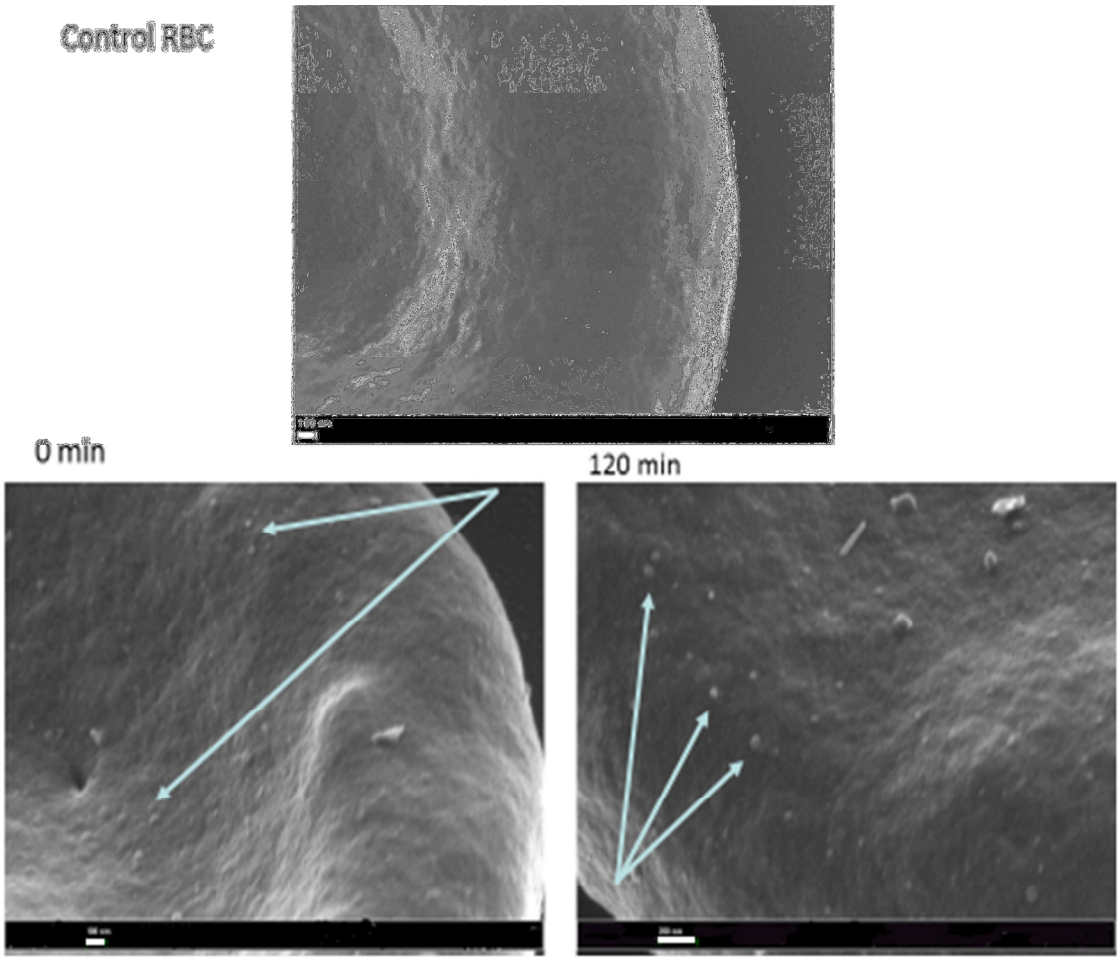


Figure 7.5: **High machine magnification SEM of RBC at 100 000 x magnification of a diabetic subject with CVD showing detail of membrane at baseline (0 min) and after a 2 hours earthing session (120 min).**

Microparticles, indicated by the arrows, are present at 0 min and 120 min. It would seem that there is an increase in the number of microparticles at 120 min. The globular appearance present at 0 min is not visible at 120 min. A very clear difference between the control and the diabetic RBC membrane (0 min and 120 min) is visible.

Table 7.1: **Effects of earthing on axial rations in the 2 diabetic groups**

Axial Ratio	Diabetic without CVD	Diabetics with CVD
0 min	1.238 ± 0.22	1.183 ± 0.17
120 min	1.245 ± 0.24	1.194 ± 0.19

Values are mean ± standard error of mean

Table 7.2: Effects of earthing on fibrin fibre diameters in the 2 diabetic groups

Fibrin fibre diameters	Diabetics without CVD	Diabetics with CVD
0 min	113 ± 41.71	124.5 ± 40.70
120 min	115.5 ± 39.95	124.1 ± 35.58

Values are mean ± standard error of mean in nm

Table 7.3: Analysis of diabetic groups (with or without CVD). Results presented as mean ± Standard Deviation (SD) of *MCFI and percentage activated platelets at 0 min and after a 2 hour earthing session (120 min)

MoAb	Diabetes without CVD (n=30)		Diabetes with CVD (n=30)	
	0 min	**120 min**	**0 min**	**120 min**
CD41-PE	40.18 ± 14.56	39.53 ± 15.16	47.81 ± 24.49	45.12 ± 16.43
CD42b-PE	24.78 ± 11.98	25.26 ± 6.63	24.09 ± 7.27	24.85 ± 24.50
CD62P-PE X-mean	17.96 ± 1.95	17.95 ± 1.99	17.79 ± 2.27	16.93 ± 3.89
CD62P % Activated Platelets	92.65 ± 3.67	92.81 ± 3.85	92.11 ± 4.31	88.24 ± 18.48
CD63-PE X-mean	13.05 ± 3.68	12.33 ± 41.10	13.38 ± 3.12	13.57 ± 2.81
CD63 % Activated Platelets	54.39 ± 32.92	52.14 ± 34.77	61.24 ± 29.73	61.16 ± 28.27

n = 10,000 platelets total analysed for each participant of each group

*MCFI: mean channel fluorescence intensity

Table 7.4: Characteristic morphological findings of RBC's in diabetic subjects with and without cardiovascular disease before and after earthing

RBC Membrane characteristic	Diabetic without CVD		Diabetic with CVD	
	0 min	**120 min**	**0 min**	**120 min**
Roughness	√	less	√	same
Smooth	x	√ (some areas)	x	x
Globular	√	√	√	√ (less)
Microparticles	√	√√	√	√√

√ = present and x = absent, √√ = greater in number

7.5 Discussion

7.5.1 Changes in RBC induced by diabetes and earthing

In order to better understand the complications of diabetes mellitus, the rheological and electrical properties of RBC's have been researched intensively (Desouky 2009). An array of abnormalities in diabetic RBC's have been identified and include amongst others: (a) altered membrane phospholipid asymmetry (Wali et al. 1988), (b) increased aggregation (Babu 2009), (c) reduced deformability (Popel & Johnson 2005), (d) decreased membrane fluidity (Bernhardt & Ellroy 2003). 'Cellular deformability' defines the RBC's ability to undergo deformation during flow. In order to accomplish oxygen delivery, it is critical that the RBC has the ability to undergo considerable cellular deformation since its diameter is 8 µm in comparison to the 2-3 µm of the capillaries through which it squeezes (Mohandas & Chasis 1993). The deformation response is guided by a number of characteristics including cell geometry, the cytoplasmic viscosity due to the presence of haemoglobin and the viscoelasticity of the cell membrane (Agrawal et al. 2016). Figure 7.2 shows the morphology of a healthy RBC and shows a discoid shape when not exposed to external stress. The biconcave disc-shaped RBC affords the benefit of a flexible membrane with a high surface-to-volume ratio that facilitates large reversible elastic deformation of the RBC (Diez-Silva et al. 2010).

Analysis of the axial ratios, as illustrated in Table 7.1, in this study shows that after a two-hour earthing session, the RBC increased in size in both diabetic groups, though this difference was not statistically significant. The study by Agrawal et al, showed that in type 2 diabetic subjects the RBC's were significantly larger when compared to controls. The researchers in the latter study postulated that mechanisms such as increased glycosylation, cholesterol and oxidative stress may result in altered cell morphology with the subsequent formation of swollen RBC's with impaired deformability (Agrawal et al. 2016). Previous research confirms oxidative damage of the RBC membrane with oxidation of spectrin in the RBC membrane or diminished enzyme activities due to defective Na-K-ATPase pumps causing impaired deformability in type 2 diabetic subjects (Schwartz et al. 1991), (Buys et al. 2013). Spectrin as can be seen in Figure 7.1, is one of the 3 principal components (other are actin and protein 4.1) of the peripheral membrane proteins located on the cytoplasmic surface of the lipid bilayer which facilitates release from the membrane by simple manipulation of the ionic strength of the variation in the concentrations of other proteins (Mohandas & Chasis 1993).

A mechanism postulated for the erythrocyte aggregation is that of reduced negative surface charge. It has long been known that red blood cells (RBC's) are negatively charged and that they move to the anode in an applied electric field. In Human RBC's the negative surface charge is largely attributed to the presence of ionogenic carboxyl groups of sialic acids on the cell surface (Kung-Ming & Chien 1973). The surface sialic acid (seen in Figure 7.1) is responsible for 80% of the charge. Furthermore, there appears to be a link between the degree of hyperglycaemia and the decreased surface charge (Gandhi & Chodbury 1979). It is not only hyperglycaemia that is implicated but studies show that hypercholesterolemia also affects the hemorheological and morphological characteristics of erythrocytes (Babu 2009). In this study, as can be seen in the demographics of the study participants (chapter 4), 33% of diabetic subjects without CVD and 83% with CVD were taking cholesterol-lowering agents.

From a clinical perspective, aggregation of erythrocytes is significant as it is observed in many pathological conditions such as diabetes, hypercholesterolemia, thrombosis, myocardial infarction, vascular diseases and haematological

pathology. The presence of large plasma proteins, particularly fibrinogen is a major cause of aggregation (Rampling 1999) and (Rampling et al. 2004). The intricate proteins found on the erythrocyte membrane serve specific functions, as Band 3, seen in Figure 7.1, the most abundant transmembrane protein provides for anion exchange at the level of plasma membrane (Desouky 2009). Erythrocyte glycosylation results in numerous effects and includes: decreasing the negative surface electric charge (Raz I, 1998) due to cleavage of terminal sialic acid components of glycophorin A, increasing erythrocyte aggregation (Mazzanti et al. 1992) and accumulating advanced glycation end products (Brown et al. 1993).

Another participant in the process of atherothrombosis in diabetes is microparticles. Microparticles are membrane-coated vesicles that emerge by budding from their parental cells upon activation or apoptosis (Burnier et al. 2009). They retain some functions of their cells of origin, which can include platelets, endothelial cells and various leucocytes. Microparticles have the ability to activate the coagulation cascade with consequent thrombosis formation (Morel et al. 2010). Review of the RBC membrane as seen in Figure 7.4 and Figure 7.5, show the presence of microparticles. Cell-derived microparticles support coagulation and inflammation and they may be involved in accelerated atherosclerosis in diabetic patients (Morel et al. 2006).

Experiments performed by Sokal and Sokal showed that earthing of the human body had a direct beneficial effect on the regulation of blood glucose in subjects with type 2 diabetes mellitus. In their research, the glucose levels decreased from 10.6 mmol to 7.4 mmol after 72 hours of earthing. This decrease was statistically significant. A mechanism postulated is that earthing the human body during relaxation and during physical activity increases glucose utilization by the cells in type 2 diabetes mellitus thus lowering blood glucose (Sokal & Sokal 2011a). This could be one possible mechanism for change in RBC structure after a two-hour earthing session as hyperglycaemia (Babu 2009) has been reported to affect morphological structures.

When an electric field is applied to a cell suspension, ions in the electrolytic medium migrate towards the electrodes. In addition, the cells of the cytoplasm are also conducting, but the charged ions inside the cells are constrained to the cell

volume since the plasma membrane is non-conducting. Thus, the ions are confined inside the membrane and accumulate at the sides of the cell resulting in the cell becoming polarized (Cole & Cole 1941) and (Debye 1929). One of the parameters that characterizes the dielectric properties of the biological membrane is relative permittivity. The permittivity is a measure of its polarizability in the electric field and many factors regulate it. These include: (a) structural arrangement of the lipid bilayer, (b) conformation and localization of proteins in the membrane, (c) spatial distribution of charge and (d) dipolar groups at the hydrophobic interface (Bonincontro et al. 1989). Research conducted in 1999 by Hillier and co-workers indicated that both decreases and increases in glucose levels results in changes in the electrolyte balance in blood (a decrease in sodium ion concentration and an increase in potassium ion concentration), cells and interstitial fluid in healthy subjects and in subjects with diabetes mellitus. More importantly, these variations affect the RBC's membrane potential (Hillier, Abbott & Barrett 1999).

7.5.2 Changes in platelets induced by diabetes and earthing

Evaluation of the physiochemical properties of the platelets show that under physiological conditions blood platelets have an overall negative charge (Stoltz, Muller & Labrador 1999). Similar to the measurement of zeta potential in the RBC, the electrokinetic charge of the surface region of a platelet is estimated from its zeta potential. In the erythrocyte, the strong net negative charge (zeta potential) is produced by the scialoglyco-protein coat (Adak, Chowdhury & Bhattacharyya 2008). Blood begins to coagulate when zeta potential values of the bilayer membrane are very low, typically found in diabetics with CVD (Adak, Chowdhury & Bhattacharyya 2008). Evidence shows that earthing increases the zeta potential of RBC's thereby decreasing aggregation (Chevalier et al. 2010). To date, there are no studies on zeta potential of platelets and earthing. Features of a healthy platelet can be seen in Figure 7.2. When platelets are not stimulated they circulate as thin discs with smooth surface membranes. However, when stimulated they undergo irreversible shape changes characterized by protrusion of spikes (filopodia), veils (lamellae) or both, to secrete bioactive products with subsequent aggregation (Hartwig 1992) and (Hartwig & DeSisto 1991). It is well known that he central regulatory molecule of blood coagulation, thrombin is regulated by platelet

membrane phosphatidylserine (PS) (Lentz 2003). However, research is suggesting that PS plays a key role in both normal haemostasis and in thrombotic disease (Lentz 2003).

7.5.3 Changes in fibrin fibres induced by diabetes and earthing

Our research team has shown in previous studies the profound changes visually discernible in fibrin fibres in many inflammatory conditions (Pretorius & Kell 2014). As is seen in many inflammatory conditions, here in the diabetic subjects no individual thick and thin fibres can be identified. Instead fibres form a thickened mass giving a typical netted appearance to form dense-matted deposits (DMD). These are clinically significant as these DMD suggest a hyper-coagulable state (Pretorius & Lipinski 2013b). More importantly and of particular interest to diabetes, DMDs may contribute to increased prevalence of thrombotic events (Lipinski & Pretorius 2012). In a normal healthy clot, both thick and thin fibres are prevalent with thick fibres the more dominant ones (Pretorius et al. 2011b). From the results of the fibrin fibre diameters whereby an increase in diameter is observed after earthing in the diabetic group without CVD, and a decrease in diameter in the group with CVD. Even though the difference was not statistically significant it would appear that earthing could have the potential to form clots that are more in keeping with the profile of healthy fibrin fibres.

7.6 Conclusion

The primary results of the study indicated that there is major visual, differences between the diabetic patients with and without cardiovascular disease. Significant differences were also noted between these two groups and the controls. These changes in the platelets, red blood cells and fibrin networks. High magnification of the RBC membrane also provides insights between the differences in the ultra-pathology of the two diabetic groups. Statistical analysis confirms differences in the axial ratios and fibrin fibres between the diabetic group with CVD and those without. Unfortunately, no statistical difference in axial ratios and fibrin diameters were noted when earthing was administered.

A secondary objective was to determine whether earthing may change the coagulation profile visually as well as physiologically. The finding of this study

illustrates clear differences in the ultrastructure of the blood and coagulation profile of both the diabetic subjects' groups, when compared to their own at baseline and at a post earthing session. Of fundamental importance was that these ultrastructural changes were noted in almost all subjects. Although this is a controversial technique, we believe that the interesting findings that we noted, particularly regarding the stabilizing of the fibrin network. The coagulation system forms a prominent part of the general health of an individual. Furthermore, equilibrium of the human body is greatly dependent on this system. In inflammatory diseases, including diabetes, cell membrane anatomy and physiology is impaired. Here we suggest that earthing changes the cell membrane potential to restore it to its optimal functionally levels. We show that within a short period of time, it may change and adjust to new conditions. This is an important and novel scientific observation.

It appears as if earthing as an adjunct modality of treatment could still offer new hope for diabetes. The visual differences in morphology between the 0 min and 120 min can clearly not be ignored. Other benefits of earthing offered to diabetics include: reduction of inflammation, electrical stability, improvement of the autonomic nervous system, optimisation of blood glucose levels and enhancement of erythrocyte's zeta potential. Long-term effects of earthing on morphology will be needed in the future to assess its impact on morbidity and mortality in diabetes.

8. CHAPTER 8: CONCLUSION

8.1 Summary of results and recommendations

In some high-income countries where improved and better implementation of quality of care is present, winning the battle against type 2 diabetes is evident as complications of the disease is postponed or prevented. However, this should not be viewed as some form of victory as the incidence of diabetes and the explosion of type 2 diabetes globally remains persistently high and we are thus far from winning the war against diabetes. Of concern too, is that the vast majority of the disease burden is located in low-and-middle income countries, whilst research on diabetes is concentrated in some high-income countries. Thus, our ability to truly understand the difference in the pathophysiology of the disease in different population groups is impaired (Narayan 2016). This has major implications for diagnosis, prevention and treatment. The need to perform research in our own population thus becomes a pressing issue and is one of the main reasons for undertaking this research project.

Figure 8.1 provides a summary of the objectives and findings of the study. With diabetes viewed as an inflammatory condition (Khansari, Shakiba & Mahmoudi 2009), serum ferritin was chosen as the inflammatory marker to measure in this study. Review of the literature confirms a link between iron and diabetes. It is also shown that iron adds to the inflammatory milieu which is highlighted in chapter 2 (Rajpathak et al. 2009). Distinct morphological alterations are observed when ferritin is elevated, especially in the fibrin networks. Even though these changes are prominent, it is not possible to deduce to what extent the iron can be accused as the culprit, as in diabetic subjects, the pattern of dense-matted deposits (DMD's) are present almost in all diabetic subjects. Previous studies by our research team have shown that the pattern of DMD's is present in many other inflammatory conditions such as Parkinson's, Alzheimer's, hereditary haemochromatosis and rheumatoid arthritis.

Chapter 3 provides extensive detail on previous research completed on diabetic RBC, platelet and fibrin networks. Physiological and biochemical abnormalities are discussed with implications on the pathogenesis of thrombosis in diabetes. The

morphological changes in RBC, platelets and fibrin networks are highlighted in Chapter 6, with abnormalities shown in Figure 6.1 as 0 min. In addition to the ultrastructural changes observed, the chapter also shows that the role of microscopy is perhaps neglected in clinical medicine. Information provided by the ultrastructural changes adds to a better understanding of the pathogenesis of atherosclerosis.

The ultrastructural findings in the coagulation system of the diabetic subjects was correlated with physiological parameters viz., platelet markers and activation using flow cytometry (chapter 4) and plasma tissue factor (chapter 5) was measured using ELISA. In our population of diabetic subjects, the platelets were indeed confirmed to be 'angry' and correlated well with the morphological findings in chapter 6. As can be viewed in Figure 6.1 there are clear visual differences between the morphology at baseline (0 min in the figure) and after a two-hour earthing session (120 min). Chapter 7 defines what earthing is and reveals changes in the morphology of RBC's, platelets and fibrin networks. A much larger proportion of subjects showed more profound changes in the platelets and almost all in the fibrin networks. The effect of earthing on the RBC viscosity has been completed by Chevalier et al, and showed that blood viscosity improves after an earthing session (Chevalier et al. 2010). The long-term effects of earthing on the morphology was not included as an objective so its effects on morbidity and mortality in diabetic subjects is unknown. But with remarkable changes observed after a mere two-hour session of earthing, it is assumed that it is likely to impact on the prevalence of thrombosis in diabetic subjects.

Medical treatment guidelines are written from a clinical perspective, to guide clinical care and are evidenced based. Even though there is no evidence on the long-term effects of earthing on the morphology of RBC's, platelets and fibrin networks, these explicit visual differences observed in nearly 60 diabetic subjects cannot be ignored and recommending earthing as a form of adjunct therapy to diabetic patients is good medical judgement and should be considered to promote best practices and to improve health in diabetic patients.

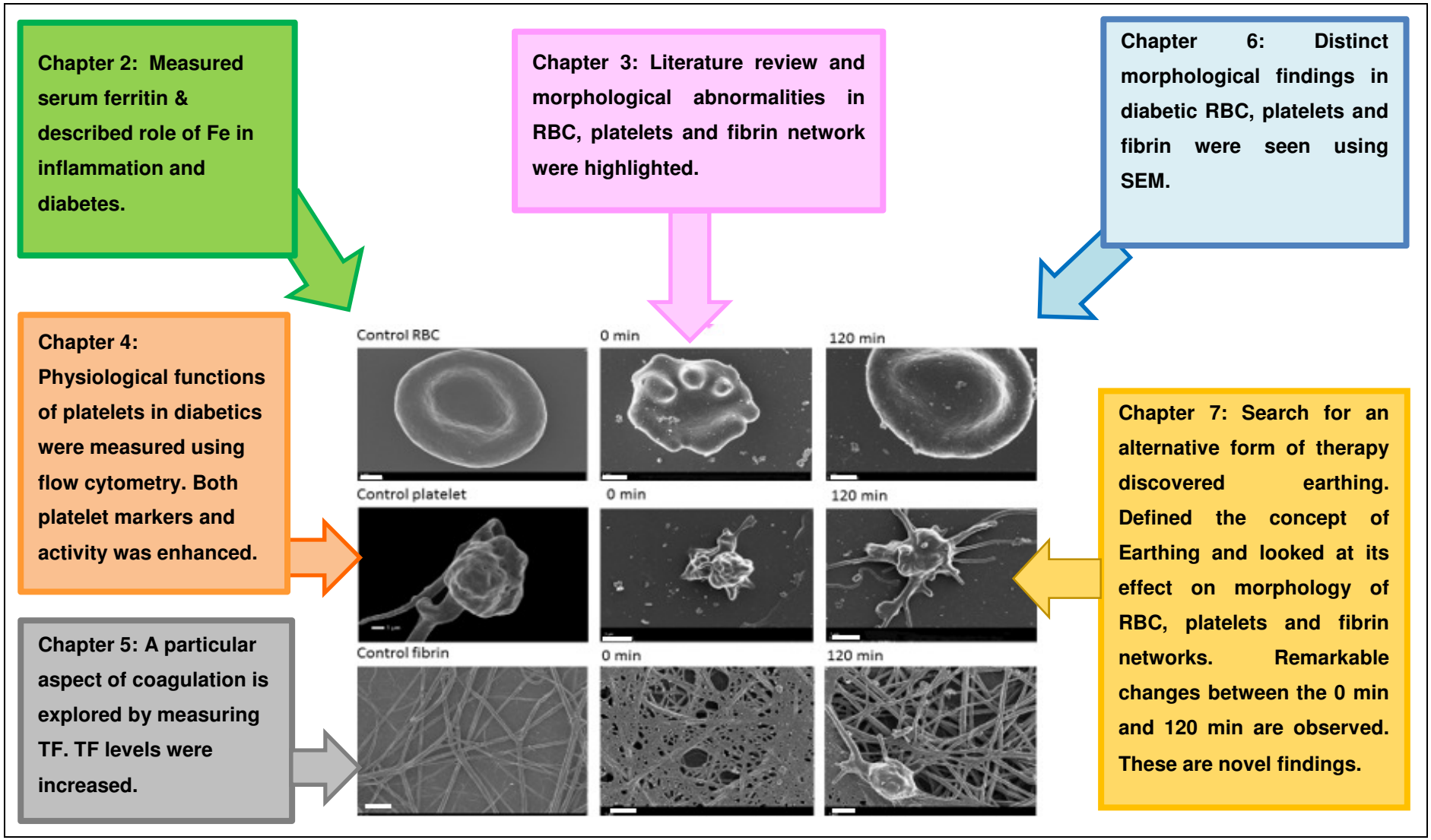


Figure 8.1: **Summary of the chapters and finding in the thesis**

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10. ANNEXURES

10.1 Annexure A: Informed Consent Form

Patient / participant's information leaflet & informed consent form for intervention research

Title of study: Inflammatory markers and ultrastructure of the coagulation profile in Diabetes Mellitus. The effects of grounding/earthing on the coagulation profile will also be evaluated.

Introduction

You are invited to volunteer for a research study. 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 me. You should not agree to take part unless you are completely happy about all the procedures involved.

What is the purpose of this study?

The study is been done to look at the effects of grounding/earthing on certain blood cells, in particular, cells involved in blood clotting, in patients with Diabetes. Earthing refers to the process of connecting by walking barefoot outside, or sitting, working or sleeping grounded indoors. In this study earthing will be achieved by using earthing ECG patches. Four patches will be used; 2 on each palm and 2 on the soles of the feet. The patches will be connected to a stainless rod that will be inserted in moist soil.

What is the duration of this trial?

If you decide to take part you will be one of approximately 30 patients. The study will last for up to 2 hours.

Explanation of procedures to be followed

This study involves answering some questions with regard to your illness, like type of diabetes and duration, measurement of your blood pressure and blood tests. Blood pressure and blood test will be done prior to earthing and repeated after 40 min and just prior to the 2 hour earthing session.

Has the trial received ethical approval?

This clinical trial Protocol was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria and written approval has been granted by that committee. The study has been structured in accordance with the Declarationof Helsinki (last update: October 2008), which deals with the recommendations guiding doctors in biomedical research involving human/subjects. A copy of the Declaration may be obtained from the investigator should you wish to review it.

What are your rights as a patient/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 current admission to hospital. The investigator retains the right to withdraw you from the study if it is considered to be in your best interest.

May any of these study procedures result in discomfort or inconvenience?

Drawing blood may result in a bruise at the puncture site, or infection and bleeding from the site. Your protection is that the procedures are performed under sterile conditions by experienced personnel. A total of 10 ml of blood (i.e. 1 tablespoon) will be collected over the course of the entire study.

Some people have reported tingling feeling while being grounded, but this subsides once the grounding is removed.

Source of additional information

For the duration of the study, you will be under the care of Dr P Soma. If at any time during the study you feel that any of your symptoms are causing you any problems, or you have any questions during the study, please do not hesitate to contact her. The telephone number is0832921170 through which you can reach her.

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 trial.

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 in this study but this information will not be disclosed to any third party without your written permission.

Informed consent

I hereby confirm that I have been informed by the investigator, Dr P Soma about the nature, conduct, benefits and risks of this study. I have also received, read and understood the above written information (Patient Information Leaflet and Informed Consent) regarding the study.

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

Patient's name _____
(Please print)

Patient's signature _____ Date _____

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

Investigator's name _____
(Please print)

Investigator's signature _____ Date _____

10.2 Annexure B: Ethics Approval Letter

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 13/04/2011 and Expires 13/04/2014.

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Faculty of Health Sciences Research Ethics Committee
Fakulteit Gesondheidswetenskappe Navorsingsetiekkomitee

DATE: 31/07/2012

NUMBER	82/2012
TITLE OF THE PROTOCOL	Inflammatory markers and ultrastructural coagulation profiles in Diabetes Mellitus
PRINCIPAL INVESTIGATOR	Dr P Soma **Dept:** Physiology,Steve Biko Academic Hospital; University of Pretoria. **Cell:**0832921170 **E-Mail:** prashilla.soma@up.ac.za
SUB INVESTIGATOR	Not Applicable
STUDY COORDINATOR	Not Applicable
SUPERVISOR (ONLY STUDENTS)	Prof E Pretorius **E-Mail:** resia.pretorius@up.ac.za
STUDY DEGREE	PhD
SPONSOR COMPANY	Not applicable
MEETING DATE	30/05/2012

The Protocol and Informed Consent Document were approved on 30/05/2012 by a properly constituted meeting of the Ethics Committee subject to the following conditions:

1. Including a pilot study on 15 people regarding the 'grounding' component, and
2. The approval is valid for 2 years period [till the end of December 20], and
3. The approval is conditional on the receipt of 6 monthly written Progress Reports, and
4. The approval is conditional on the research being conducted as stipulated by the details of the documents submitted to and approved by the Committee. In the event that a need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Members of the Research Ethics Committee:

Prof M J Bester	(female)BSc (Chemistry and Biochemistry); BSc (Hons)(Biochemistry); MSc(Biochemistry); PhD (Medical Biochemistry)
Prof R Delport	(female)BA et Scien, B Curationis (Hons) (Intensive care Nursing), M Sc (Physiology), PhD (Medicine), M Ed Computer Assisted Education
Prof JA Ker	MBChB; MMed(Int); MD – Vice-Dean (ex officio)
Dr NK Likibi	MBB HM – Representing Gauteng Department of Health) MPH
Dr MP Mathebula	(female)Deputy CEO: Steve Biko Academic Hospital; MBCHB, PDM, HM
Prof A Nienaber	(female) BA(Hons)(Wits); LLB; LLM; LLD(UP); PhD; Dipl.Datametrics(UNISA) – Legal advisor
Mrs MC Nzeku	(female) BSc(NUL); MSc(Biochem)(UCL, UK) – Community representative
Prof L M Ntlhe	MbChB (Natal) FCS (SA)
Snr Sr J Phatoli	(female) BCur(Eet.A); BTec(Oncology Nursing Science) – Nursing representative
Dr R Reynders	MBChB (Pret), FCPaed (CMSA) MRCPCH (Lon) Cert Med. Onc (CMSA)
Dr T Rossouw	(female) MBChB (cum laude); M.Phil (Applied Ethics) (cum laude), MPH (Biostatistics and Epidemiology (cum laude), D.Phil

2012/07/31MS: dd 2012/07/31: C:\Documents and Settings\user\My Documents\Protokolle\Grade briewe\Letters 2012\82.2012-Including a pilot.doc

DR R SOMMERS; MBChB; MMed(Int); MPharmMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

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10.3 Annexure C: Statistician Letter

UNIVERSITEIT VAN PRETORIA
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YUNIBESITHI YA PRETORIA

DEPARTMENT OF STATISTICS

LETTER OF STATISTICAL SUPPORT

Date: 16 April 2012

This letter is to confirm that **Dr P Soma**, studying at the University of Pretoria, discussed the project with the title **THE EFFECTS OF GROUNDING ON THE COAGULATION PROFILE OF DIABETIC PATIENTS** with me.

I hereby confirm that I am aware of the project and also undertake to assist with the statistical analysis of the data generated from the project.

The data analysis will consist of descriptive statistics to summarise the data at baseline and after the intervention; McNemar's test for paired proportions to assess the platelets morphology and paired t-tests (or nonparametric equivalent Wilcoxon signed rank tests) to evaluate the changes in the numerical measurements (fibrin networks patterns, CRP or ESR, tissue factor and fibrinogen levels).

A sample size of between 26 and 64 is recommended to detect a medium to large effect at 0.80 power and using a level of significance of 0.05.

L. Fletcher

Dr Lizelle Fletcher
Department of Statistics
Internal Consultation Service
Tel 012 420 3967

10.4 Annexure D: Article 1

Soma and Pretorius. *Cardiovasc Diabetol* (2015) 14:96
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CARDIO VASCULAR DIABETOLOGY

REVIEW Open Access

Interplay between ultrastructural findings and atherothrombotic complications in type 2 diabetes mellitus

Prashilla Soma and Etheresia Pretorius*

Abstract

Accelerated atherosclerosis is the main underlying factor contributing to the high risk of atherothrombotic events in patients with diabetes mellitus and atherothrombotic complications are the main cause of mortality. Like with many bodily systems, pathology is observed when the normal processes are exaggerated or uncontrolled. This applies to the processes of coagulation and thrombosis as well. In diabetes, in fact, the balance between prothrombotic and fibrinolytic factors is impaired and thus the scale is tipped towards a prothrombotic and hypofibrinolytic milieu, which in association with the vascular changes accompanying plaque formation and ruptures, increases the prevalence of ischaemic events such as angina and myocardial infarction. Apart from traditional, modifiable risk factors for cardiovascular disease like hypertension, smoking, elevated cholesterol; rheological properties, endogenous fibrinolysis and impaired platelet activity are rapidly gaining significance in the pathogenesis of atherosclerosis especially in diabetic subjects. Blood clot formation represents the last step in the athero-thrombotic process, and the structure of the fibrin network has a role in determining predisposition to cardiovascular disease. It is no surprise that just like platelets and fibrin networks, erythrocytes have been shown to play a role in coagulation as well. This is in striking contrast to their traditional physiological role of oxygen transport. In fact, emerging evidence suggests that erythrocytes enhance functional coagulation properties and platelet aggregation. Among the spectrum of haematological abnormalities in diabetes, erythrocyte aggregation and decreased deformability of erythrocytes predominate. More importantly, they are implicated in the pathogenesis of microvascular complications of diabetes. The morphology of platelets, fibrin networks and erythrocytes are thus essential role players in unravelling the pathogenesis of cardiovascular complications in diabetic subjects.

Keywords: Diabetes, Morphology, Platelets, Fibrin, Erythrocytes, Atherosclerosis

Background

The trend regarding the incidence of type 2 diabetes mellitus is that it is increasing in the general population because of increasing obesity and is likely to subsequently increase the incidence of coronary artery disease. It is also known that risk factors such as obesity, hypertension and hypercholesterolemia are crucial to the development of atherosclerosis which results in inflammation [1]. Fundamental to contributory factors of morbidity and mortality in diabetes is atherothrombotic complications [2]. Despite advances in antiplatelet therapies and control of modifiable risk factors: like hypertension, obesity, smoking and dyslipidaemia, the risk of ischaemic events remains high in patients with type 2 diabetes. There is thus a pressing need to understand the complexity of mechanisms contributing to atherothrombotic complications so that more effective therapies can be developed. Evidence shows that diabetes has been considered to be a prothrombotic status. Characteristic findings in type 2 diabetes includes: increased coagulation, impaired fibrinolysis, endothelial dysfunction and platelet hyperreactivity [3].

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All stages in the pathophysiology of plaque formation with atherosclerosis are widespread and accelerated in type 2 diabetes. This is attributed to the imbalance in endothelial damage and repair mechanisms that are usually exhausted. The plaque is made up of erythrocytes, fibrin fibres and platelets [1]. Plaques are more susceptible to rupture and commonly referred to as "vulnerable plaque" syndrome. In particular, hyperglycaemia causing platelet activation, the increase in fibrinogen and hypofibrinolysis related to insulin resistance, all play a significant role in the development of angiopathy [4]. One research method that is used to study platelet structure and activation, is electron microscopy. This review defines ultrastructural findings in diabetic platelets, fibrin network and erythrocytes that can contribute to accelerated atherosclerosis. The outline of the review is highlighted in Fig. 1. The next paragraphs focus on structure and function of platelets, fibrin networks and erythrocytes in relation to type 2 diabetes.

Review

Platelets

An important element postulated in the pathogenesis of the prothrombotic state in diabetic patients is platelet hyper-reactivity. The prothrombotic condition that is characteristic in diabetes, in turn, can be ascribed to the following factors: (1) increased coagulation, (2) impaired fibrinolysis, (3) endothelial dysfunction and (4) platelet hyper-reactivity [3, 5]. In diabetes, several mechanisms contribute to platelet dysfunction, such as those due to hyperglycaemia, insulin deficiency and insulin resistance, associated metabolic conditions and other cellular mechanisms [6]. Even though platelets perform multiple functions, one of their primary functions is to respond to blood vessel injury by utilizing some of its unique characteristic like changing shape, secreting granule contents and aggregating to form a platelet clot. Secondary functions include: maintenance of vascular tone, inflammation, host defence and tumour biology. Two major storage granules in platelets are α- and dense granules. Alpha-granules are most abundant and contain proteins essential for platelet adhesion [7], while the dense granules function to recruit additional platelets to sites of vascular injury. Dense granules store molecules that are secreted upon platelet activation. Contents of dense granules include substances such as catecholamine, serotonin, calcium, adenosine 5′-diphos-phate and adenosine 5′-triphosphate [8].

Like with all cells, the plasma membrane lies beneath the outermost layer and its main component is a

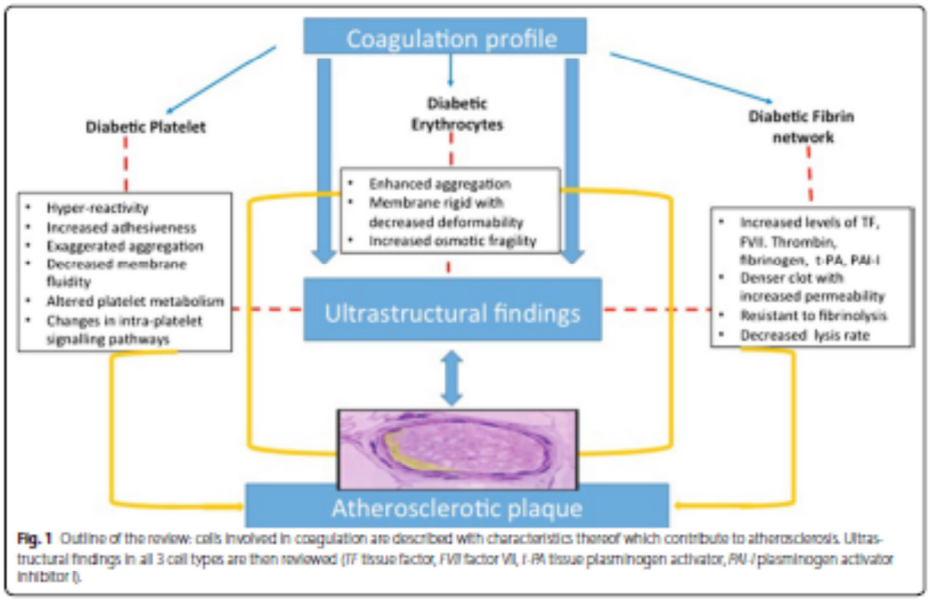

Fig. 1 Outline of the review: cells involved in coagulation are described with characteristics thereof which contribute to atherosclerosis. Ultrastructural findings in all 3 cell types are then reviewed (*TF* tissue factor, *FVII* factor VII, *t-PA* tissue plasminogen activator, *PAI-I* plasminogen activator inhibitor I).

phospholipid bilayer in which cholesterol, glycolipids and glycoproteins are embedded. Unlike erythrocytes, platelets present these molecules on their surface. The organization of the phospholipids between the inner and outer leaflets is asymmetrical and this regulates coagulation. There is an abundance of negatively charged phospholipids in the inner leaflet of the plasma membrane which maintains the platelet surface in a non-procoagulant state [9]. The phospholipids contribute to coagulation by stimulating the coagulation factor X to Xa and prothrombin to thrombin, both integral steps in the coagulation cascade [10]. Other protein components of the resting platelets include CD36, CD63, CD9 and GLUT-3 [7]. These are platelet activation markers and Eibl et al. found that subjects with type 2 diabetes exhibit increased expression of CD31, Cd36, Cd49b, CD62P and CD63 [11]. Research has shown that enhanced platelet activation, aggregation and increased expression of CD63 and CD62 contribute to atherosclerosis and thrombosis in diabetes [12]. A cross section of the discoid platelet also reveals that the platelet membrane is densely packed with highly specific surface receptors that tightly controls signal-dependent platelet activation and may modify α-granule release to coagulation, inflammation, atherosclerosis, antimicrobial host defence, angiogenesis, wound repair or malignancy [7].

Insulin resistance is a common finding in subjects with type 2 diabetes as are the complications in the macrovascular and microvascular circulation. The insulin resistance is responsible for numerous alterations both at the metabolic and cellular levels. Included in the target tissues of insulin, in particular, the cellular systems that are affected is the endothelial cells, platelets, monocytes and erythrocytes [13]. Insulin's action on platelets is to sensitize platelets to the inhibitory effects of prostacyclin and nitric oxide on aggregation and to diminish the proaggregatory properties of agonists such as prostaglandin E1, and E2. During platelet aggregation and activation, mechanisms like phospholipase C-induced hydrolysis of inositol phospholipids and opening of ion channels are activated. This results in various physiological responses by inducing changes in the phosphorylation state, activity of enzymes and structural properties of key platelet proteins [13].

Structural abnormalities found in diabetic platelets

Previous research has shown that diabetic platelets are characterised by increased adhesiveness and exaggerated aggregation. An array of mechanisms for the platelet hyper-reactivity has been postulated: decreased membrane fluidity, altered platelet metabolism (impaired calcium and magnesium homeostasis), elevated glycoprotein receptors, increased thromboxane A2, non-enzymatic glycation of surface proteins, enhanced generation of reactive oxygen species and decreased antioxidants with decreased prostacyclin and nitric oxide [2, 14].

Our research team has looked at the ultrastructure of platelets and fibrin networks in diabetic patients and confirmed a changed platelet membrane ultrastructure [15]. In our studies, platelets seemed shrunken and the membranes showed blebbing [15]. Barely no pseudopodia were seen, which normally develop spontaneously from platelets, as can be seen in control platelet. This blebbed morphology is typical of apoptosis as shown in Fig. 2d [15]. Due to the blebbed morphology, suggesting apoptosis, the integrity and surface of the platelet membrane is impaired, implying functional impairment. The finding of membrane blebbing is critical as it may cause an increase in microparticles in diabetes. Microparticles are membrane-coated vesicles that originate by budding from their parental cells upon activation or apoptosis [16]. Cell-derived microparticles support coagulation and inflammation and they may be involved in accelerated atherosclerosis in diabetic patients [17].

Fibrin networks

There is an increased prevalence of atherothrombotic complications in subjects with diabetes. Prominent features contributing to premature atherosclerosis in this group include: increased platelet reactivity and activation of coagulation factors with associated fibrinolysis [18]. During blood vessel injury, the normal physiological response is that fibrin is deposited at the atherosclerotic lesion. Thus the structure of the deposited fibrin has become significant and is viewed as a probable risk factor for increased affinity for cardiovascular events in subjects with atherosclerosis [19]. Investigating ultrastructural morphological changes in activated platelets, as well as that of fibrin networks, is emerging as an important tool when studying different medical conditions. An aetiologic factor postulated to be causing ultrastructural changes in fibrin networks is abnormalities in the coagulation process [20, 21].

The role of coagulation proteins in the coagulation cascade

The coagulation cascade includes both clot formation and fibrinolysis. Coagulation proteins play significant roles in both the processes. Diabetic subjects are known to have higher levels of circulating tissue factor (TF), FVII (factor VII), thrombin, fibrinogen, tPA (tissue plasminogen activator) and PAI-1 (plasminogen activator inhibitor-I) [18]. TF initiates the thrombotic process with the ultimate production of thrombin which is crucial for the conversion of fibrinogen to fibrin. The increased TF levels in diabetes are under the control of both glucose

and insulin. In fact, the two controlling factors tend to have an additive effect [22]. Another mechanism implicated for the elevated TF levels is through the formation of advanced glycation end products and reactive oxygen species [23]. During the process of plaque rupture TF/FVII complex is formed. Together with the underlying platelet stimulation, this complex activates different coagulation factors with the ultimate generation of thrombin [18]. Like TF, FVII is also elevated in subjects with diabetes and in those with the metabolic syndrome [5]. Early work has shown that FVII coagulant activity has been associated with fatal cardiovascular events and more importantly, increased FVII coagulant activity is directly correlated with raised blood glucose [18].

In both type 1 and type 2 diabetes thrombin generation is enhanced [22]. The hyper-glycaemia found in diabetic subjects, is the culprit causing increased thrombin production and when the hyperglycaemia is controlled, thrombin production is reduced, proving the prothrombotic nature of hyperglycaemia. [24] High concentration of thrombin results in altered clot structures as they are denser and less permeable making them more resistant to lysis [22]. Fibrinogen, the precursor of fibrin is described as an independent risk factor for cardiovascular disease and is often used as a surrogate marker for cardiovascular risk [25]. High fibrinogen levels are known to have a predictive value in the setting of silent myocardial ischaemia especially in subjects with type 2 diabetes [25].

Description of fibrin networks in diabetes

Diabetic subjects have been shown to have altered fibrin network structures as illustrated in Fig. 2e, and this was first confirmed by Jörneskog and colleagues [26]. Their findings indicated that plasma clots from Type 1 diabetes subjects have reduced permeability suggestive of a more compact structure which is independent of the presence of microvascular complications. Using confocal microscopy techniques, Alzahrani and colleagues found that clots made from pooled plasma-purified fibrinogen in diabetic and insulin resistant subjects have a more compact fibrin network structure compared with controls, supporting earlier findings [18]. Figure 2b, e compares fibrin fibres of a healthy individual and that of a typical diabetic individual. Healthy fibrin fibres show individual fibres (Fig. 2b) while fibrin fibres in diabetes have thickened masses of fibres with a netted morphology as indicated Fig. 2e, giving the appearance of dense-matted net. This pattern is suggestive of a systemic inflammatory profile [15] and may contribute to the hypofibrinolysis which is a frequent and significant feature of diabetes.

Mechanisms involved in changing the fibrin network architecture in diabetes cause both quantitative and qualitative changes. Hyperglycaemia and insulin resistance induce qualitative changes as a result of increased glycation and oxidation while quantitative changes are associated with elevated levels of TF, thrombin, fibrinogen and PA1-1 as discussed above. The final end result is that the clot exhibits a denser structure and resistance to fibrinolysis [18]. Clots formed at high fibrinogen concentrations show unique properties and includes: thin fibres, reduced pore size and increased tensile strength; also this clot is degraded at a lower rate by plasmin [27].

The first intervention trial by Pieters and co-workers, investigated the effect of glycaemic control on fibrin network structures of type 2 diabetic subjects using isolated fibrinogen [28]. A variety of parameters were measured to highlight their findings and included, fibrinogen glycation, clot permeability, turbidity measurements, fibre diameter, visco-elastic properties, lysis rate and effect of fibrinogen glycation on FXIIIa cross-linking. Results obtained were as follows:

- Fibrinogen glycation: higher level of fibrinogen glycation among the diabetic group with a significant decrease upon achieving glycaemic control.
- Clot permeability: this parameter reflects the clot structure, specifically the average pore size. This was increased in the diabetic subjects and a significant correlation between permeability and HbA1c was also proven.
- Turbidity measurements: analysis of this parameter results in turbidity curves which are used to characterize the kinetics of polymerization and clot structure. The slope of increase in turbidity, representative of lateral aggregation was higher in the diabetic group.
- Fibre diameter: this was measured using scanning electron microscopy (SEM), and the median fibre diameter of the clots from the diabetics and controls did not differ.
- Viscoelastic properties: were similar between the controls and diabetics however, there was a lower proportion of inelastic component in the fibrin clots of the diabetic subjects.
- Lysis rate: the diabetic subjects had a lower lysis rate.
- Effect of fibrinogen glycation on FXIIIA cross-linking: No differences were detected among the diabetic and control groups.

Subjects with diabetes have higher than normal tissue factor levels. Of significance is that tissue factor activity is controlled by both insulin and glucose. Another stimulating factor for the synthesis of tissue factor particularly in diabetes is glycation end products and reactive oxygen species (ROS). Also the elevated production of thrombin in diabetic subjects has a direct effect on the clot

formation, structure and stability. The final product is thus a denser, less permeable clot which is more resistant to lysis. Linking the concept of diabetes as a prothrombotic state and inflammation is the elevated cytokine, IL-6 which stimulates the hepatocytes to produce more fibrinogen. Increased production of fibrinogen by hepatocytes is also a common finding in insulin resistance [29].

Erythrocytes

It is no surprise that just like platelets and fibrin networks, erythrocytes have been shown to play a role in coagulation as well. This is in striking contrast to their traditional physiological role of oxygen transport. In fact, emerging evidence suggests that erythrocytes enhance functional coagulation properties and platelet aggregation [30]. Also, erythrocytes have been localized in coronary atherosclerotic plaques [31]. Among the spectrum of haematological abnormalities in diabetes, erythrocyte aggregation and decreased deformability of erythrocytes feature strongly [32]. More importantly, they are implicated in the pathogenesis of microvascular complications of diabetes. The adverse effects of glucose manifests in multiple ways: rearrangement of erythrocyte membranes, defects in haemoglobin oxygen binding activity, alterations of mechanical features of the membrane and general aspects of the cell as well [33]. This is attributed to the prothrombotic nature of the erythrocytes as they increase blood viscosity and forcing platelets towards the vessel wall. Thus the integration of erythrocytes into a fibrin clot has an influence on clot structure and its mechanical properties [34–36].

Diabetic-induced changes in the erythrocytes

Alterations in erythrocyte structure

In diabetic subjects the erythrocyte membrane becomes rigid and non-deformable. A decrease in the cholesterol to phospholipid ratio is responsible for this abnormality. Not only is the cholesterol component of the membrane increased but there is a four-fold increase in the phospholipids concentration, which results in a significant decreased ratio [37]. Of interest is that the proportion of membrane cholesterol is increased and has been predicted to contribute to instability of atherosclerotic plaque [38, 39]. Cytoskeletal proteins, in particular, beta spectrin, ankyrin and protein 4.1 are heavily glycosylated [40]. Disturbances in ionic balance is attributed to the lowered Na^+/K^+—ATPase activity and this leads to complications such as increased serum and intra-erythrocyte sodium and serum potassium in diabetic subjects. This also results in an increase in the cell size and increased osmotic fragility which contributes to the development of microvascular complications [41]. Elevated fibrinogen and glucagon are common findings in uncontrolled diabetes [42]. Oxidative stress plays a role in causing increased membrane lipid peroxidation and this may lead to abnormalities in composition and function. Enhanced levels of malondialdehyde (an indicator of lipid peroxidation) [43] and reduced levels of glutathione and membrane –SH group are features of the diabetic erythrocyte [44].

Despite absent mitochondria in the erythrocytes, they still depend on glucose as their energy source. However, in a hyperglycaemic environment, glycosylation of haemoglobin takes place which creates oxidative stress thereby making the cellular components of the erythrocyte more vulnerable [45]. One of the functions of a membrane is to provide protection and this includes against oxidative damage as well. However, in diabetic subjects, lipid peroxidation causes structural damage to the membrane with a subsequent decrease in the cell deformability and fluidity. SEM and AFM findings in the study by Buys et al., confirmed the correlation between the cytoskeletal protein and lipid layer damage with the ultrastructural roughness of the erythrocyte membrane found with AFM [46]. As mentioned previously, evidence points to glycosylation of cytoskeletal proteins and oxidative damage of spectrin [47].

Alterations in erythrocyte aggregation and deformability

The property of aggregation is guided by the composition of erythrocyte membrane and plasma proteins fibrinogen and globulin. When fibrinogen levels are increased and albumin is decreased, aggregation is enhanced. A mechanism that favours increased tendency of erythrocyte aggregation is the decreased ionic charge of the membrane. Microscopic examination of the erythrocyte aggregate reveals an increase in aggregate shape and size when compared to healthy controls. The parameter erythrocyte deformability unlike aggregation is modified by the composition of the membrane, cytoplasmic contents and age of the erythrocytes [42]. It is the measure of the ability of the cells to deform under applied shear stress. [47] Advanced technological measurements confirm that in diabetes, deformability is significantly decreased. This abnormality is attributed to the specific changes in the membrane structure. The consequence of altered deformability is the increase in blood viscosity which can lead to increase in shear stress on the endothelial wall [42]. Investigation of diabetic erythrocytes with cardiovascular complications is associated with lowered membrane fluidity when compared to healthy controls. The diffusion of protein and lipid molecules within the membrane is known as membrane fluidity and is dependent on the presence of saturated and polyunsaturated fatty acid [48]. The increased tendency of erythrocyte from subjects with diabetes to adhere to cultured human

vascular endothelial cells was confirmed by research performed by Wali et al. Their results suggest that a possible mechanism for the latter is a loss of lipid asymmetry and or less ordered packing in the outer leaflet of the diabetic erythrocyte membrane [49].

Diabetes, a multifactorial disease has a marked effect on the rheological and electrical properties of the erythrocyte. The erythrocyte is commonly described as more rigid than normal with a reduced deformability. However diabetes also causes profound changes on the ultrastructure of erythrocytes, fibrin networks and platelets. Pretorius and co-workers showed a changed morphology in type 2 diabetic erythrocytes using the scanning electron microscope (SEM), which showed elongated cells forming extended projections that twist around spontaneously formed fibrin fibres as indicated in Fig. 2f. The causative role of iron overload and subsequent non-enzymatic fibrinogen polymerization on altering the ultrastructure of erythrocytes in type 2 diabetes, has been detailed by Lipinski et al. [50, 51].

In the process of a thrombotic event many parameters (which are increased) play a role, however, increased levels of fibrinogen feature strongly. This is of significance as it causes abnormal fibrin fibre formation visible as dense matted deposits (DMD) and the resulting coagulum causes blood cells to change shape and to be trapped in the abnormal mesh [52]. An array of inflammatory diseases, including type 2 diabetes mellitus are associated with increased fibrinogen levels and hypercoagulability which results in markedly changed fibrin morphology, DMDs [53]. DMDs may therefore reflect a hypercoagulable profile. Of note is the close link between this hypercoagulability of fibrin and involvement of erythrocytes [54, 55]. Our research team has also shown that when fibrin clots abnormally, erythrocytes are entrapped more tightly inside the clot as indicated in Fig. 2f [51, 56]. The factors of both erythrocyte aggregation and fibrinogen interactions cause a changed viscosity which impacts on the optimal functioning of erythrocytes. It is thus difficult to ignore the influence of increased blood viscosity and increased fibrin concentration, not only in their role as strong predictors of cardiovascular diseases but also as important factors in the development of atherosclerosis [56–59].

Conclusion

Platelet dysfunction poses an increased risk for thrombotic vascular events. The significance of platelet abnormalities in the atherothrombotic process has been highlighted by the use of antiplatelet drugs that form part of the therapeutic regime in reducing cardiovascular risk [2]. The multifactorial aetiologies for platelet dysfunction is beyond the scope of this review, however, diabetes and the associated hyperglycaemia cannot be ignored. Hyperglycaemia induces a hypercoagulable condition and contributes to micro- and macrovascular disease. Studies have shown that optimal control of both fasting and post-prandial glucose levels will reduce the impact. It remains to see if the latter will improve morphological findings of the platelet ultrastructure in diabetic subjects. Suppression of fibrinolysis and increased fibrinogen are among the group of haemostatic abnormalities in type 2 diabetes. Increased fibrinogen adds to the burden of cardiovascular risk by increasing blood viscosity, increasing the size of the clot, tissue deposition is increased and stimulation of atherosclerosis and vascular thickening. Erythrocytes too contribute to the high incidence of atherosclerotic diseases in diabetes partly due to the association of abnormalities of erythrocyte composition and rheological function with increased oxidative stress.

The combination of blebbed platelets, abnormal fibrin in the form of dense matted deposits and changed erythrocytes morphology creates a thrombotic risk cluster which underpins the development of cardiovascular disease. Analysis of ultrastructural findings in diabetic platelets, fibrin network and erythrocytes can reveal significant findings which may add to a better understanding of the pathogenesis of atherosclerosis and thrombosis.

Authors' contributions
PS: wrote manuscript; EP: research group leader, editing of paper. All authors read and approved the final manuscript.

Compliance with ethical guidelines

Competing interests
The authors declare that they have no competing interests.

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Ethical clearance
Ethical clearance was obtained for blood sample collection from diabetes type II and healthy individuals, from the Human Ethics Committee of the University of Pretoria. Informed consent forms were filled in by all participants.

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10.5 Annexure E: Article 2

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ORIGINAL INVESTIGATION Open Access

Flow cytometric analysis of platelets type 2 diabetes mellitus reveals 'angry' platelets

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Abstract

Background: The function of platelets have extended way beyond the horizon of haemostasis and thrombosis, and are recognised as active participants in vascular inflammation, as well as in prothrombotic complications of cardiovascular diseases. We describe and compare platelet function in type II diabetes (with and without cardiovascular manifestation) and healthy individuals using scanning electron microscopy and flow cytometry.

Methods: Thirty subjects were recruited per group and informed consent was obtained from all participants. Diabetic patients were recruited from the diabetic clinic of the Steve Biko Academic Hospital (South Africa). Blood samples were drawn from all participants so that platelet specific antigens were analyzed in citrated whole blood. The platelet parameters used in the study were platelet identifiers (CD41 and CD42) and markers of platelet activation (CD62 and CD63).

Results: Results show that, compared to healthy individuals, both diabetic groups showed a significant difference in both platelet identifiers (CD41-PE, CD42b-PE) as well as markers indicating platelet activation (CD62P-PE and CD63-PE).

Interpretation: The flow cytometric data shows that the platelet surface receptors and platelet activation are statistically elevated. This is suggestive of enhanced platelet activation and it appears as if platelets are displaying 'angry' behaviour. The lysosomal granules may play a significant role in diabetes with cardiovascular complications. These results were confirmed by ultrastructural analysis.

Background

It is plausible to underestimate the impact of platelets in clinical medicine, when one considers that these blood cells are only 1.5–3 μm in size, survive for approximately 8–10 days, and are mere fragments of megakaryocyte cytoplasm [1–3]. The function of platelets have extended way beyond the horizon of haemostasis and thrombosis. In fact, they are now recognised as active participants in initiating and sustaining vascular inflammation as well as in prothrombotic complications of cardiovascular diseases [1]. Platelets have been assigned multiple attributes and have been described in inflammatory conditions such as atherosclerosis, arthritis and tumour metastasis [1]. Due to the multifunctional role of platelets, they are an accessible and important inflammatory marker for disease pathophysiology [4, 5]. Platelets are activated when they are in contact with damaged vascular endothelium [5], and once activated, they are able to secrete a wide spectrum of inflammatory mediators that exert both local and systemic effects [6].

Platelet activation is also the mechanism implicated in the pathogenesis of chronic medical conditions such as atherosclerosis, coronary vascular disease and cerebrovascular disease [3]. Due to inflammation there is an imbalance between procoagulant and anticoagulant properties of the endothelium with subsequent local stimulation of the coagulation cascade [7]. Another feature of inflammation is a multitude of interactions between leukocytes, endothelial cells and platelets. More importantly, regardless of its aetiology, inflammation causes endothelial activation [7]. In diabetes mellitus, endothelial dysfunction is one of the mechanism ascribed to increased atherothrombotic risk [8]. With

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cigarette smoking, the endothelium 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 [9]. Abnormal platelet activation, platelet count and volume have been implicated as risk factors of ischaemic stroke [10].

Once platelets are activated, they initiate reactions whereby changes in the level of expression of surface glycoproteins (GP) results, which act as receptors for platelet agonists and for adhesive proteins, involved in platelet aggregation. Platelet activity can be measured using various fluorescently labelled markers in flow cytometry. As flow cytometry allows the simultaneous detection of surface antigens in a sensitive and specific manner, it is therefore possible to examine aspects of the platelet membrane activity—see Table 1 for examples of available platelet markers.

Platelets in inflammatory conditions

There is a strong indication that platelets also have relevant functions in inflammation [11]. In fact, it was shown that thrombosis and inflammation share many key molecular mechanisms and that they are fundamentally linked processes [7]. It is now recognised that vascular inflammation is the key underlying mechanism in atherogenesis and atheroprogression. The evidence of platelets being fundamental mediators in the initiation and maintenance of a chronic proinflammatory milieu is provided by the direct interactions with inflammatory cells and secretion of autocrine and paracrine effector molecules [12]. Another emerging concept is the significant role of platelet-mediated recruitment of leucocytes in the propagation, progression and pathogenesis of atherosclerotic disease. Platelets can interact with leucocytes: (a) during haemostasis, when there is vascular damage and recruit leucocytes to the growing thrombus, (b) when endothelial cells are stimulated thereby adhering and activating platelets and then bridge blood-borne leucocytes to the vessel wall and (c) in the formation of heterotypic aggregates prior to contact with endothelial cells when adhesion between platelets and leucocytes occur in the blood [13].

It is well known that in subjects with type 2 diabetes mellitus, function of platelets is impaired. In fact, a sub-threshold stimuli is needed to activate platelets which are constantly in activation despite the lack of a major plaque event and have thus been defined as 'angry platelets' [14]. This is significant as it has been postulated that circulating platelets in subjects with untreated type 2 diabetes mellitus are in a hyperactive state and are implicated as etiologic factors in thrombotic complications [15] which are accelerated in diabetics [16]. Of note is the finding of hyperactive platelets in metabolically controlled diabetics without cardiovascular complications [17]. In addition, expression of P-selectin is increased on the surface of platelets in patients presenting with symptomatic coronary artery disease, making it a marker of 'angry platelets' [18].

Diabetes with cardiovascular complication may also lead to acute conditions like thrombo-embolic ischemic stroke. Multiple studies regarding the activity of platelets in acute stroke have been performed. Results obtained from these studies (acute ischaemic stroke) showed increased mean platelet volume, platelet aggregation enhancement in post-ischaemic stroke, increased α-and dense granule release and statistically significant increase in expression of P-selectin (CD62P), CD63, and thrombospondin [19]. The study by Marquardt and co-workers investigated the time course of platelet activation after ischaemic stroke. They found a significant increase in CD62P and CD63 expression within 24 h post cerebral ischaemia. In addition, it was also shown that CD62P expression declines during the first weeks after stroke, whereas CD63 expression remains increased for at least 3 months after stroke [20].

Another confounding factor together with diabetes is cigarette smoking. Multiple studies provides evidence on the many adverse effects of smoking on the cardiovascular system. This includes: (a) it causes endothelial

Table 1 Platelet parameters measured in this study

Cluster of differentiation	Glycoprotein (GP)	Function
CD41	GPIb	Anti-CD41a reacts with platelet membrane GPIb in the intact complex with GPIIa but not with GPIb or GPIIa separately. Useful in the identification and enumeration of platelets
CD42	GPIb	Anti-CD42b reacts with platelet membrane GPIb
CD62	P-Selectin	Anti-CD62 reacts with α-granule membrane protein which is expressed on the surface of activated platelet
CD63	GPIV	Anti-CD63 reacts with lysosomal granule-membrane glycoprotein that is expressed on surface of activated platelet

In this study CD41-FITC (fluorescein isothiocyanate) and CD41, Cd42b, CD62, CD63-PE (phycoerythrin) was used

dysfunction [21, 22]; (b) increases inflammation [23]; (c) it alters the lipid profile and creates an atherogenic setting; (d) promotes atherosclerotic progression by enhancing oxidative stress, lipid peroxidation and mitochondrial damage [23, 24]; (e) it destabilizes atherosclerotic plaque by increasing matrix metalloproteinases [25]; (f) increases platelet activation and activates coagulation cascade with subsequent atherothrombosis [26, 27]. Flow cytometric findings in the research by Al-Dahr, showed a decrease in CD41b with an increase in CD40 and CD62 [28]. This paper, therefore investigates the functional role of platelets in diabetes, with and without cardiovascular involvement using flow cytometry and scanning electron microscopy to look at platelet ultrastructure.

Methods

Participants

Thirty healthy individuals were used as controls. These individuals were non-smokers, who did not use any chronic medication and did not have a history of thrombotic disease. Sixty diabetic subjects (type 2) were recruited from the Steve Biko Academic Hospital, diabetic clinic in South Africa. Inclusion criteria included: (a) subjects older than 18 years and willing to provide informed consent, (b) subjects with known diagnosis of diabetes, (c) for the cardiovascular group, history of previous myocardial infarction, peripheral arterial disease, stroke or coronary arterial bypass grafting. Exclusion criteria included: (a) subjects hemodynamically unstable and (b) subjected with documented life threatening disease (malignancy, HIV/AIDS). Two groups of thirty each were distinguished, with and without cardiovascular complications. Five milliliters of blood was drawn into a citrate tube, from each participant. Ethical clearance was obtained for this study from the University of Pretoria Human Ethics Committee. Informed consent was obtained from all participants.

Ultrastructural analysis

Scanning electron microscopy was used to prepare platelets from platelet rich plasma (PRP) according to previously described methods [29]. Platelets from individuals with diabetes, cerebrovascular disease and smoking were compared to platelets from healthy individuals.

Flow cytometry

For each blood sample taken four tubes was prepared; each tube containing 1 ml sheath fluid from Beckmann and Coulter and 20 µl of blood. The various tubes were stained with 20 µl of CD41-FITC (fluorescein isothiocyanate) and 20 µl of one of the following probes: CD41-PE (phycoerythrin), CD42b-PE, CD62P-PE and CD63-PE (from Beckman Coulter). The samples stained with different probes, were incubated at room temperature in the dark for 20 min before being analyzed by a flow cytometer (FC 500, Beckman Coulter). The surface expression of platelet receptors was determined by flow cytometry using the different monoclonal antibodies as indicated in Table 1.

Forward scatter and 90° 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).

Statistical analysis

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 groups. GraphPad Prism 5 was employed to perform one-way ANOVA for all statistical analysis, with a p value of ≤ 0.005 considered significant. Post-hoc Dunnett's Multiple Comparison Test was performed to compare the two diabetic groups to the controls.

Results

Table 2 shows the demographic data of our study population. SEM analysis of the platelets from the three groups showed that there is a progressive change in platelet structure between the groups. Representative micrographs of the ultrastructure of platelets from healthy individuals, and individuals with diabetes (with and without cardiovascular manifestations), are shown in Fig. 1. Healthy platelets prepared for SEM, typically show slight contact activation, where minimal pseudopodia formation is visible (Fig. 1a). However, during inflammation, platelets form numerous pseudopodia, with microparticle formation, as well as spreading and extensive clumping, which is the hallmark of over-, or hyperactivation. This hyperactivation is seen in platelets from individuals with diabetes with and without CVD (Fig. 1b, c). However, diabetic patients with CVD are characterised by an increased presence of hyperactivation and microparticle formation Fig. 1c. Following the ultrastructural analysis we performed flow cytometry on the control and two diabetic groups. We found that the ultrastructural results were fully supported by the flow cytometry results discussed below.

Table 2 Baseline demographic data and clinical characteristics of the study population

Variable	Controls (n = 30)	Diabetics without CVD (n = 30)	Diabetics with CVD (n = 30)
Age, years	25 ± 9.64	53 ± 13.7	61 ± 9.4
Males, n (%)	6 (20)	11 (37)	20 (67)
Females, n (%)	24 (80)	19 (63)	10 (33)
Hypertension, n (%)		17 (57)	26 (87)
Diabetic treatment			
Insulin, n (%)		5 (17)	3 (10)
Oral agents only, n (%)		16 (53)	12 (40)
Oral and insulin, n (%)		9 (30)	15 (50)
HBA1c %*		9.0 ± 2.6	8.5 ± 1.7
Cardiovascular complications			
Previous MI, n (%)			19 (63)
PAD, n (%)			2 (7)
CABG, n (%)			9 (30)
Essential medication			
ACEI, n (%)		10 (33)	20 (67)
Ca-antagonist, n (%)		8 (27)	3 (10)
B-blocker, n (%)		3 (10)	11 (37)
Nitrates, n (%)		1 (3)	14 (47)
Statins, n (%)		10 (33)	25 (83)
Disprin, n (%)		6 (20)	20 (67)
Warfarin, n (%)		2 (7)	5 (17)

Data expressed as mean ± (SD) or n (%)

MI myocardial infarction, *PAD* peripheral arterial disease, *CABG* coronary arterial bypass grafting, *ACEI* angiotensin converting enzyme inhibitor, *Ca-antagonist* calcium antagonist, *HBA1c* haemoglobin A1c

* Not all 60 subjects had this test completed (results are available for 50 % of the subjects)

CD41-PE and CD42b-PE MCFI were significantly elevated in both diabetic groups when compared to healthy individuals (p value <0.001). CD62P-PE and CD63-PE MCFI were significantly decreased for both diabetic groups. The percentage activated platelets indicated with CD62P-PE and CD63-PE were significantly increased in both the diabetic groups. It should be noted that the platelet activation indicated CD63-PE showed the diabetic group with cardiovascular complication to have the largest percentage of activated platelets.

Discussion

Diabetes

Increased expression of platelet activation markers CD31, CD36, CD49b, CD62P and CD63 was confirmed by Eibl and co-workers when type 2 diabetics were compared with normal individuals [30]. In fact, increased expression of CD63 and CD62, enhanced platelet activation, and aggregation are viewed as one of the major causes of atherosclerosis and thrombosis in diabetes [31]. CD62P is found in the α-granules of platelets and are used as markers for activated platelets, while their absence suggests a resting state [32]. A feature that appears strongly in diabetics is that of platelet hyperaggregation. This is prevalent in both type 1 and type 2 diabetics [33]. From a pathophysiological view, this is significant as hyperaggregated platelets have a tendency to block blood vessels [5], contributing to atherothrombotic complications in diabetics. CD63 is a 53 kDa lysosomal membrane protein identified on surface of activated platelets after release reaction [34, 35].

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 [36]. The CD42b MoAb used in this research, specifically binds to the platelet GPIbα. 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. According to De Meyer and co-workers in 2011, 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 [37]. Furthermore, 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 attacks and stroke [38].

Flow cytometric analysis found a significant increase in platelet activation regarding CD62 (P-selectin) for the diabetics group while the CD62 MCFI values decreased compared to the controls as shown in Table 3. 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 [39]. However, studies have shown an increase in both P-selectin on the surface of platelets and sP-selectin indicating that diabetes with or without cardiovascular complications are associated with chronic activation of platelets as P-selectin is being shed from activated platelets and as new P-selectin is being expressed on recently activated platelet [40]. This study finding echoes results found by Véricel et al. [17] whom also discovered hyperactive platelets in metabolically controlled diabetics without cardiovascular complications. The diabetic subjects with cardiovascular disease, recruited in this study were those

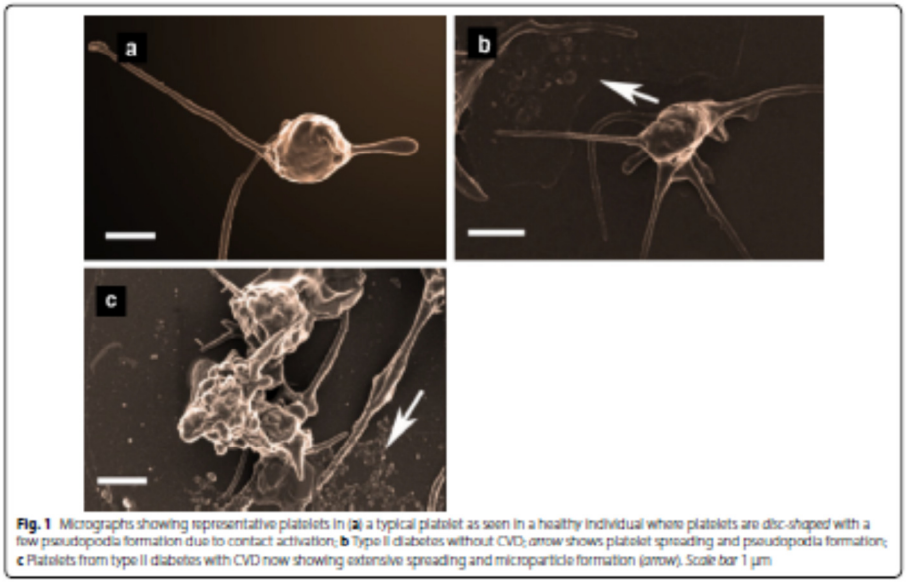

Fig. 1 Micrographs showing representative platelets in (**a**) a typical platelet as seen in a healthy individual where platelets are disc-shaped with a few pseudopodia formation due to contact activation; **b** Type II diabetes without CVD; arrow shows platelet spreading and pseudopodia formation; **c** Platelets from type II diabetes with CVD now showing extensive spreading and microparticle formation (arrow). *Scale bar* 1 μm

with ischaemic events many months and years prior to recruitment into this study. Our finding is in keeping with persistently hyperactivated platelets.

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 [41]. In our study CD63 percentage activated platelets were significantly increased compared to the healthy controls. The diabetic group with cardiovascular complication showed the greatest percentage of platelet activation (61.24 % activation) while the diabetic group without cardiovascular complications showed a slightly lower activation percentage (54.39 % activation), as shown in Fig. 2. It appears as if CVD may play a role in platelet hyperactivation with lysosomal involvement.

Conclusion

This study adds to the body of evidence that diabetic patients have 'angry platelets'. Platelet membrane markers and percentage activated platelets were increased in

Table 3 Analysis of control and diabetic groups (with or without CVD), results presented as mean ± standard deviation (SD) of MCFI and percentage activated platelets

MoAb	Controls (n = 30)	Diabetes without CVD[a] (n = 30)	Diabetes with CVD (n = 30)
CD41-PE	21.08 ± 8.74	40.18 ± 14.56*	47.81 ± 24.49*
CD42b-PE	14.43 ± 1.84	24.78 ± 11.98*	24.09 ± 7.27*
CD62P-PE X-mean	30.86 ± 11.37	17.96 ± 1.95*	17.79 ± 2.266*
CD62P-PE % activated platelets	71.01 ± 16.20	92.65 ± 3.67*	92.11 ± 4.31*
CD63-PE X-mean	16.40 ± 3.32	13.05 ± 3.68**	13.38 ± 3.12**
CD63-PE % activated platelets	37.84 ± 17.59	54.39 ± 32.92**	61.24 ± 29.73**

n = 10,000 platelets total analysed for each participant of each group

Statistically significant differences: * $p < 0.001$, ** $p < 0.005$

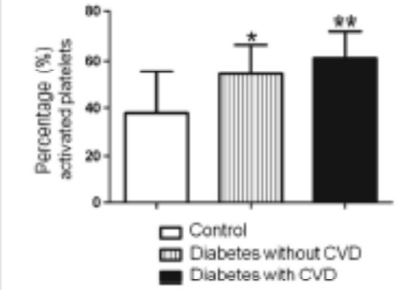

Fig. 2 Percentage activated platelets identified with CD63-PE. The *asterisk* represent the q-value as calculated with the Newman–Keuls multiple comparison test: *3.291; **6.653. The q-value represents the studentized range value

diabetics with and without CVD. These results support and confirm the numerous papers suggesting that platelets play an important and possibly key role in the inflammatory profile of diabetic and cardiovascular patients and their health should play a key role in a patient-orientated precision medicine approach.

Authors' contributions
JNP, ACS and TM prepared the blood sample for microscopy and flow cytometry analysis. PS and EP contributed equally to microscopy analysis and writing of the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Ethics, consent and permissions
Ethical approval was granted at the University of Pretoria (Human Ethics Committee: Faculty of Health Sciences): E. Pretorius and P. Soma. All participants filled in informed consent forms.

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