Cape Peninsula
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PLATELET FUNCTION AND ACTIVATION IN MIXED ANCESTRY SUBJECTS WITH HYPERGLYCEMIA, FROM THE WESTERN CAPE, SOUTH AFRICA

by

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DECLARATION

I, Zibusiso Mkandla, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed **Date**

ABSTRACT

Background: Type 2 diabetes mellitus (T2DM) is a metabolic disorder which is characterised by insulin resistance, defective insulin secretion or both. The chronic state of hyperglycemia in diabetes is associated with microvascular complications and macrovascular complications which account for an estimated 80% of deaths as a result of cardiovascular complications. Type 2 diabetes mellitus is an inflammatory disease and pro-inflammatory stimuli in the form of activated endothelial cells, render the vascular endothelial surface attractive for both platelets and leukocytes. Activated platelets bind to the exposed extracellular matrix (ECM) and also to each other in both physiological haemostasis and pathological thrombosis. They can also adhere to leukocytes. Platelet leukocyte interactions are divided into 3 stages; these include the initiation of the interaction, stabilization of the aggregates and amplification of leukocyte activation. This study attempts to contribute to the current knowledge of T2DM by investigating the percentage of monocytes and neutrophils forming aggregates with platelets in pre-diabetes and diabetes and comparing this to non-diabetes individuals as well as the up regulation of pro-thrombotic and activation antigens on the surface of monocytes and neutrophils (Tissue Factor and CD69). Levels of platelet activation and function will be determined by both plate monocyte aggregates (PMA) and platelet neutrophil aggregates (PNA).

Methods: A total of 124 individuals were recruited from Bellville South, Cape Town, South Africa. This comprised of diabetes (DM) (n=15), pre-diabetes (pre-T2DM) (n=25) and controls (n=84). All individuals were screened for diabetes using the oral glucose tolerance test (OGTT). Platelet leukocyte measurements were performed using the Navios 8-colour flow cytometer.

Results: The median percentage of circulating platelets bound to monocytes (%PMAs) was significantly increased in the T2DM 49.04[36.78-62] and the Pre-T2DM 48.96[36.72-61.2],groups, compared to the control group 7.2[5.4-9], $p<0.0001$. The median %PNAs, which show interactions between neutrophils and platelets, were significantly increased in the T2DM group 13.56[10.17-16.95] compared to the control group 6.01[4.51-7.51] $p<0.0001$.

Conclusion: Platelet monocyte aggregates (PMAs) were higher in both the pre-T2DM and T2DM groups when compared to the control group indicating increased interactions between platelets and monocytes. In addition to forming aggregates with leukocytes, the platelets were able to initiate activation and phenotypic change to the leukocytes by increasing the expression of CD69 and TF (CD142). This finding provides further evidence that there is a link between the inflammatory process and the prothrombotic activity evident in diabetes and

pre-diabetes individuals. Furthermore, we describe elevated levels of circulating activated neutrophils which directly correlate with increased PNA formation in both the pre-T2DM and T2DM group.

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DEDICATION

To my young brother Nyasha M. Mkandla,
we were not born for mediocrity.

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LIST OF ABBREVIATIONS

%PMA	Percentage platelet monocyte aggregates
%PNA	Percentage platelet neutrophil aggregates
ADA	American diabetes association
ADP	Adenosine diphosphate
AGEs	Advanced glycation end products
ATP	Adenosine triphosphate
BMI	Body mass index
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
CCE	capacitative calcium entry
cGMP	Cyclic guanosine monophosphate
CRP	C-reactive protein
CVD	Cardiovascular diseases
DTS	Dense tubular system
EC	Endothelial cells
ECM	Extracellular matrix
FFA	Free fatty acids
glycLDL	glycated low-density lipoproteins
HbA1C	Glycated haemoglobin
IFG	Impaired fasting glucose
IFN	Interferon
IGT	Impaired glucose tolerance
IL-1	Interleukin-1
IL-6	Interleukin-6
IR	Insulin Resistance
LDLs	low density lipoproteins
MCP-1	Monocyte chemoattractant protein-1

MEIA	Microparticle enzyme immunoassay
MI	Myocardial infarcts
MU	methylumbelliferone
MUP	4-methyl umbelliferone phosphate
NCCE	non capacitative calcium entry
NO	Nitric oxide
OCS	Open canicular system
PAL-1	Plasminogen activator inhibitor-1
PF4	platelet factor 4
PFA	Platelet activating factor
PGI_2	Prostaglandin
PKC	Protein kinase C
PLAs	Platelet leukocyte aggregates
PMA	Platelet monocyte aggregates
PNA	Platelet neutrophil aggregates
Pre-T2DM	Pre-Diabetes
RAGE	Receptor for advanced glycation end products
RANTES	Regulated upon activation normal T-cell expressed and secreted
RR	Relative risk
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TF	Tissue Factor
TLR	Toll like receptors
TNFα	Tumour Necrosis Factor alpha
TXA_2	Thromboxane
WCC	white cell count
WHO	World Health Organisation
β-TG	β-thromboglobulin

Chapter 1

1 Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disorder which is characterised by insulin resistance, defective insulin secretion or both. The chronic state of hyperglycemia in diabetes is associated with microvascular complications affecting the eyes, kidneys, and nerves (Goldenberg & Punthakee, 2013). Macrovascular complications are represented by atherosclerotic disease and about 80% of deaths are as a result of cardiovascular complications (Matsuda et al., 1996; Paneni et al., 2013). Accelerated atherosclerosis remains the main underlying factor contributing to the high risk of atherothrombotic events in these individuals (Vazzana et al., 2012). Individuals withT2DM have a 2-4 fold increased risk of coronary artery disease (CAD) and in the majority of individuals T2DM may remain undetected until an advanced stage when vascular complications have occurred (Vazzana et al., 2012; Goldenberg & Punthakee, 2013). Pre-diabetes is defined as an impaired fasting glucose (IFG) and /or impaired glucose tolerance (IGT) (Ford et al., 2010).

Increased coagulation system activation may be a reason for the increased thrombotic risk. Glucose and insulin seem to play a role in the pathogenesis of the prothrombotic state and it has been demonstrated, that *in vitro,* thrombin generation is increased in platelet rich plasma from individuals with diabetes (Vazzana et al., 2012). Furthermore, platelets from these individuals exhibit deregulated signaling pathways with an increased tendency to aggregate and become activated in response to stimuli (Kakouros et al., 2011). In support of this, experiments in healthy individuals have demonstrated that the induction of hyperglycemia leads to increased platelet reactivity which can be demonstrated *in vivo* by increased expression of markers such as P-selectin and CD40-ligand (CD40L) (Kakouros et al., 2011). Animal studies using rats in which diabetes has been induced using streptozotocin, have also shown enhanced platelet aggregation in response to endogenous agonists and increased thromboxane (TXA_2) synthesis. However, these changes occur well before vessel wall changes (Vazzana et al., 2012).

Insulin, which controls blood glucose, binds to the insulin receptor (IR) which is also found on platelets (Kakouros et al., 2011). Under normal physiological conditions, insulin inhibits platelet aggregation and thrombosis due to tissue factor (TF) inhibition and enhanced fibrinolytic action which is as a result of the modulation of plasminogen activator inhibitor-1 (PAL-1) (Paneni et al., 2013). However, in individuals with T2DM this interaction is disrupted which could explain the increased platelet activity. Studies have further demonstrated that in

T2DM, platelet sensitivity increases when exposed to the action of various platelet agonists such as adenosine diphosphate (ADP), thrombin and collagen (Suslova et al., 2015).

Acute hyperglycemia has been shown to increase *in vitro* collagen induced platelet aggregation via enhanced mitochondrial superoxide production (Sudic et al., 2006). In support of this, Sudic et al demonstrated that ADP induced platelet P-selectin expression was increased in high glucose concentrations. In contrast however, ADP-induced fibrinogen binding was not elevated. From this, the authors suggest that fibrinogen induced platelet aggregation, is not influenced by hyperglycemia (Sudic et al., 2006). Therefore blood glucose control may not be enough to curb platelet aggregation and other factors may therefore be involved in the pro-inflammatory and pro-coagulant state observed in these individuals.

Type 2 diabetes is an inflammatory disease and pro-inflammatory stimuli render the vascular endothelial surface attractive for both platelets and leukocytes which leads to a well-defined and regulated multistep signalling cascade process involving both blood cells and the endothelium (Cerletti et al., 2010). Leukocytes bind to activated platelets and become enmeshed in platelet thrombi or form a monolayer on top of the adherent aggregated platelets (Ghasemzadeh & Hosseini, 2013). Platelets communicate to leucocytes via biochemical signals, adhesive receptors and secreted soluble mediators (Totani & Evangelista, 2012) and in turn the leukocytes release factors such as proteases and nitric oxide which are able to modulate platelet responses (Totani & Evangelista, 2012).

The above introduction implies that there is a large body of evidence to support the theory that there is a relationship between platelet activation, the inflammatory process, hyperglycemia and the resulting thrombotic disease observed in individuals with diabetes. However, the mechanisms and detail of this association are still unclear and require further investigation.

1.2 Rationale and Importance of Study

Type 2 Diabetes Mellitus (T2DM) is a global disease with a prevalence particularly striking in the developed world. The global health burden of T2DM was estimated to be 177 million in 2000 and projections estimate this figure to increase to 366 million by the year 2030 (Shaw et al., 2010). This projected increase is as a result of population growth, increased life expectancy, increase prevalence of obesity and decreased physical activity as well as urbanization.

Clinicians often encounter this condition at an advanced state when vascular complications have already occurred (Paneni et al., 2013). The abnormal metabolic state that accompanies T2DM renders arteries susceptible to atherosclerosis, and is thought to be responsible for altering the functional properties of multiple cell types, including endothelial cells, leukocytes and platelets (Ferroni et al., 2004; Cerletti et al., 2010).

Because of the significant increase in the prevalence of diabetes and the resulting complications there is an urgent need for research to be undertaken which would propose solutions to avert an increase in the prevalence of T2DM and its related complications.

This study attempts to contribute to the current knowledge of T2DM and hyperglycemia by investigating the relationship between platelet function and the immune system in the initiation and progression of cardiovascular disease within individuals with pre-diabetes and diabetes.

1.3 Aim

Therefore the objectives of this project are:

1) To investigate the baseline levels of platelet activation in pre-diabetes and diabetes and compare this to normal controls.

2) To investigate the percentage of monocytes and neutrophils forming aggregates with platelets (PMA's and PNA's) in pre-diabetes and diabetes and comparing this to normal individuals.

3) To investigate the up regulation of pro-thrombotic and activation antigens on the surface of monocytes and neutrophils (Tissue Factor and CD69) in individuals with pre-diabetes and diabetes.

4) To correlate PMA and PNA formation and function with other biochemical and haematological markers (glycated haemoglobin, insulin, fasting glucose, CRP, triglycerides).

Chapter 2

2 Literature Review

2.1 Introduction

Diabetes represents a group of heterogeneous conditions in which hyperglycemia is a common feature (Maraschin, 2012). Type 2 diabetes mellitus (T2DM) is a metabolic condition associated with hyperglycemia due to insulin resistance or the lack of insulin secretion due to beta cell dysfunction (Kahn, 2003). It is a major contributor to cardiovascular disease because it is involved in the acceleration of atherosclerosis which is one of the most important factors contributing to the high risk of atherothrombotic events (Ferreiro et al., 2010).

The prevalence of diabetes has increased dramatically and it is estimated that by the year 2030 about 350 million people will be affected (Hess & Grant, 2011). The burden of this disease in Cape Town was demonstrated by a study conducted in the Bellville South community. In that study, it was shown that a high prevalence (28.2%) of the mixed ancestry population in this area suffered from T2DM (Erasmus et al., 2012).

Deficient insulin function is the basis of the abnormalities seen in carbohydrate, fat and protein metabolism and results from inadequate secretion and /or an abnormal tissue response to this hormone (American Diabetes Association, 2014). The consequence of the hyperglycemia is an increased risk of cardiovascular disease and other clinical manifestations which are well described (Ford et al, 2010).

The World Health Organisation (WHO) has classified diabetes by incorporating fasting and 2 hour glucose load as shown in table 2.1. The classification of diabetes has resulted in better treatment and four categories have been identified; (1) type 1 diabetes mellitus (T1DM), (2) T2DM, (3) gestational diabetes and (4) other types (Maraschin et al., 2010). Pre diabetes is defined as IFG, IGT or glycosylated haemoglobin (A1C) of 5.7%-6.5%. This condition indicates that the individual is at a high risk of developing diabetes and its complications (Goldenberg & Punthakee, 2013). Research has demonstrated that 7% of individuals with pre-diabetes progress to diabetes within a year if no changes are implemented in order to correct the condition (Kahn, 2003). Table 2.1 gives the diagnostic criteria used for diabetes according to the world health organisation department of non-communicable disease surveillance.

Table 2.1: Diagnostic criteria of diabetes mellitus

Diabetes Mellitus	• Fasting >7.0mmol/l or 2-hr post glucose load > 11.1mmol/l • A1c ≥6.5%
Impaired glucose tolerance	Fasting < 7.0mmol/l and 2-hr post glucose load >7.8mmol/l and <11.1mmol/l
Impaired fasting glucose	Fasting >6mmol/l and 2-hr post glucose load <7.8mmol/l

*Glucose load = 75g oral glucose (Gabir et al., 2000; World health organization department of non-communicable disease Surveillance, 2006)

Type 2 diabetes mellitus (T2DM) is a condition that involves both microvascular disease (retinopathy and nephropathy) and macrovascular complications such as cardiovascular diseases (CVD) (Paneni et al., 2013). There is a 2-4 fold increased risk of coronary artery disease (CAD) in T2DM individuals which is as a result of the macrovascular complications which are initiated as accelerated atherosclerosis, and may result in premature CAD and acute coronary events (Vazzana et al., 2012). Evidence for this has been demonstrated in studies which report that T2DM individuals without a history of myocardial infarcts (MI) have the same incidence of acute coronary events as individuals with a history of MI (Ferroni et al., 2004). Therefore, type 2 diabetes is a strong predictor for short and long term ischaemic events and individuals with this disease have an increased risk of recurrent atherosclerotic events when compared to non-diabetes (Ferreiro et al., 2010). Although, the risk and development of atherosclerosis is well documented the mechanisms involved are not well understood. It is suspected that hyperglycemia influences the production of atherosclerotic plaques but little is known of the mechanisms influencing this(Muačević-Katanec & Reiner, 2011).

It has been suggested that hyperglycemia can lead to increased platelet activity by the alteration of the platelet structure, shape and changes to the make-up of the lipid membrane (Santilli et al., 2011). Platelets bind to insulin via the insulin receptor thereby reducing the platelet's sensitivity to pro-thrombotic stimuli or agonists such as adenosine diphosphate (ADP), collagen, thrombin and epinephrine. In obese T2DM individuals however, the anti-aggregating effect of insulin is reduced (Santilli et al., 2012). These studies support the

theory that increased glucose and decreased insulin activity increase the risk of atherosclerosis and cardiovascular disease.

Obesity is a chronic disease caused by many factors associated with an increase in body fat (Fernández-Sánchez et al., 2011). It has been associated with a pro-thrombotic state which also enhances the susceptibility of obese individuals to acute cardiovascular disease as it elevates the risk of plaque formation. This is primarily due to hyperlipidaemia and inflammation (Bordeaux et al. 2010;Badimon et al. 2013). Further evidence suggests that the pro-thrombotic state characteristic of obesity and the metabolic syndrome are as a result of a combination of elevated thrombin generation, platelet hyperactivity and decreased fibrinolysis (Santilli et al., 2012). Platelet activation has been identified as the primary mechanism of the increased cardiovascular risk observed in obese individuals. This has been demonstrated by the increased adhesiveness and activation of platelets both *in vitro* and *in vivo,* together with a reduced sensitivity to physiological agonists (Graziani et al., 2011; Santilli et al., 2012).

Under normal physiological conditions, endothelial derived nitric oxide (NO) and prostaglandin (PGI_2) counteract platelet activation through the increase of intra platelet cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) which are responsible for maintaining vascular smooth muscles in blood vessels (Badimon et al., 2013). Both obesity and diabetes result in increased levels of insulin resistance in endothelial cells and platelets. This subsequently results in atherothrombosis due to an increase in free fatty acids (FFA) leading to the activation of toll like receptors (TLR) (Paneni et al., 2013). Toll like receptors (TLR) recognise different pathogen components and elicit an immune response resulting in inflammation. In a cohort study of individuals with diabetes, it was observed that several inflammatory platelet mRNA including transcripts for TLR2 and TLR4 were associated with a higher BMI which is a known risk factor for type 2 diabetes (Dasu et al., 2010; Santilli et al., 2012). In addition, specific toll like receptors (TLR2 and TLR4) were found to recognise patterns expressed on innate immune cells such as monocytes and have consequently been shown to be involved in the pathogenesis of diabetes, insulin resistance and artherosclerosis(Dasu et al., 2010).

Oxidative stress and chronic inflammation have been associated with T2DM. These processes could result in endothelial dysfunction and decreased production of nitric oxide. Nitric oxide plays a role in vasodilation and therefore lower levels would promote vasoconstriction and consequently platelet activation (Schneider, 2009). Platelet activation and inflammation have a reciprocal relationship, in which inflammation promotes platelet activation which in turn also promotes inflammation (Randriamboavonjy & Fleming, 2009; Endoza, 2010). Evidence now indicates that platelets from individuals with diabetes are

hyperactive and express increased adhesion molecules on the cell surface such as glycoprotein Ib-IX (GPIb-IX) and GPIV which bind von Willebrand factor and collagen on the extracellular matrix respectively (Sudic et al., 2006; Li et al., 2010). Insulin resistance also leads to increased formation of coagulation cascade factors VII and XII as well as fibrinogen as shown in figure 2.1 (Grant 2005).

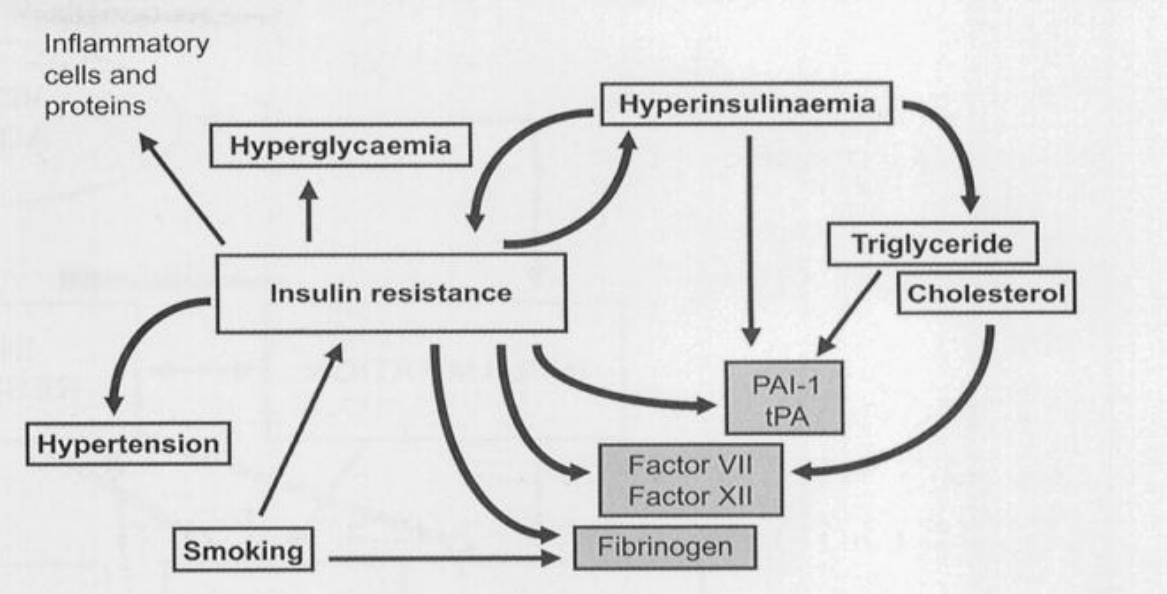

Figure 2.1: Insulin resistance as an inflammatory atherothrombotic condition. The figure shows the causes of insulin resistance such as smoking, the subsequent hyperinsulinaemia and how this leads to hyperglycemia and hypertension. Insulin resistance also leads to increased formation of coagulation cascade factors VII and XII as well as fibrinogen (Grant 2005).

Platelets, when activated, release a number of vasoactive mediators which damage blood vessels and obstruct blood flow. By doing this, they play a major role in the development of ischemic heart disease and contribute to acute coronary syndromes (Rauch et al., 1999; Brouns & De Deyn, 2009). The combination of hyperglycemia and elevated platelet activation enhances thrombotic processes through non-enzymatic protein glycation and glucose being metabolised to compounds that alter the oxidation-reduction potential and intracellular signalling pathway (Sudic et al., 2006). In addition, activated platelets cause obstruction of the microcapillaries, release both constrictive and oxidative molecules that increase the rate of formation of vascular lesions and contribute to the cardiovascular problems in T2DM individuals (Kakouros et al., 2011).

In summary, the above introduction indicates that there is plenty of evidence suggesting that platelets and platelet activation play a significant role in the cardiovascular disease characteristic of T2DM; however the mechanisms and their relationship with the immune system still require clarity.

2.2 Inflammation

Inflammation is the body's attempt to return to a pre-injury state and may be divided into; acute and chronic inflammation (Serhan et al., 2010). This process involves increased blood flow, activation of clotting factors and accumulation of phagocytic cells. Once activated, haemostasis which involves both platelet activation and coagulation, traps pathogens thereby localising the infection and attracting phagocytic cells (granulocytes and monocytes) (Elgert, 2009; Abbas et al., 2012). The inflammatory response consists of four main role players: the inducers, sensors, mediators and target tissues (figure 2.2). Once initiated it can result in redness, swelling, heat and pain on and around the affected area (Scott et al., 2004; Medzhitov, 2010; V. Stankov, 2012).

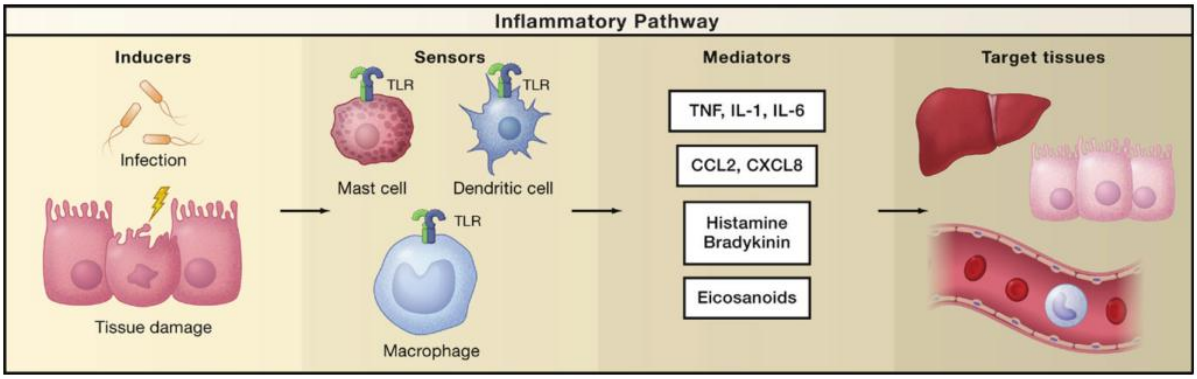

Figure 2.2 shows the four components of inflammation. The inflammatory inducers maybe be bacteria of tissue injury/damage, they activate inflammatory sensors/cells with TLRs to detect bacterial infection. The sensors release inflammatory mediators such as tissue necrosis factor and interleukins-1 and 6 which act on target tissues to optimise conditions and adapt to the inflammatory condition (Medzhitov, 2010)

Acute inflammation is defined as the body's initial response to injury which occurs within a few hours and declines unless the inducer or pathogen cannot be cleared by phagocytosis (Serhan et al., 2010). Endothelial cells and platelet activation play an important role in the acute phase (Serhan et al., 2010). Acute inflammation may be caused by tissue injury or infection which leads to the production of inflammatory mediators; chemokines, cytokines, and growth factors. These are necessary for tissue repair and eradication of the infectious organism (Eming et al., 2007; Monteiro & Azevedo, 2010). Immediately after injury, platelets and polymorphonuclear leukocytes release factors which initiate the coagulation cascade and attract inflammatory cells (Eming et al., 2007). As shown in figure 2.3, at sites of acute inflammation there is a higher proportion of neutrophils and activated T-cells (Male et al., 2013). Macrophages, at the site of inflammation, play an important role in maintaining homeostasis and recruit more cells (monocytes) to the site of inflammation (Medzhitov, 2010). Pro-inflammatory cytokines activate endothelial cells which become adhesive allowing leukocyte diapedesis and transmigration to the site of injury (Eming et al., 2007).

The transmigration is controlled by increased blood flow, vascular permeability and the leakage of fluid together with leukocytes inflammatory mediators at the site of inflammation/injury (Feghali & Wright, 1997; Nathan & Ding, 2010). Macrophages and neutrophils which have migrated to the site of infection clear the infection by phagocytosis and can therefore resolve the inflammation. However, the inflammatory cells themselves must be cleared form the site of infection and this is achieved by systemic recirculation or death followed by monocyte derived macrophage phagocytosis (Serhan et al., 2007).

When a prolonged inflammatory trigger is not eliminated by the acute response, a chronic inflammatory response is initiated which is localised at the area of infection or injury (Medzhitov, 2010). Both acute and chronic inflammation may co-exist for long periods with the cells involved in the acute phase being present in the chronic phase resulting in on-going re-initiation of the inflammatory process (Nathan & Ding, 2010). Unlike acute inflammation, sites of chronic inflammation have increased numbers of macrophages, cytotoxic T-cells and B-cells (Male et al., 2013) as shown in figure 2.3. However in both acute and chronic inflammation the arrival of immune cell populations is dependent on endothelial cells which secrete cytokines which are responsible for activating lymphoid cells and other specific leukocyte populations which consequently migrate to the site of inflammation (Male et al., 2013).

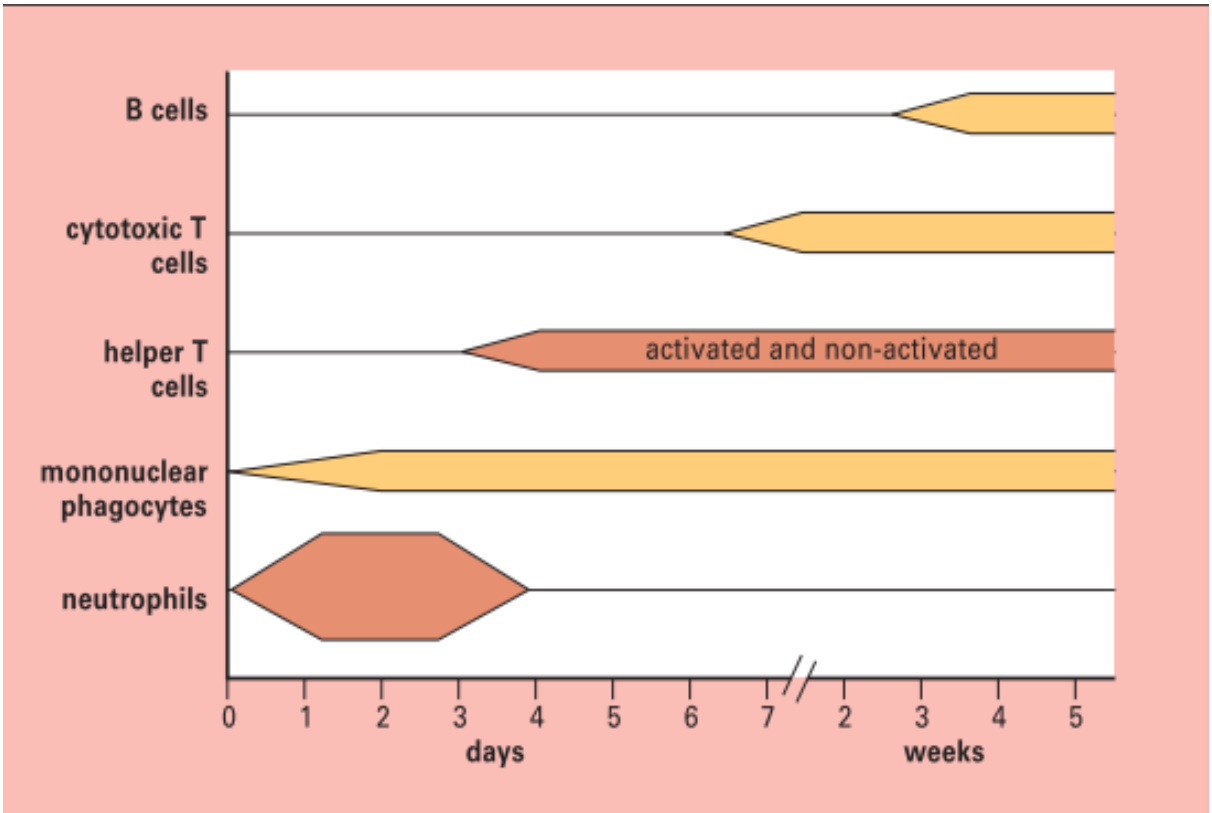

Figure 2.3: This figure shows the phases in the arrival of leukocytes to the sites of inflammation. Neutrophils and mononuclear phagocytes (monocytes) are the first to arrive at the site of inflammation; neutrophils are cleared within 4 days but more monocytes are recruited to the site of inflammation by chemokines released from the mononuclear phagocytes. This continues in cases of chronic inflammation. If inflammation persists, cells involved in the chronic inflammatory state such as T-helper cells, cytotoxic T cells and B-cells will also become involved(Male et al., 2013).

2.2.1 Type 2 diabetes as an inflammatory condition

Type 2 diabetes mellitus (T2DM) is thought to be a manifestation of an on-going chronic low grade inflammation which has been defined as a 2-3 fold increase in plasma concentrations of cytokines and acute phase proteins (Xie & Du, 2011). Chronic inflammation has been associated with chronic diseases such as atherosclerosis, hypertension, polycystic ovarian syndrome, non-alcoholic steatosis, obesity and metabolic syndrome (Petersen & Pedersen, 2005; Calle & Fernandez, 2012; Goldfine et al., 2012). In T2DM the chronic inflammatory environment has been proposed due to the reported increase in plasma concentrations of C-reactive protein (CRP), interleukin-6 (IL-6), interleukin-1 (IL-1) and Tumour Necrosis Factor alpha (TNFα) (Folsom et al., 1999; Shurtz-Swirski et al., 2001; Simsek et al., 2010). In support of this Dasu *et al.,* demonstrated an association between elevated levels of

circulating CRP and pro inflammatory cytokines such as (interleukin (IL)-1β and interferon (IFN)-γ) (Dasu et al., 2010).

It has been suggested that increased white cell counts precede and may predict the risk of T2DM and coronary heart disease. This leucocytosis is due to an increase in neutrophils or granulocytes rather than lymphocytes or monocytes (Goldfine et al., 2012). In contrast to this however, a further report indicated that neutrophils are rarely seen in individuals with atherosclerosis. The authors indicated that the reason for this was their short life span and the inadequate detection methods in human and murine atherosclerotic lesions (Drechsler et al., 2011). These reports not only attempt to explain the role of the immune cells in creating an inflammatory environment but also highlight some of controversies in this area.

During atherogenesis, the pro-inflammatory process alters the function of endothelial and monocyte/macrophage as well as lipid concentrations. The inflammation results in smooth muscle cell migration which promotes plaque formation and instability (Hess & Grant, 2011). In addition hyperglycemia in T2DM induces the production of advanced glycation end products (AGEs) (Muačević-Katanec & Reiner, 2011). Exogenous sources of these AGEs are found in many diets and these can increase the plasma CRP and TNF-α in the mononuclear cells of individuals with T2DM (Calle & Fernandez, 2012). Advanced glycation end product modified proteins bind to AGE receptors on macrophages, vascular endothelial cells and smooth muscle cells and initiate pro-oxidant and pro-inflammatory responses which could lead to endothelial dysfunction (Calle & Fernandez, 2012).

Platelets are the first responders to vascular injury and endothelial dysfunction and play a crucial role in thrombosis and inflammation (FerrerAcosta, 2014). Previous research has indicated that individuals with type 2 diabetes mellitus (T2DM) with macrovascular disease have an increased number of circulating activated platelets. In contrast, some studies have shown that platelet activation is not related to the presence of T2DM but rather to the presence of vascular disease (Davì et al., 1997; Coppola et al., 1997; FerrerAcosta, 2014).

Hyperglycemia, which is a major risk factor for atherosclerosis and vascular disease may cause vessel damage through three pathways; AGE formation, activation of protein kinase C (PKC) as shown in figure 2.4 and sorbitol accumulation through the polyol pathway (Ferroni et al., 2004). Although unrelated, these pathways can be linked by the elevated production of superoxide. Activation of the receptor for advanced glycation end products (RAGE) and increased oxidative stress increase hyperglycemia. This in turn leads to activation of the transcription factor-ƙB (NF-ƙB) in the endothelial and smooth muscle cells which are responsible for regulating the expression of genes that encode mediators of atherogenesis and inflammation (Yerneni et al., 1999; Ferroni et al., 2004).

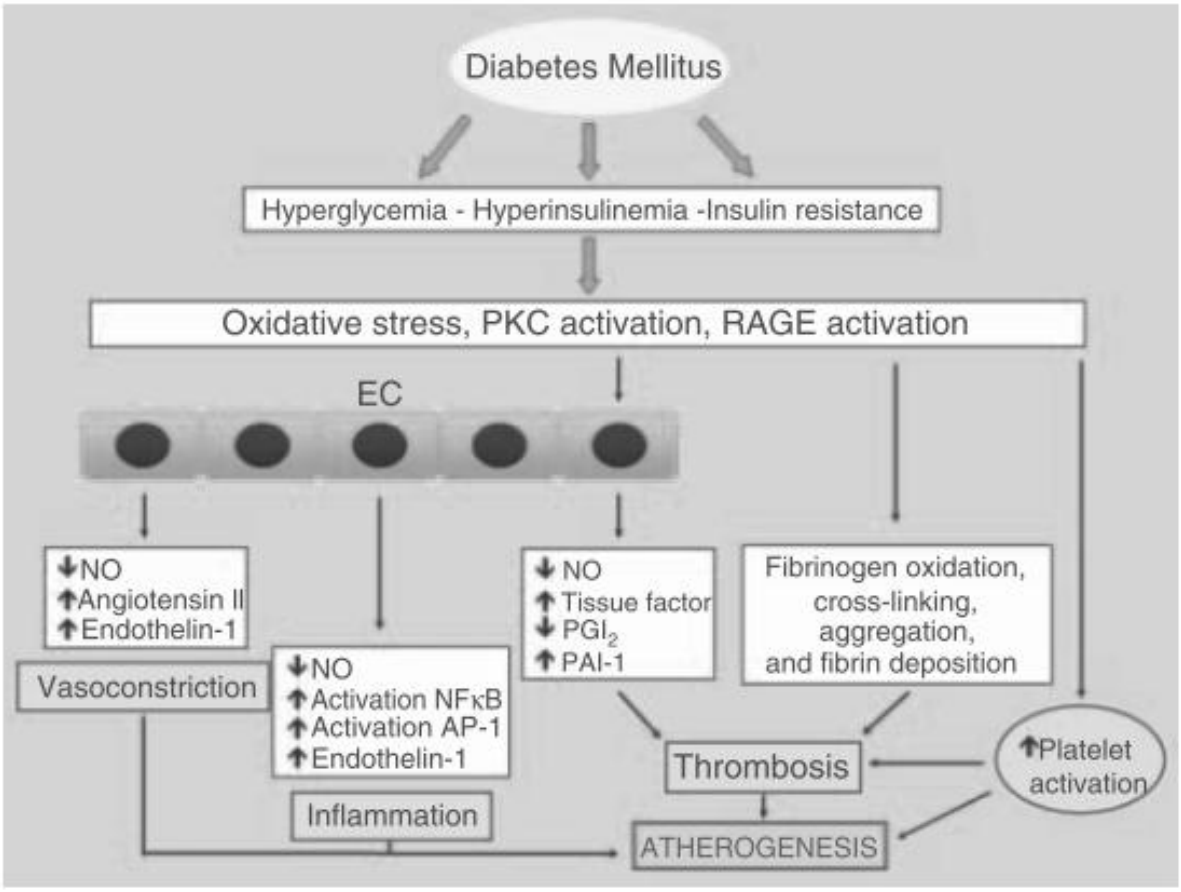

Figure 2.4: The figure shows diabetes as a major cause of downstream activation of various cells and pathways including the activation of endothelial cells (EC) and platelets due to metabolic dysfunction. Metabolic dysfunction is able to cause the activation of EC and platelets through increased oxidative stress, PKC activation and receptor for advanced glycation end products (RAGE) activation. This leads to a pro-thrombotic and pro-inflammatory environment which contributes to the pathogenesis of atherosclerosis (Ferroni et al., 2004).

2.3 Platelet activation and aggregation in T2DM

2.3.1 Platelet structure

Platelets are non-nucleated cells which are formed from the cytoplasm of megakaryocytes (Bambace & Holmes, 2011). The platelet membrane consists of phospholipids, glycoproteins and integrins which are utilised in the process of platelet adhesion, aggregation and activation (Bambace & Holmes, 2011). They have a diameter of 2-4μm and their concentration in the peripheral blood ranges from 150-400 x 10^9/L (Harrison, 2005). Three types of storage granules have been identified within the platelet. These are the α-granules

which contain coagulation factors fibrinogen, fibronectin, von Willebrand Factor, thrombospondin and vitronectin, the dense granules which contain platelet agonists adenosine triphosphate (ATP), calcium, serotonin and ADP and the lysosomes which contain glycosidases and proteases. These granular contents are released upon platelet activation (Gupta et al., 2007; Symth et al., 2009; Bambace & Holmes, 2011). The membrane of the platelet contains receptors which include glycoprotein Ib-IX-V (GPIb-IX-V) CD42b, glycoprotein VI (GPVI) and glycoprotein IIb-IIIa (GPIIb-IIIa/integrin αIIIbβ3) and CD36. These receptors are essential for complete adhesion and aggregation (Bambace & Holmes, 2011). The structure of the platelet is depicted in Figure 2.5.

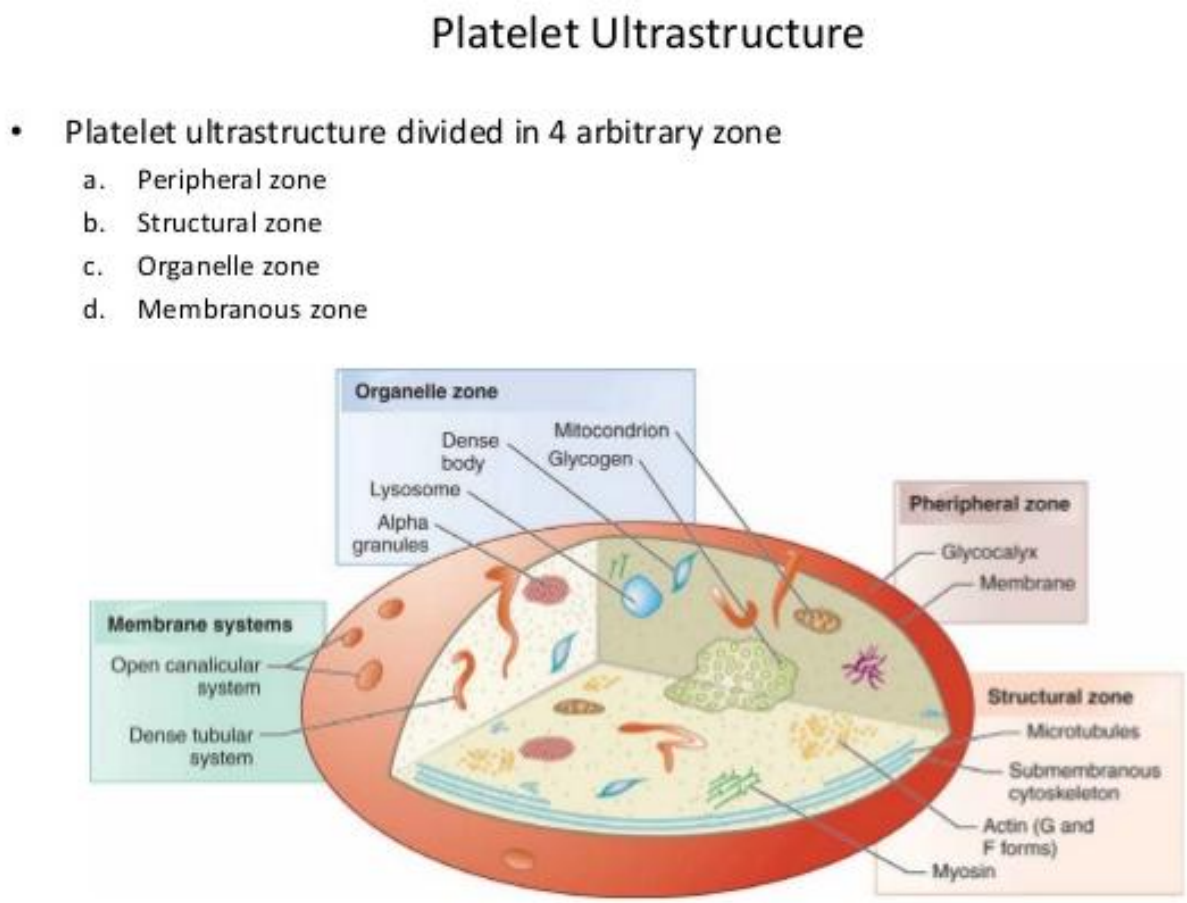

Figure 2.5: Platelet structure. Platelets have 4 different zones. A-Peripheral zone: which includes the outer membranes, closely associated structures and the walls of the open canicular system (OCS). The OCS provides access to the interior of the platelet to the plasma substances and also acts as an outlet channel for platelet products. B-Structural zone: consists of the framework of the platelet and cytoskeleton. The cytoskeleton maintains the discoid shape of the platelet as well as the contractile system that allows shape change, pseudopod extension, internal contraction and release of granular constituents upon activation. C-Organelle zone: contains the granules and cellular components of the lysosomes. The organelles help in the metabolic processes of the platelet and store enzymes and a large variety of other substances important in platelet function. D- Membrane system: Includes the dense tubular system (DTS), this is where calcium is concentrated which is important for triggering contractile events. The enzymatic systems for prostaglandin systems is also found in this zone (Anjali A. Sharathkumar, 2008).

2.3.3 Platelet adhesion and activation

The extracellular matrix (ECM) contains several adhesive macromolecules such as collagen, von Willebrand factor (vWF), laminin, fibronectin and thrombospondin which serve as ligands for different platelet receptors (Broos et al., 2011). Platelet adhesion receptors play a key role in initiating platelet activation when the platelets become exposed to the ECM adhesive proteins (Li et al., 2010). Platelet adhesion and activation occurs in a coordinated process

that involves tethering, rolling, activation and firm adhesion (Broos et al., 2011; Stegner & Nieswandt, 2011). Platelet adhesion to the ECM is an important step in the adhesion and eventual extravasation of leukocytes to sites of infection, this forms an integral part of the inflammatory response (Cerletti et al., 2010). After exposure to the damaged sub endothelial extracellular matrix (ECM), circulating platelets are rapidly decelerated which enables them to maintain prolonged contact with platelet receptors on the ECM (Stegner & Nieswandt, 2011). An inflamed vessel wall releases von Willebrand factor and collagen which are essential for the initial binding of platelets to the endothelial wall. Collagen receptor GPVI and von Willebrand receptor GPIb are both found on platelets and once bound they initiate integrin activation (van Gils et al., 2009). This results in platelet activation as shown in figure 2.6.

The consequent activation of the platelet is mediated by signalling processes which are divided into 3 stages (figure 2.6). Firstly agonists interact with their respective platelet receptors causing the activation of signalling events and integrin activation which constitutes inside out signalling. This is consequently followed by outside in signalling (El Haouari & Rosado, 2008; Li et al., 2010)

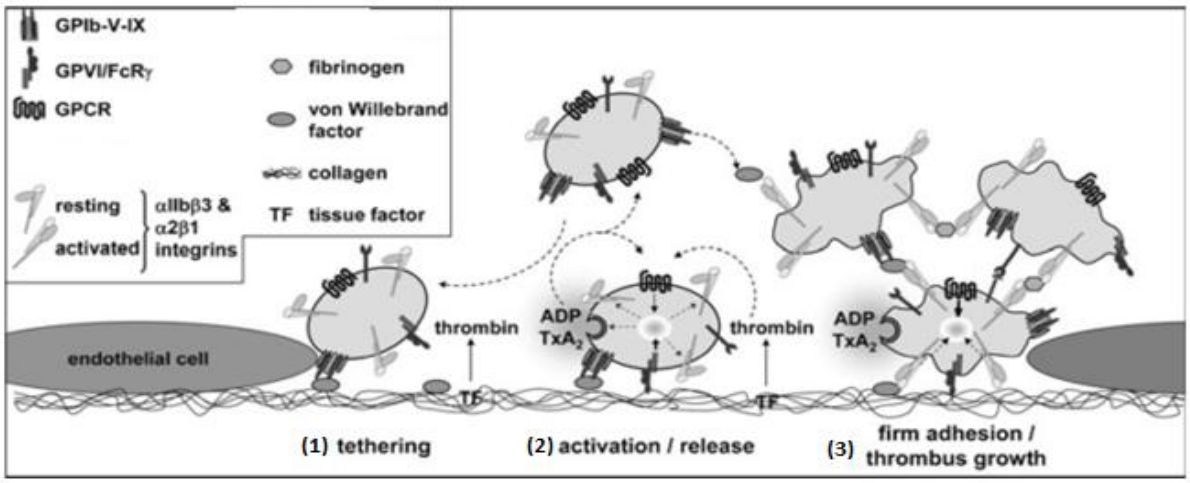

Figure 2.6: Platelet –ECM interactions. The ECM becomes exposed to platelets which rapidly decelerated through the initial contact (tethering) (1) which is mediated by the GPIb-vWF interactions. GPVI collagen interactions are involved in the second step (2), which initiates cellular activation and results in the release of a second wave of agonists (ADP and TXA2). Tissue factor (TF) triggers the formation of thrombin which also mediates cellular activation. The final step (3) is firm adhesion of platelets to collagen through activated α2β1 (directly) and αIIbβ3 (indirectly using vWF), this leads to continued GPVI signalling, increased release of soluble agonists and procoagulant activity. Adapted from (Stegner & Nieswandt, 2011).

Platelets are activated through various signal transduction pathways one of which involves the activation of the tyrosine kinase cascade (Broos et al., 2011). During vascular injury soluble vWF is rapidly bound to exposed collagen on the ECM via the collagen binding site A3 domain for immobilization. After binding, vWF binds platelets in the circulation via their A1 domain (Broos et al., 2011). Binding of immobilized vWF to platelet receptor GPIb-IX results in platelet activation as well as aggregation (Li et al., 2010; Broos et al., 2011). GPIb-IX also binds to thrombin and sensitizes platelets to low-dose thrombin (Li et al., 2010). After activation platelets de-granulate releasing contents from their α- and dense granules which bind to platelet receptors activating integrins α2β1 and αIIbβ3 (Clemetson, 2012). Activation of these integrins causes stable binding of the platelets and the consequent activation of pathways involving tyrosine kinases and signal transduction through G-coupled receptors which leads to increased cytostolic calcium (Ca^{2+}) levels, cytoskeletal rearrangements and integrin activation (Broos et al., 2011). G-coupled receptors include the ADP receptors P2Y1 and P2Y12, thrombin receptors, proteinase-activated receptor 1 (PAR1) and PAR4 and thromboxane receptor for TXA2 as shown in figure 2.7 (Clemetson, 2012).

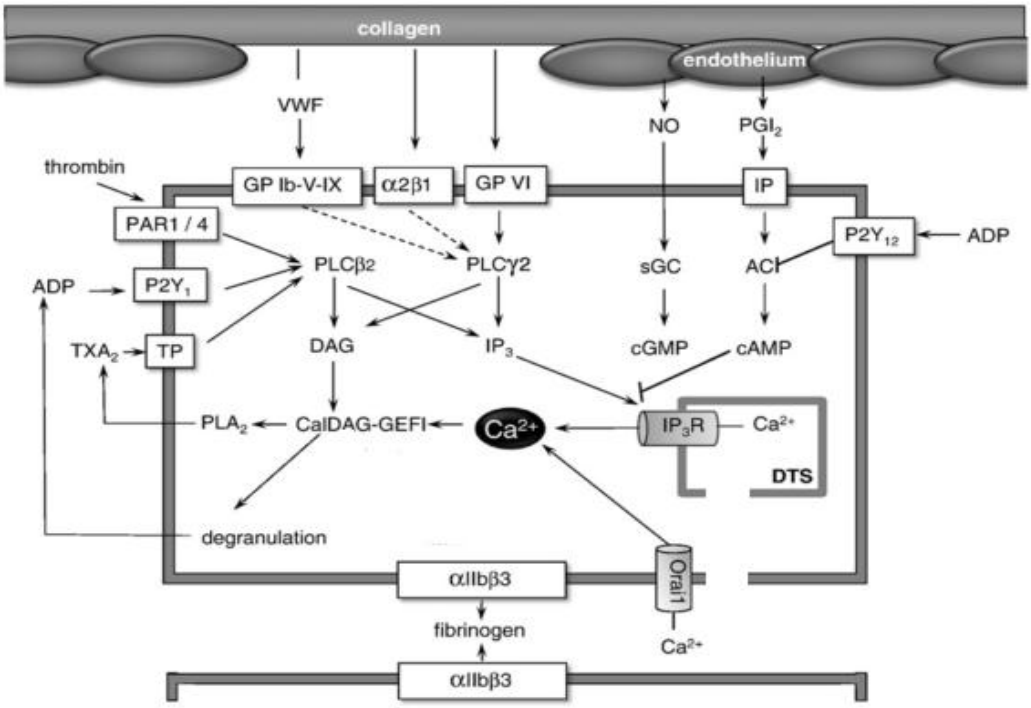

Figure 2.7: This figure shows the main platelet receptors and the pathways involved in signalling. Under physiological conditions the endothelial surface releases Nitric oxide (NO) and prostacyclin (PGI_2) which inhibit platelet binding. Platelets are bound to the exposed collagen on the ECM, during vascular injury or inflammatory conditions, through its receptors GPIb-V-X via vWF, α2β1 and GPVI. This leads to activation of phospholipase Cγ2 (PLCγ2) which hydrolyses phosphatidylinositol 4,5 biphosphate to produces inositol 1,4,5 triphosphate (IP3) and diaglycerol (DAG) IP3 binds to its IP3 receptor IP_3R on the dense tubular system causing it to release Ca^{2+} into the cytoplasm. Extracellular calcium enters the cell through the calcium channel Orai1. DAG and Ca^{2+} function as an internal receptor for protein kinase C which consequently moves from the cytosol to the membrane before being activated. Elevated concentrations of Ca^{2+} and DAG cause the activation of phospholipase A2 (PLA2), change in platelet shape, degranulation and ultimately aggregation. (Broos et al., 2011).

Outside in signalling initiates activation of platelets and the release of granules which results in the recruitment and activation of other platelets. Adhesion and aggregation of platelets leads to aggregation and the presentation of a pro-coagulant surface promoting formation of a fibrin haemostatic plug (Broos et al., 2011). Apart from aggregating with each other (homogenous aggregation), platelets once activated also bind to leukocytes (heterogeneous aggregation) (Wang et al., 2014).

2.3.4 Platelets and the immune system

The immune system consists of two mechanisms in its response to harmful stimuli these are the innate and adaptive immunity. The innate immune response is mediated by macrophages and neutrophils which distinguish between self and pathogen by utilising

signals from toll like receptors (TLRs) (FerrerAcosta, 2014). Platelets also form part of this response and have been shown to use TLRs to identify bacterial proteins and regulate platelet immunity and function (Beaulieu & Freedman, 2010). The expression of TLRs (TLR2, TLR4 and TLR9) on the platelet surface allows them to directly eliminate blood pathogens both by secreting microbiocidal molecules and other immune cells by phagocytosis (FerrerAcosta, 2014).

Platelets also interact with various cell types through receptors constitutively expressed on their surface or after activation, as well as by autocrine and paracrine pathways (Santilli et al., 2012). Platelet activation has been demonstrated in a variety of inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, arterial thrombosis, asthma and transplant rejection (Klinger & Jelkmann, 2002). Once activated platelets release several cytokines which are able to enhance immune function; these include, platelet factor 4 (PF4), platelet activating factor (PAF), monocyte chemoattactant factor (MPC-1), interleukin (IL) 1-β and regulated on activation normal T-cell expressed and secreted (RANTES) (Cognasse et al., 2007; Antoniades et al., 2009; Li et al., 2011). Other agonists such as thrombin and adenosine disphosphate (ADP) also contribute to the inflammatory and immune response as shown in the figure 2.8 below(Rondina et al., 2013).

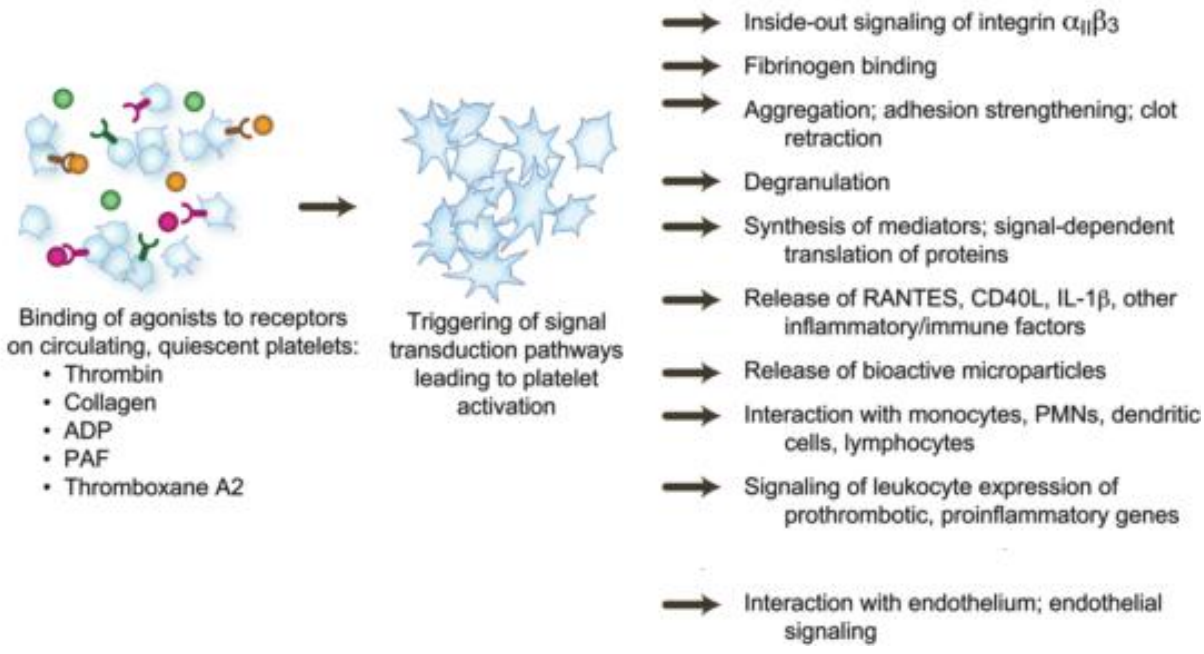

Figure 2.8: This figure shows how indigenous agonists such as thrombin, collagen, ADP, platelet activating factor (PAF) and thromboxane A2 initiate signalling pathways during platelet activation. Upon activation platelets undergo a variety of processes which include degranulation aggregation. the signalling of leukocytes which in turn express pro-thrombotic and pro-inflammatory genes, inside out signalling, interaction with the endothelium and the release of bioactive microparticles. Adapted from (Rondina et al., 2013)

2.3.5 Platelet leukocyte aggregates

Platelets do not interact with the vessel wall under normal physiological conditions as they are in an inactive state. They however become activated at sites of vascular injury or pathological alteration of the endothelial layer after they are exposed to agonists and adhesive proteins from endothelial cells (Li et al., 2010; Stegner & Nieswandt, 2011). Activated platelets bind to the exposed ECM and also to each other in both physiological haemostasis and pathological thrombosis (Cerletti et al., 2012). They can also adhere to leukocytes (Cerletti et al., 2012). Platelet leukocyte interactions are divided into 3 stages. These include the initiation of the interaction, stabilization of the aggregates and amplification of leukocyte activation (Wang et al., 2014). Platelets bind to P-selectin (CD62P) or glycoprotein ligand-1 (PSGL-1) expressed on leukocytes via the P-selectin antigen which is expressed on activated platelets (Freedman & Loscalzo, 2002). The activation of platelets induces platelet leukocyte aggregation and binding of TF, Factor Xa and fibrinogen on the surfaces of the neutrophils and monocytes. Therefore these aggregates may be vehicles for local and systematic delivery of coagulation factors (Rondina et al., 2013).

2.3.5.1 Platelet-monocyte aggregates (PMAs)

Platelet monocyte aggregates have been identified in clinical conditions such as peripheral vascular disease, hypertension, acute or stable coronary syndromes, stroke or diabetes (Neumann et al., 1997; Furman et al., 2001; Nomura et al., 2003; McCabe et al., 2005; Elalamy et al., 2008; van Gils et al., 2009). In acute coronary syndromes, the binding of platelets and monocytes suggests a relationship between thrombosis and inflammation in cardiovascular disease (Freedman & Loscalzo, 2002).

Furthermore monocytes are known to have the highest affinity for platelet CD62P (Schrottmaier et al., 2015) and the interaction of CD62P with PSGL-1 leads to the increased expression of CD11b/CD18 (Mac-1) on leukocytes. This further supports the theory that monocytes actively interact with platelets (Freedman & Loscalzo, 2002). The extent of PMA formation is dependent on the extent of platelet activation and experiments which block CD62P on platelets resulted in the reduction of PMA formation (van Gils et al., 2009). In humans, studies have shown that platelet monocyte interactions were able to induce the elevation of $CD14^{high}CD16^{+}$ monocytes which have been identified as the major leukocyte population which enters atherosclerotic lesions and forms foam cells. Therefore the up-regulation of CD16 on monocytes by platelets may drive inflammation (Wang et al., 2014; Schrottmaier et al., 2015). Furthermore, platelets induce the pro-coagulant monocyte phenotype in which TF, elevated levels of coagulation factor FXa and fibrinogen are

expressed by platelet bound monocytes (Freedman & Loscalzo, 2002; Gorbet & Sefton, 2003; Schrottmaier et al., 2015).

2.3.5.2 Platelet neutrophil aggregates (PNAs)

Platelets are deposited and activated at the sites of damaged vessel walls and release chemokines which recruit neutrophils to the site of inflammation. Through P-selectin/PSGL-1 interaction, the neutrophil and platelet will bind to each other forming PNAs (Ghasemzadeh & Hosseini, 2013). The activated platelets will initiate leukocyte degranulation and the release of pro-inflammatory factors such as IL-1β, IL-8, and matrix metalloprotease 9 (MMP-9) (Schrottmaier et al., 2015). MMP-9 plays a role in degrading fibrin collagen in atherosclerotic plaques which leads to instability of the plaque (Franks et al., 2010). Integrin $\alpha_M\beta_2$ (Mac-1) activation which is initiated by P-selectin/PSGL-1 leads to a firm adhesion of the neutrophil and platelet (Ghasemzadeh & Hosseini, 2013)which in-turn triggers kinase cascades and chemokine synthetic pathways in the neutrophils (Rondina et al., 2013).

2.3.6 Vascular injury and platelet activation in diabetes

In healthy conditions platelets do not interact with the intact vessel wall because of substances such as NO and PGI_2 which are released by the vessel wall and inhibit platelet adhesion (Broos et al., 2011; Kaplan & Jackson, 2011). Endothelial dysfunction is associated with functional alterations in the vascular endothelium such as abnormal regulation of vasodilation and vasoconstriction, impaired and excessive angiogenesis, reduced barrier function and increased inflammatory activation (Hall et al., 2011). In normal physiological conditions, NO prevents the adhesion of leukocytes to the endothelium and maintains the endothelium in a resting state (Tabit et al., 2010). In response to thrombin and bradykinin, NO and PGI_2 synthesis is elevated in the area near to aggregating platelets thereby limiting the growth of the platelet plug (Suslova et al., 2015).

However, in individuals with diabetes, there is a reduced synthesis of these two molecules which consequently promotes aggregation and vasoconstriction. Studies have also shown that in diabetes, platelets have reduced sensitivity to NO and PGI_2 (Hall et al., 2011). In addition, angiotensin II and TXA, which promote vasoconstriction and are pro-thrombotic are increased in individuals with diabetes. Hyperglycemia and insulin resistance further contribute to the pro-thrombotic state by inhibiting NO production and increasing reactive oxygen species (ROS) production. This consequently leads to an increased expression of pro-inflammatory cytokines and platelet adhesion molecules (Hess & Grant, 2011).

2.3.7 Oxidative stress and platelet activation in diabetes

The underlying mechanisms are not clearly understood but it is recognized that oxidative stress due to hyperglycemia may play a significant role in the etiology of diabetic complications (Ferroni et al., 2004). Pitocco *et al* demonstrated that both glucotoxicity and lipotoxicity are diabetes related entities that initiate oxidative stress and can be identified as the a cause of beta cell dysfunction (Pitocco et al., 2010). Glucose and lipids are harmful to beta cells and studies have shown that lipotoxicity is only present together with elevated glucose levels. Hyperglycemia may be a prerequisite for the negative effects of lipotoxicity hence glucolipotoxicity describes the harmful relationship between lipids and beta cell function (Pitocco et al., 2010).

Hyperglycemia may induce ROS production directly through glucose metabolism and auto oxidation or indirectly through the formation of advanced glycation end products (AGEs) and their receptor binding proteins (Ferroni et al., 2004). The major consequence of hyperglycemia is excess oxidative stress which has been identified as a possible mechanism for the increased vascular disease in a diabetic state (Pitocco et al., 2010). These proteins (AGEs) are involved in the development of atherosclerosis by mechanisms involving the activation of the receptor for AGEs (RAGE) and by enhancing platelet aggregation through the serotonin receptor (Ferreiro et al., 2010). Non-enzymatically glycated low-density lipoproteins (glycLDL) render platelets susceptible to oxidative stress (Hess & Grant, 2011). This may explain the platelets' increased sensitivity to aggregation agents (Hess & Grant, 2011). As hyperglycemia has been demonstrated to cause oxidative stress it is very likely that it may lead to the activation of platelets (Freedman & Loscalzo, 2002). This results in a reduction in the production of NO and PGI_2 which ultimately leads to endothelial dysfunction and increased platelet activation. In addition, it has been shown that platelets from individuals with diabetes have a reduced sensitivity to NO and PGI2 which would further increase their activation, (Ferreiro et al., 2010).

2.4 Conclusion

Type 2 Diabetes Mellitus is a non-communicable disease associated with hyperglycemia and a high incidence of cardiovascular disease. The incidence of the disease is expected to dramatically increase by 2030 particularly in developing countries(Shaw et al., 2010).

T2DM is considered to be a low grade inflammatory condition (Xie & Du, 2011), and it is hypothesised that inflammation may play a part in the development of CVDs. However, the role of activated platelets in diabetes and their link to inflammatory cells such as monocytes and neutrophils in the progression of CVDs via the tissue factor (TF) pathway has not been extensively studied. It is therefore the objective of this study to examine the increased levels

of platelet activation in diabetes, their connection with the immune response and attempt to explain how this could lead to the pro-thrombotic state occurring in individuals with diabetes.

Chapter 3

3 Materials and Methods

3.1 Participants and sampling

The study was conducted on a group of individuals of mixed ancestry residing in the Bellville South community of Cape Town, South Africa. Written informed consent was obtained from each participant after details of the project were explained in the language of their choice. The study was approved by the Cape Peninsula University of Technology ethics committee (REC-230408-014) and conducted according to the guidelines set forth in the Helinski Declaration. It is part of a larger study with ethical approval (CPUT/HW-REC 2008/002 and CPUT/HW-REC 2010). Participants were only identified by means of codes, which were used throughout the study. The researcher was blinded in that the glucose tolerance status of participants was not revealed at the time of acquisition and data analysis. Trained phlebotomists and nurses were involved in the collection of blood. In addition, they were trained for the specific needs of the study such as measuring blood pressures and measurement of hip to waist circumferences. Other clinical parameters that were measured included weight, height, body fat measurements as well as the calculation of the body mass index (BMI). The participants were separated into three groups based on the glycaemic status.

3.1.1 Sample collection and analysis

Blood samples were collected from all participants after an overnight fast, and two hours after a 75 g oral glucose tolerance test (OGTT) following the WHO recommendations (World health organization department of non-communicable disease Surveillance, 2006). Fasting status was verified in all participants. Blood was also collected into serum separator tubes (SST), heparin and EDTA for both biochemical and haematology testing. For the platelet studies venous blood was collected into 4.5ml sodium citrate (blue top) tubes containing 3.2% sodium citrate instead of the EDTA containing purple top tube. The samples were analysed within 1 hour of collection.

3.1.2 Inclusion criteria

Participants, who had fasted overnight, refrained from consuming alcohol the day before sampling and had not taken any aspirin or any non-steroidal anti-inflammatories (1 week/7 days prior) were included. Participants who had not taken any medication for recent infection were included in this analysis.

3.1.3 Exclusion criteria

Participants were excluded if they were not fasting and had reported a recent infection. Those who had taken aspirin medication were also excluded as aspirin inhibits the cyclooxygenase-2 enzyme involved in inflammation and platelet activation resulting in platelets becoming dysfunctional for their entire 10 day lifespan (Mason et al., 2007; Korbecki et al., 2014). Participants who had used steroids prior to blood being drawn were excluded.

3.2 Flow cytometry analysis

3.2.1 Instrument set-up

Data acquisition was performed using the Navios 8-colour Flow Cytometer (Beckman Coulter, Miami, Florida). To ensure reporting of standardized results, flow check pro fluorescent labelled beads (Beckman Coulter, Miami, Florida) were used to standardise the optical path and laminar flow of the instrument.

3.2.2 Detector settings

To detect small particles, the forward (FS) and side scatter (SS) parameters were set at a log scale. An unstained sample was used to set the voltages for the FS/SS photomultiplier tubes in order to allow for the clear separation of red blood cells and platelets based on size and granularity. Stained samples for each panel were used to perform colour compensation and to prevent spectral overlap.

Antibody titrations were performed to determine the optimal antibody concentrations to be used when making up the antibody cocktails for each of the panels in the analysis (see appendix 3).

3.2.3 Fluorescence minus one (FMO)

Fluorescence minus one controls were used to distinguish between negative and positive events and also correct for any auto-fluorescence and non- specific binding. An FMO control contains all the fluorochromes except that which is being measured. Quadrants were then set around the negative population so as to exclude any non-specific binding from the analysis. Figure 17 in appendix 2 shows an example.

3.4. Platelet leukocyte aggregate (PLA) measurements

Antibodies used for PLA measurements were anti-human CD42b-FITC (clone SZ2), CD69-PC5 (clone TP1.55.3), CD16-PC7 (clone 3G8), CD14-APC (clone RMO52) from Beckman Coulter, Miami, Florida and CD142-PE (clone NY2) from Biocom Biotech, South Africa. Fifty

microliters (50µl) of citrated blood was incubated for 10 minutes in the dark at room temperature with 5µl of an antibody cocktail containing the antibodies CD42b-FITC, CD142-PE, CD69-PC5, CD16-PC7 and CD14-APC. The sample was then lysed with 500µl Versalyse lysing solution for 15 minutes in order to breakdown the red blood cells. Five hundred microliters (500µl) of PBS was added to the sample and it was immediately analysed on the flow cytometer.

3.4.1 PLA panel with ADP activated platelets

This assay was done to measure the functionality of platelets when an agonist (ADP) is introduced into the sample. Fifty microliters (50µl) of citrated blood was incubated with 10µl of a 20µM ADP solution for 15 minutes which will cause irreversible aggregation and activation of platelets. The concentration was prepared by reconstituting a ready-made powdered concentration of ADP with 500ml of distilled water. The sample was then stained with the titrated PLA antibody cocktail (see 3.4) and incubated in the dark at room temperature for 10 minutes. Five hundred microliters (500µl) of versalyse solution was then used to lyse the red blood cells. After 15 minutes, 500µl of Lonza PBS without calcium and magnesium was added and the sample was immediately analysed by flow cytometry.

3.4.2 PLA measurements gating

To ensure that no autofluorescence was included in the analysis, unstained blood samples were run and these plots were used to set the quadrants for the PLA panel. Neutrophils and monocytes were gated based on side scatter and the expression of CD16 and CD14 respectively. We further gated on classical monocytes which were defined as $CD14^{+}CD16^{-}$. Platelet monocyte aggregates (PMAs) were defined as CD14+CD42b+ and platelet neutrophil aggregates (PNAs) were defined as CD16+CD42b+. The percentage of cells expressing CD69 (quantitative measure) and the MFI (qualitative measure) were used as a measure of monocyte and neutrophil activation. Tissue factor expression on PMAs and PNAs were measured as the % of cells expressing CD142. See figure 3.2.

After stimulation with ADP the expression of CD142 and CD69 on both bright and dim CD14+ monocytes and CD16+ neutrophils populations was determined in order to see if there was further PLA formation and to see if platelet activation was induced by an agonist. See figures 3.3, 3.4 and 3.5.

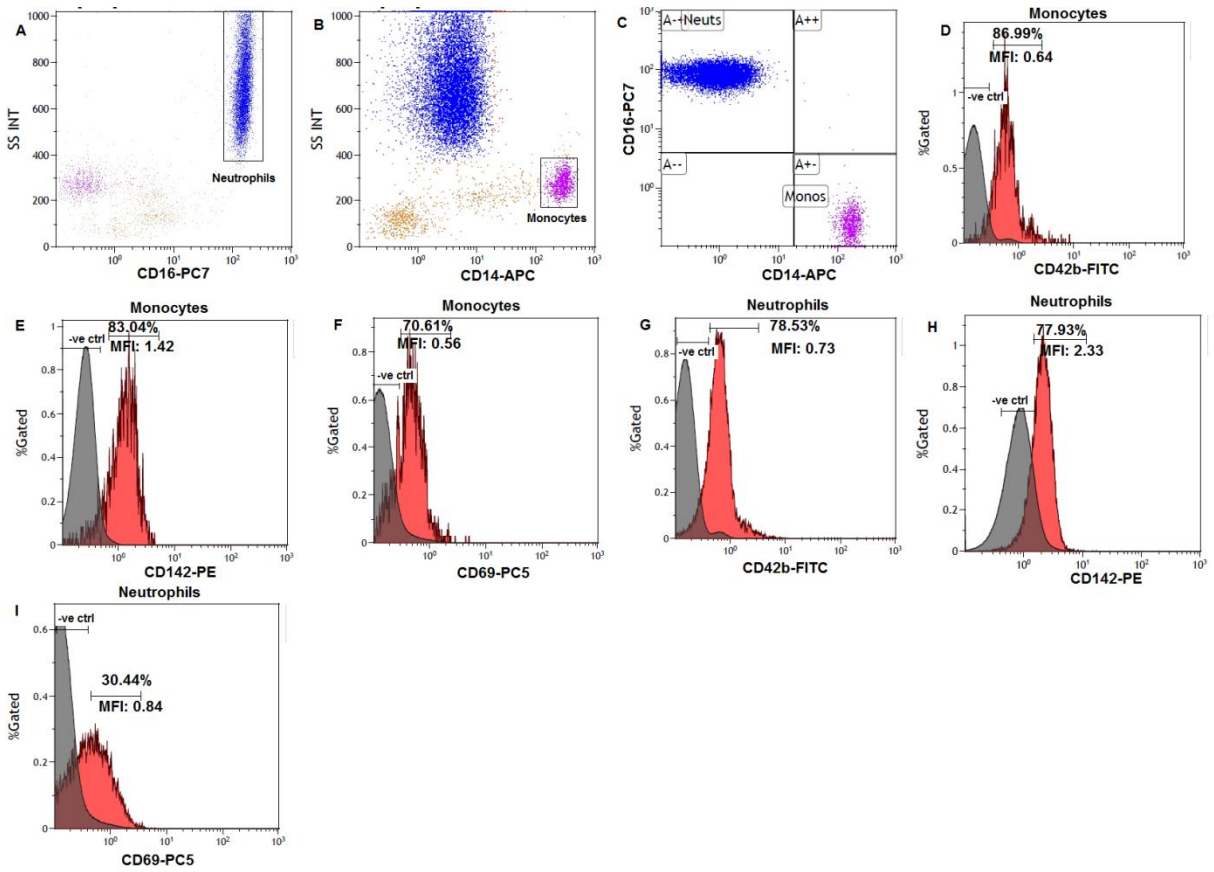

Figure 3.2: In this figure, the gating strategy used for the PLA measurements is shown. Cytograms A and B show the primary gating on neutrophils (CD16+) and monocytes (CD14+) using SS properties. Classical monocytes were gated on as shown in C. PMAs were regarded as CD14+CD42b+ (D) events and PNAs as CD16+CD42b+ (G). Histograms E and F show the percentage expression and MFIs of CD142 and CD69 on PMAs, H and I show the percentage expression and MFIs of CD142 and CD69 on PNAs. The use of the negative control to control for autofluorescence is shown in each of the histograms.

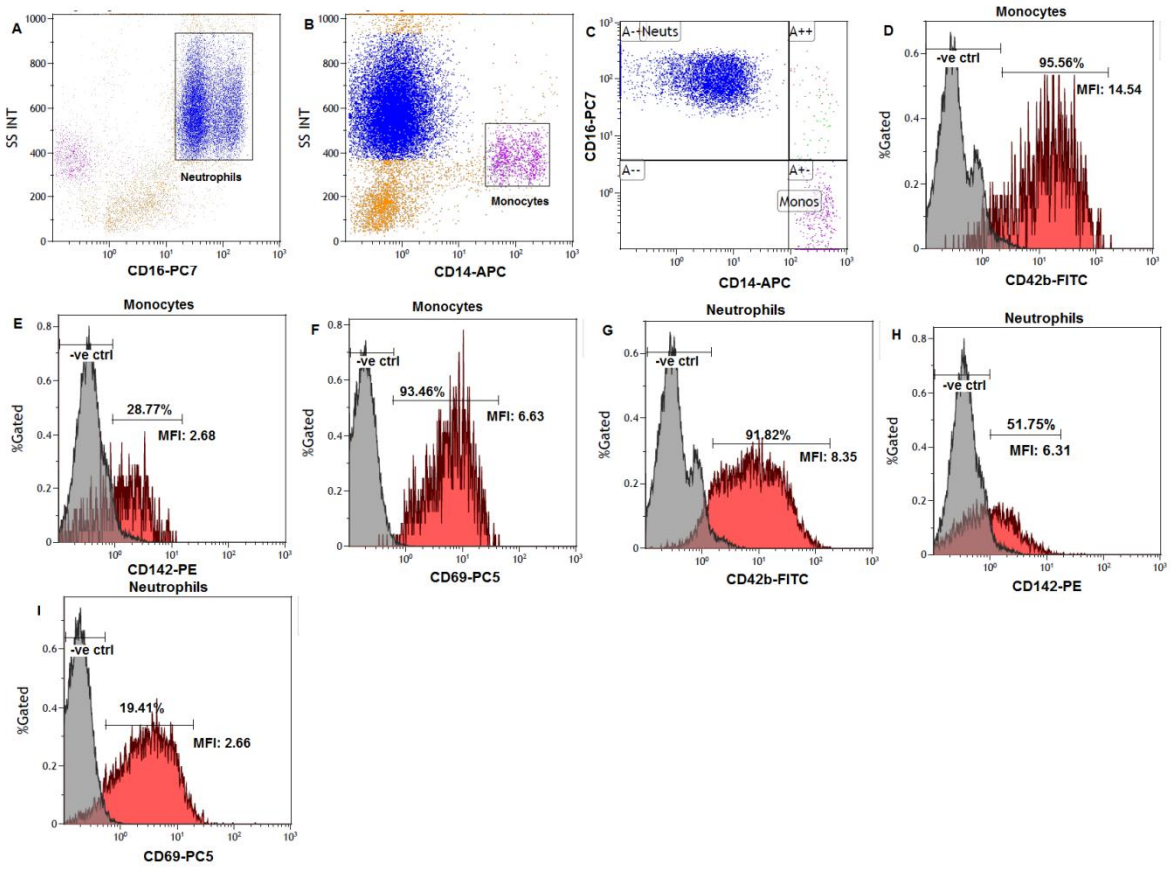

Figure 3.3: In this figure, the gating strategy used for the analysis of PLA after 20µM ADP stimulation is shown. Colour dot plots A and B show the primary gating on neutrophils (CD16+) and monocytes (CD14+) using their SS properties. Classical monocytes were gated on as shown in C. PMAs were regarded as CD14+CD42b+ (D) events and PNAs as CD16+CD42b+ (G). Histograms E and F show the percentage expression and MFIs of CD142 and CD69 on PMAs, H and I show the percentage expression and MFIs of CD142 and CD69 on PNAs. The use of the negative control to control for autofluorescence is shown in each of the histograms.

3.5 Immune activation panel

The anti-human CD95-FITC (clone UB2), CD4-PE (clone 13B8.2), CD3-PC7 and CD8-APC (B9.11) were purchased from Beckman Coulter, Miami, Florida. Antibody titrations were performed to determine the optimal antibody concentrations to be used in each of the panels.

3.5.1 Gating strategy used to analyse Immune activation

An unstained sample was used as the negative control to set the quadrants for this panel.. Target T-lymphocytes were identified using CD3 (Beckman Coulter Miami, Florida) and side scatter. T-helper cells were defined as CD3+CD4+ events and T-cytotoxic cells as CD3+CD8+. To measure the level of activation, the percentage expression and MFI of CD95 was measured. See figure 3.6

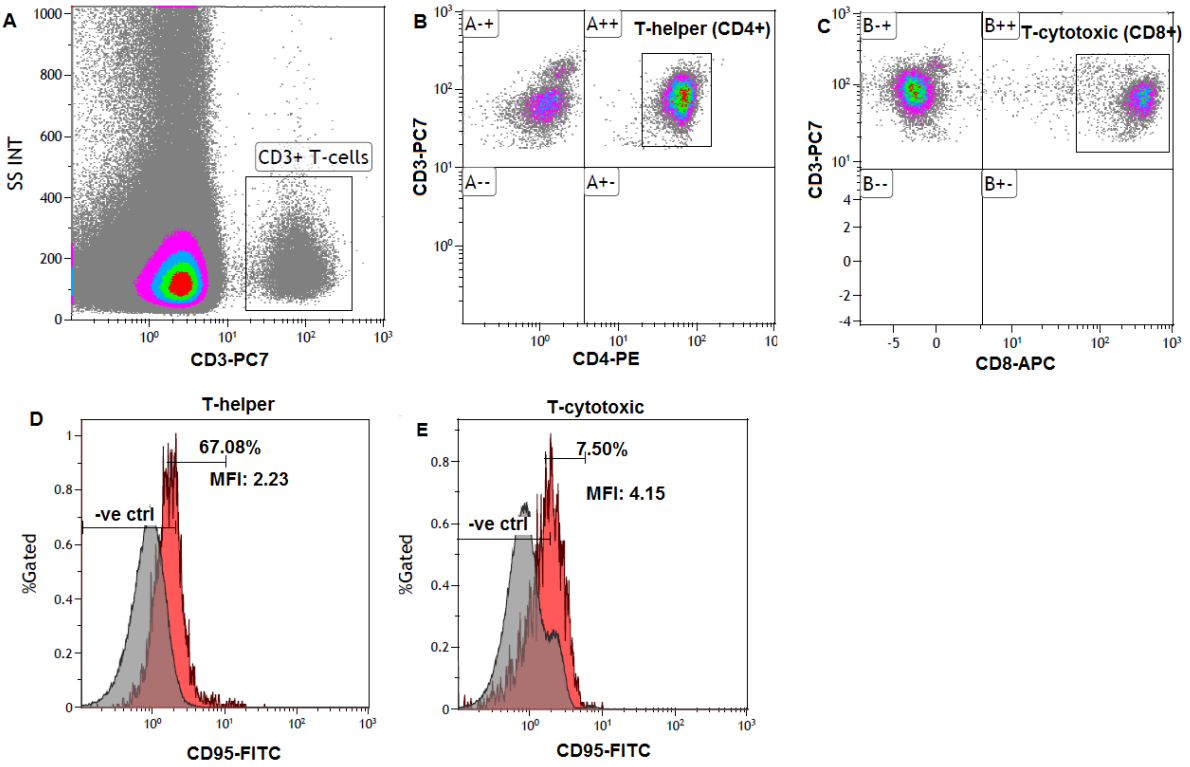

Figure 3.6: Illustrates the gating strategy for measurement of CD95 on CD4+ and CD8+ T cells. Figure A shows the primary gate for T lymphocytes (CD3+T-cells) using the side scatter properties and CD3+ events. In plots B and C CD3+ T cells are further sub classified into T-helper (CD4+) and Cytotoxic (CD8+) T-cells. Histograms D and E illustrate how the percentage expression and MFI of CD95 were measured on the CD3+ T-cells so as to determine the level of lymphocyte activation. . The negative controls are also shown on how they were used to correct for autofluorescence in analysis.

***3.6* Biochemical and haematological measurements**

Blood was collected into Serum Separator Tubes (SST), Ethylenediaminetetraacetic acid (EDTA) and Heparin in order to perform biochemical and routine haematological measurements. These tests were performed at an ISO 15189 accredited Pathology practice (PathCare, Reference Laboratory, Cape Town, South Africa) that had no access to the clinical information of participants. Pathcare are also accredited by the South African National Accreditation Systems (SANAS).The analytes and parameters measured are shown in table 3.1.

Table 3.1: Table showing the laboratory techniques used for the measurement of parameters, the respective instruments used and references values.

3.7 Statistical Analysis

Analyte	**Method**	**Equipment used**	**Reference values**
Plasma glucose (mmol/L)	enzymatic hexokinase	Cobas 6000 Roche Diagnostics	4.1-5.6
HbA1c (%)	Turbidimetric inhibition immunoassay	Cobas 6000 Roche Diagnostics	4.50-6.30
LDL (mmol/L)	Friedwald's formula	Cobas 6000 Roche Diagnostics	1.0-3.0
Insulin (uU/mL)	microparticle enzyme immunoassay	Axsym, Abbot	0.2-9.4
-White Cell Count (cells/µl)	Impedance method	Coulter counter	Male:4.32 x 10^6-5.72 x 10^6 Female:3.90 x 10^6-5.03 x 10^6
Platelet count (cell/µl)	Impedance method	Coulter counter	150 x 10^3-400 x 10^3

For non-parametric, data the researcher used the Mann-Whitney U test and the data was presented as median and interquartile ranges. The student t-test was used for parametric data and the results presented as mean (±2SD) and standard deviation. The spearman correlation coefficient was used to perform correlations. Statistical significance was represented by a p value of $p< 0.05$. Graph pad prism 5 statistical software was used for all statistical analysis.

Chapter 4

4 Results

4.1 Individual demographics

This study consisted of one hundred and twenty four (124) participants (27% male) recruited from the Bellville South community in Cape Town (See table 4.1) The results were age matched for age as well as sex. According to the WHO glucose tolerance categories, 12% had diabetes, 20% prediabetes, that is, IFG and/or IGT and/or both, and 68% nomortolerant. The mean +/- standard deviation (SD) age of the individuals was 53 (+/-SD) years in the overall sample, mean (SD) diabetes, mean (SD) prediabetes, and mean (SD) normotolerant. The mean body mass index (BMI) was above the normal reference range (18.50-24.99kg/m^2) for all 3 groups, however it was significantly higher in individuals with diabetes or prediabetes ($p<0.001$).

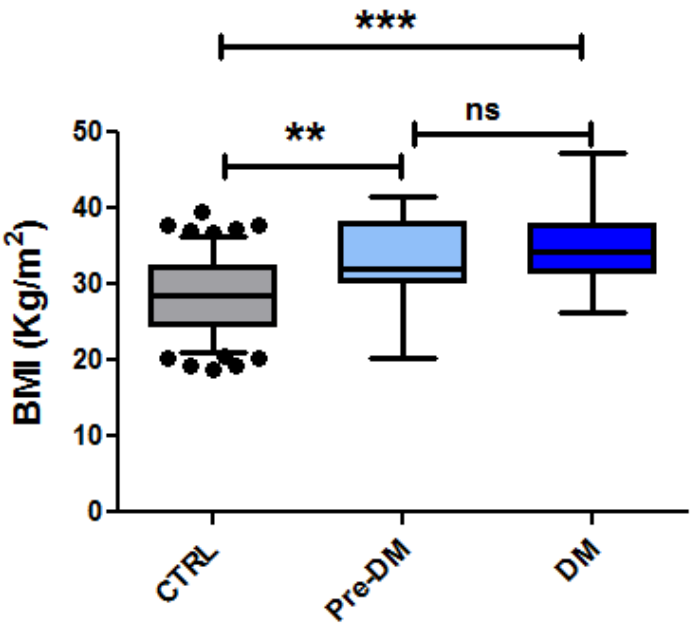

Figure 4.1: This figure shows the graphs for BMI between the three study groups. Although all three groups from this population had BMIs above the reference range, the T2DM group had significantly higher BMIs compared to the control (CTRL) group. Pre-T2DM individuals also had significantly increased BMIs when compared to the control group. ns: no significance; *: p=0.009-0.05; **: p=0.0001-0.009; ***: p<0.0001. Data on plot presented as mean ±2SD.

Table 4.1: Participant demographics

	Control Group (n=84)	Pre-Diabetes (n=25)	Diabetes (n=15)	Reference ranges	P-value
Age(years)[1]	49.93 ±13.83	57.68 ± 13.65	59.73 ± 11.32	-	**0.0054**
Sex- Male: Female	1:2	1:4	1:4	-	-

Significant values (p<0.05) are in bold text, [1]One Way ANOVA, [2]Kruska Wallis test, Median [IQR], Mean ±SD

4.2 Biochemical parameters

As expected, the T2DM group showed elevated median levels of plasma glucose 6.9 [5.18-8.63] compared to the pre-T2DM (5.4[4.05-6.75]) and the control group (4.85[3.64-6.06] p<0.0001). In addition, HbA1c levels within the diabetes (DM) group were significantly increased (7.69±1.01) compared to the pre diabetes (pre-T2DM) group (6.02±0.50) and control group (5.70 ±0.35), p<0.0001.

The median insulin level was elevated in both the diabetes 7.265[5.45-9.08] and pre-T2DM 8.75 [6.56-10.94] compared to the control group (median 5.55[4.16-6.94)], p<0.0001), however, although the-insulin levels were increased compared to the controls, they still fell within the normal reference range of 0.2-9.4 uU/mL See table 4.2.

Triglyceride levels were significantly increased in individuals with diabetes (median 1.73[1.30-2.16]) compared to the control group (median 0.98[0.74-1.23]), p<0.0001. The T2DM group showed increased levels of low density lipoprotein cholesterol (LDL-C) (median 4.5[3.38-5.63]) compared to the normotolerant group (median 3[2.25-3.75]) whose levels were within the reference range.

Table 4.2: Biochemical parameters

	Control Group (n=84)	Pre-Diabetes (n=25)	Diabetes (n=15)	Reference ranges	P-value
HbA1c (%)[1]	5.70 ±0.35	6.02±0.50	7.69±1.01	4.50-6.30%	**P<0.0001**
Insulin Fasting (uU/mL)[2]	5.55[4.16-6.94]	8.750[6.56-10.94]	15.25[11.44-19.06]	0.2-9.4 uU/mL	**P<0.0001**
Glucose (mmol/L)[2]	4.85[3.64-6.06]	5.4[4.05-6.75]	6.9[5.18-8.63]	4.1-5.6 mmol/L	**P<0.0001**
Triglycerides (mmol/L)[2]	0.98[0.74-1.23]	1.42[1.07-1.78]	1.73[1.30-2.16]	1.8-2.2 mmol/L	**P<0.0001**
Low density lipoprotein (LDL) (mmol/L)[2]	3[2.25-3.75]	3.4[2.55-4.25]	4.5[3.38-5.63]	1.0-3.0 mmol/L	**P<0.0001**
Calcium (mmol/L)[2]	2.33[1.75-2.91]	2.36[1.77-2.95]	2.39[1.79-2.99]	1.1-1.35 mmol/l	0.0556

Significant values (p<0.05) are in bold text, [1]One Way ANOVA, [2]Kruska Wallis test, Median [IQR], Mean ±SD

4.3 Inflammatory and haematological parameters

Active inflammation was determined by measuring the baseline levels of C-reactive protein (CRP). The levels of CRP within the T2DM group was significantly increased 7.265[5.45-9.08] compared to the control group 2.74[2.06-3.425], p=0.0006), indicating an elevated level of inflammation. See table 4.3.

Table 4.3: Inflammatory and haematological parameters

	Control Group (n=84)	Pre-Diabetes (n=25)	Diabetes (n=15)	Reference ranges	P-value
CRP (mg/L)[2]	2.740[2.06-3.425]	3.470[2.60-4.68]	7.265[5.45-9.08]	<3.00 mg/L	**0.0006**
Platelet count (x10^{3}cells/µl)[1]	276.6±59.44	284.8±60.98	290.4±62.86	150-400 x10^{3}cells/µl	0.9692
Haemoglobin (g/dl)	13.50[12.83-14.18]	13.80[13.05-14.40]	13.40[12.60-14.00]	13.00-17.00 g/dl	0.4150
White cell count (x10^{3}cells/µL)[2]	6.85[5.14-8.56]	8.1[6.08-10.13]	7.4[5.55-9.25]	3.5-10.5 x 10^3 cells/µl	**0.0220**
Abs Monos x10^{3}cells/µL[2]	0.5[0.38-0.63]	0.5[0.38-0.63]	0.4[0.3-0.5]	0.0-0.8 x10^{3}cells/µL	0.7984
Abs Neuts x10^{3}cells/µL[2]	3.75[2.81-4.69]	4.5[3.38-5.63]	4.9[3.68-6.13]	1.80-7.00 x10^{3}cells/µL	**0.0263**

Significant values ($p<0.05$) are in bold text, [1]One Way ANOVA,[2]Kruska Wallis test, Median [IQR], Mean ±SD

The study population did not have thrombocytopenia or thrombocytosis and the median platelet count in all the three groups was within the normal reference range of 150 x 10^3-400 x 10^3 cells/µl. In addition, there were no significant differences in the platelet count between the groups, $p>0.05$. An elevated white cell count (WCC) can also be used to determine the inflammatory status of individuals however participants from this cohort had WCCs which were all within the normal reference ranges (3.5 x 10^3 – 10.5 x 10^3 cells/µl). None of the study population exhibited leukopenia or leucocytosis however, the median white cell count (WCC) was significantly increased in the pre-T2DM group compared to the T2DM group and the control group, $p=0.0220$. Haemoglobin levels for the individuals in the study were within the reference range (13.00-17.00 g/dl)

4.4 Tissue factor (TF) and CD69 expression on monocytes and neutrophils

Tissue Factor (CD142) expression on monocytes indicates that they are acquiring a pro-coagulant phenotype. The percentage of monocytes expressing CD142 was significantly

increased in both diabetes 61.46[46.1-76.83] % and prediabetes 75.02[56.27-93.78] % state as compared to the normal control group 10.98[8.24-13.74] %, p<0.0001. In addition, the mean fluorescent intensity (MFI) which correlates with the number of surface antigens was also significantly elevated in the pre-T2DM and T2DM groups compared to normotorelant individuals, p=0.0034. In contrast the expression of TF on neutrophils was also significantly increased within the T2DM however there was no significant difference between the pre-T2DM group and the normal controls. In the pre-T2DM group classical monocytes expressing TF (CD142+), showed a significant inverse correlation with the platelet count(r=-0.4301; p=0.0319), in contrast, a positive correlation within the T2DM group (r=0.7131; p=0.0028).

Levels of monocyte activation were determined by the percentage expression of CD69 on the cells. The hyperglycaemic groups showed significantly higher levels of monocyte activation (DM: median 24.13[18.1-30.16]); (pre-T2DM: median 32.59[24.44-40.74]) compared to the control group (median 5.23[3.92-6.54]), p<0.0001. Neutrophils were also highly activated in the pre-T2DM groups and the percentage expression of CD69 on neutrophils was significantly increased in this group. The pre-T2DM group had significantly higher levels of CD69 expression on neutrophils (median 1.54[1.16-1.93]) compared to the control group (median 0.6[0.45-0.75]). Interestingly the T2DM group had decreased levels of activated neutrophils compared to the pre-T2DM group (median 0.54[0.40-0.67]).

Table 4.4: PLA levels, levels of activation and tissue factor expression on monocytes and neutrophils

	Control Group (n=84)	Pre-Diabetes (n=25)	Diabetes (n=15)	Reference ranges	P-value
%PMAs[2]	7.2[5.4-9]	48.96[36.72-61.2]	49.04[36.78-62]	-	**P<0.0001**
MFI PMA[2]	2.13[1.60-2.66]	2.37[1.78-2.96]	2.06[1.55-2.58]	-	0.5225
%PNAs[2]	6.01[4.51-7.51]	14.26[10.7-17.83]	13.56[10.17-16.95]	-	**P<0.0001**
MFI PNA[2]	1.98[1.49-2.48]	2.06[1.55-2.58]	1.93[1.45-2.41]	-	0.8533
%CD69 Monos[2]	5.23[3.92-6.54]	32.59[24.44-40.74]	24.13[18.1-30.16]	-	**P<0.0001**
%CD69 Neuts[2]	0.6[0.45-0.75]	1.54[1.16-1.93]	0.54[0.40-0.67]	-	**0.0284**
%TF Monos[2]	10.98[8.24-13.74]	75.02[56.27-93.78]	61.46[46.1-76.83]	-	**P<0.0001**
TF Monos MFI[2]	1.84[1.38-2.3]	2.01[1.51-2.51]	2.38[1.79-2.98]	-	**0.0034**
%TF Neuts[2]	7.945[5.96-9.93]	7.9[5.93-9.88]	38.19[28.64-47.73]	-	**0.0293**

Significant values (p<0.05) are in bold text, [1]One Way ANOVA, [2]Kruska Wallis test, Median [IQR], Mean ±SD

4.5 Platelet monocyte aggregates (%PMAs)

4.5.1 Baseline levels of %PMA

At baseline, the median percentage of circulating platelets bound to monocytes (%PMAs) was significantly increased in the T2DM 49.04[36.78-62] and the Pre-T2DM 48.96[36.72-61.2], groups, compared to the control group 7.2[5.4-9], p<0.0001. Notably, there was no

significant difference in the median %PMA between the T2DM group 7.2[5.4-9] and the Pre T2DM p=0.9554. (see figure 4.2).

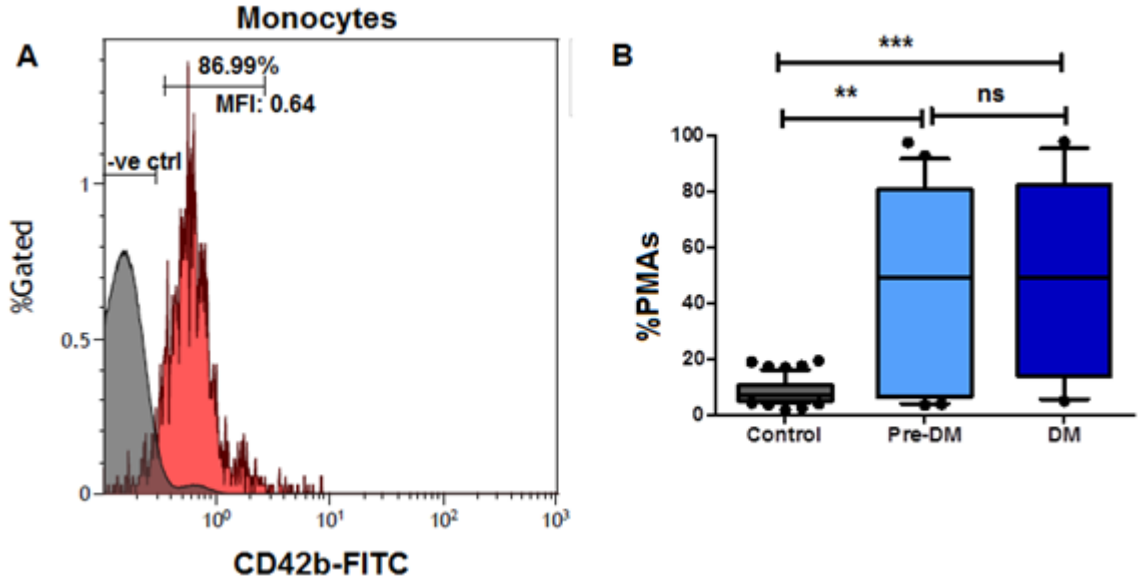

Figure 4.2: This figure shows the baseline levels of circulating platelet bound monocytes. Figure A shows how the percentage expression of platelet bound monocytes was determined and is representative of a T2DM individual with a high % of platelet bound monocytes (86.99%). An FMO was used as a negative control to distinguish platelet bound monocytes (%PMAs) from unbound freely circulating monocytes. Figure B shows whisker and box plots for the %PMAs between the three groups (control, pre-T2DM and T2DM). ns: no significance; *: p=0.009-0.05; **: p=0.0001-0.009; ***: p<0.0001, Data on plot presented as median [IQR].

4.5.2 Platelet monocyte aggregates (%PMAs): correlations with other parameters in pre-diabetes

In the pre-T2DM group, increased percentages of PMAs correlated positively with PNAs (r=0.8092; p<0.0001); activated neutrophils (reported as %CD69 positive neutrophils) (r=0.8639; p<0.0001) and TF MFI expression on neutrophils (reported as CD142 positive neutrophils) (r=0.6216; p<0.0001) (see figure 4.3). Elevated %PMAs showed no significant correlation with any markers of glucose metabolism (See table 4.5).

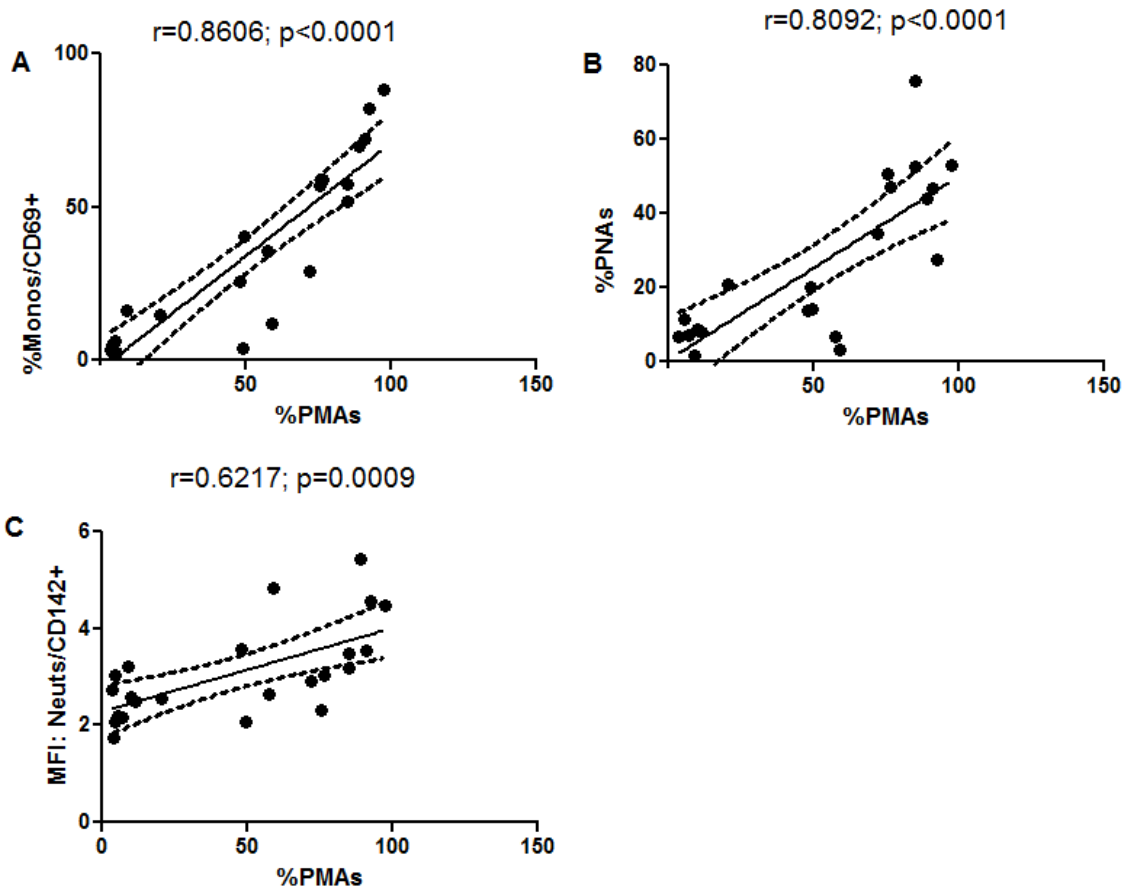

Figure 4.3: This figure shows the correlation of the %PMAs with the expression of CD69 on monocytes, the % platelet neutrophil aggregates and the MFI of CD142 on neutrophils within the pre-diabetes group of individuals. Figure A is representative of the %PMA and levels of activated monocytes (measured as %CD69 positive monocytes). Figure B shows the positive correlation between increased f %PMA vs platelet bound neutrophils (%PNA) while figure C shows a positive correlation between elevated %PMA levels and pro-coagulant neutrophils (MFI: Neuts/CD142+).

Table 4.5: PMA correlations in pre-T2DM

Parameter	r	p-value
HbA1c (%)	0.0659	0.7539
Insulin fasting (uU/mL)	0.1399	0.5245
Monocyte absolute count ($x10^3$cells/µL)	0.2419	0.2440
Platelet count ($x10^3$cells/µL)	-0.3060	0.1457
CRP (mg/L)	-0.2017	0.3443
Monos-%CD142	-0.3419	0.0942
Monos-MFI:CD142	0.2089	0.3162
Monos-%CD69	0.8606	**<0.0001**
Monos-MFI:CD69	0.8209	**<0.0001**
%PNAs	0.8092	**<0.0001**
MFI:PNAs	0.6744	**<0.0001**
Neuts-%CD69	0.8639	**<0.0001**
Neuts-MFI:CD142	0.6216	**0.0009**

Significant values ($p<0.05$) are in bold text

4.5.3 PMAs: correlations with other parameters in subjects with diabetes.

In the T2DM group, increased %PMAs showed a correlation with PNAs ($r=0.8092$; $p<0.0001$); activated neutrophils (reported as %CD69 positive neutrophils) ($r=0.8639$; $p<0.0001$) and TF MFI on neutrophils (reported as CD142 positive neutrophils) ($r=0.6216$; $p<0.0001$), shown in table 4.5. Elevated %PMA showed no correlations with any markers of glucose metabolism.

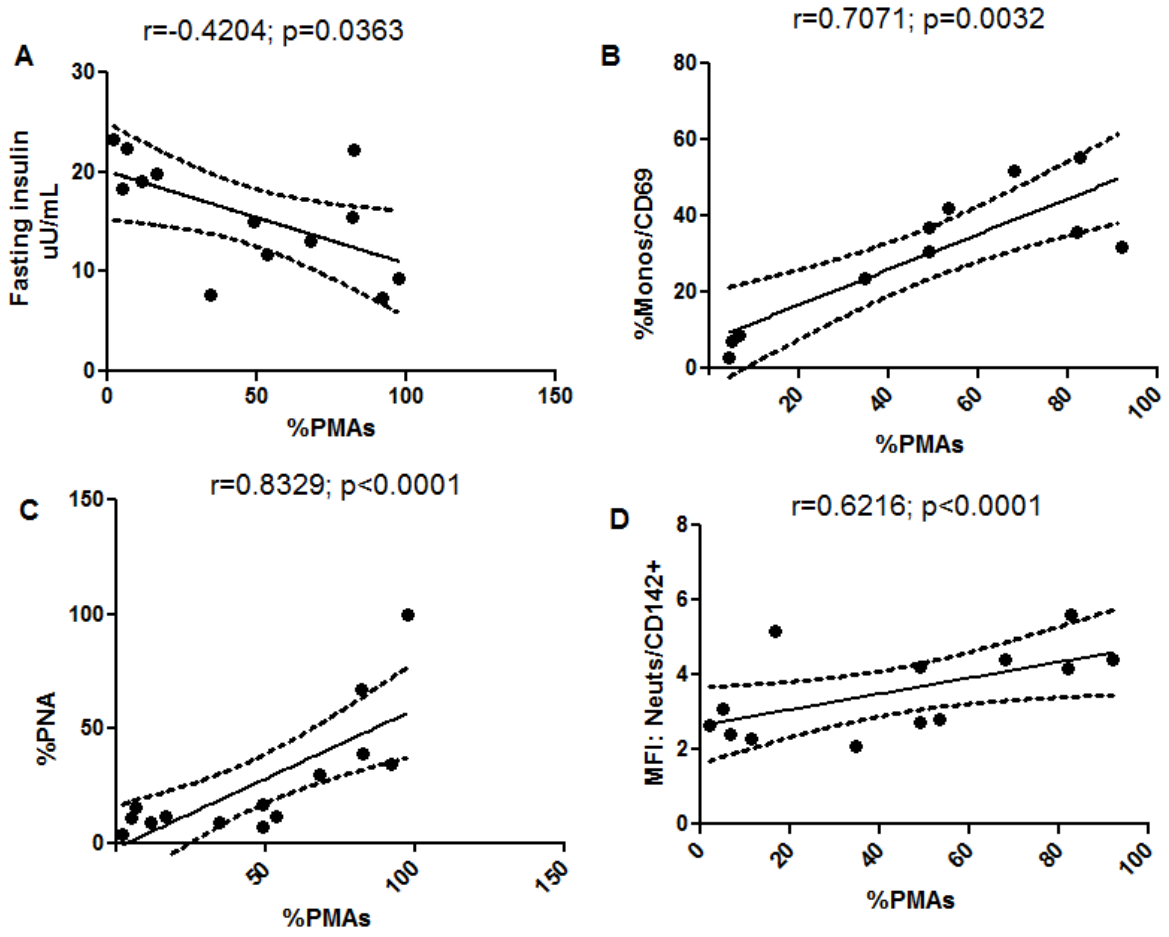

Figure 4.4: This Figure illustrates the correlation between the % PMA's and insulin, %monocyte activation, %PNAs in the T2DM group. A shows a negative correlation between %PMA and Fasting insulin; B %PMA vs activated monocytes (%Monos/CD69+), C %PMA vs platelet bound neutrophils (%PNA) and D PMA vs pro-coagulant neutrophils (MFI: CD142+ Neuts).

Table 4.6: %PMA correlations in T2DM

Parameter	r	p-value
Insulin fasting (uU/mL)	-0.5918	**0.0331**
Monos-%CD69	0.7071	**0.0032**
Monos-MFI:CD69	0.8209	**P<0.0001**
Neuts-%CD69	0.8639	**P<0.0001**
Neuts-MFI:CD142	0.6216	**P<0.0001**
%PNAs	0.8329	**P<0.0001**
MFI:PNAs	0.6744	**P<0.0001**

Significant values (p<0.05) are in bold text

4.6 Platelet neutrophil aggregates (%PNAs)

4.6.1 Baseline levels of %PNA

In order to determine the levels of platelet-neutrophil interactions in hyperglycaemic individuals the baseline levels of PNAs were measured in hyperglycaemic conditions and compared with normal controls. The %PNA and MFI were measured in order to give both a quantitative (%) and qualitative (MFI) measurement. The median %PNAs were significantly increased in the T2DM group 13.56[10.17-16.95] compared to the control group 6.01[4.51-7.51] p<0.0001. However, there was no significant differences in the %PNAs between the T2DM and pre-T2DM group [median 14.26(10.7-17.83)] p=0.7586.

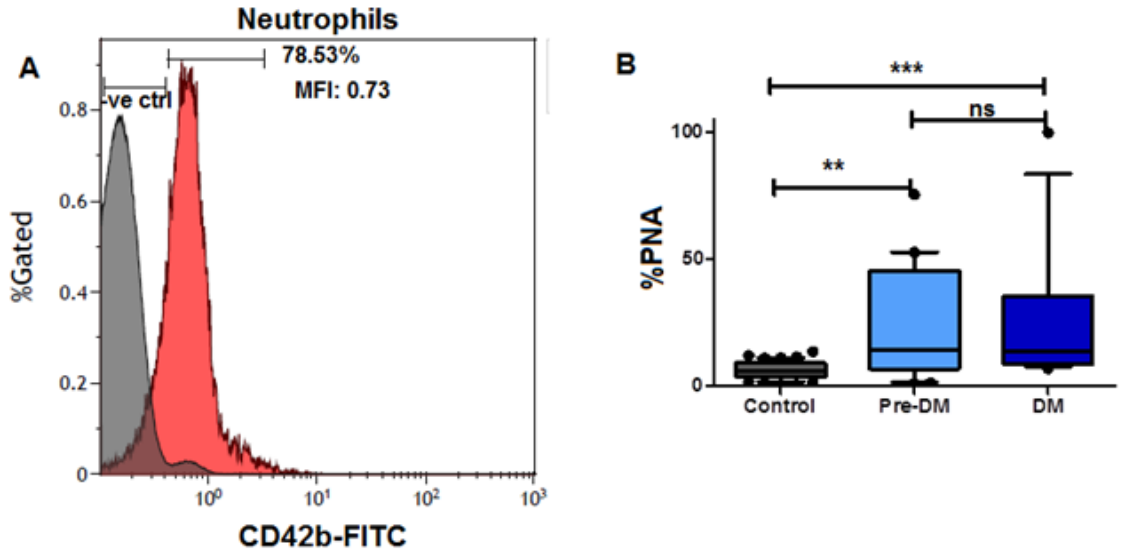

Figure 4.5: This figure demonstrates the baseline levels of circulating platelet bound neutrophils (PNAs). A shows how the percentage expression of platelet bound neutrophils was determined. An FMO was used as the negative control histogram shows the % expression of platelet bound monocytes to be 78.53% which is representative of a typical diabetes sample. B shows whisker and box plots for the %PNAs between the three groups in the study (control, pre-T2DM and T2DM). ns: no significance; *: p=0.009-0.05; **: p=0.0001-0.009; ***: p<0.0001. Data on plot presented as median [IQR].

4.6.2 PNAs: correlations with other parameters in pre-diabetes

In the pre-T2DM group, increased levels of PNAs correlated with activated neutrophils (r=0.7961; p<0.0001) figure 4.6A; TF expression on neutrophils (Neuts-MFI: CD142) (r=0.4531; p=0.0229) figure 4.6B and PMAs (r=0.8092; p<0.0001) as shown in table 4.7.

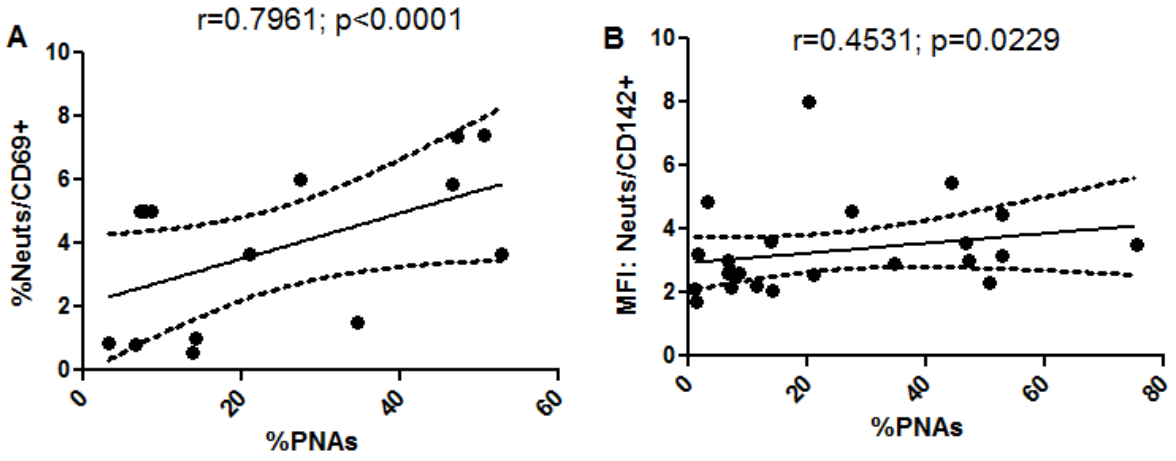

Figure 4.6: This figure shows the correlation graphs of the pre-T2DM group. A shows the correlation between %PNA and activated neutrophils (neutrophils expressing CD69), B %PNA vs pro-coagulant neutrophils (MFI: Neuts/CD142+).

Table 4.7: PNA correlations in pre-T2DM

Parameter	r	p-value
Neuts-MFI:CD142	0.4531	**0.0239**
Neuts-%CD69	0.7961	**<0.0001**
Neuts-MFI:CD69	0.4856	**0.0138**
Monos-%CD69	0.6913	**P<0.0001**
Monos-MFI:CD69	0.6638	**0.0003**
%PMAs	0.8092	**P<0.0001**
MFI:PMAs	0.6673	**P<0.0001**

Significant values (p<0.05) are in bold text

4.6.3 %PNAs: correlations with other parameters in diabetes

Hyperglycaemic T2DM individuals demonstrated a positive correlation between the %PNA and activated CD69+ neutrophils (r=0.7961; p<0.0001). In addition there was a positive correlation with pro-coagulant neutrophils expressing CD142 (TF) (Neuts-MFI: CD142) (r=0.0.5004; p<0.0001) shown in figure 4.7. Elevated %PNA levels in the diabetes also correlated with activated monocytes (r=0.6913; p<0.0001) and platelets bound to monocytes (%PMAs) (r=0.8092; p<0.0001) (shown in figure 4.4C). No further correlations were found between PNAs and other parameters for both pre-T2DM and T2DM (Table 4.8).

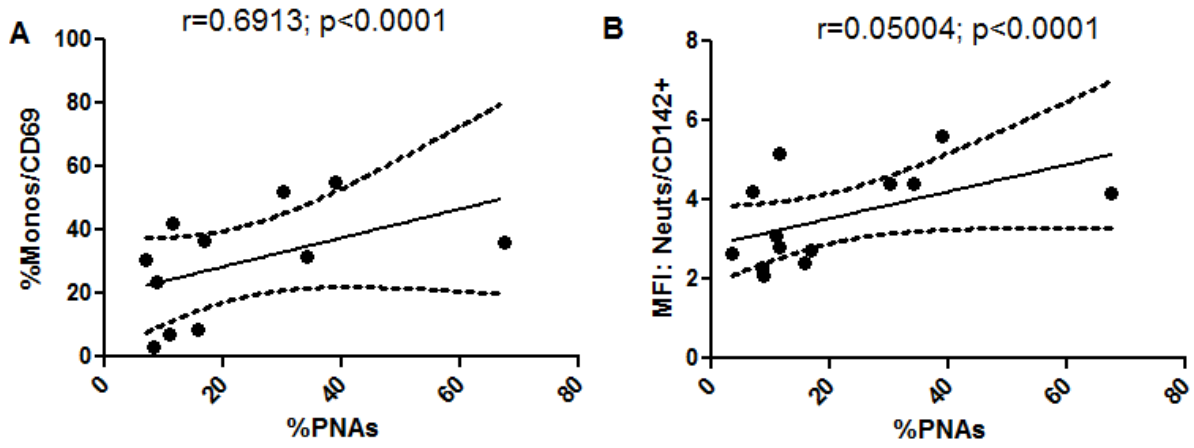

Figure 4.7: This figure shows the correlation graphs for individuals within the T2DM group. Correlations between %PNAs vs activated monocytes are shown in A and the correlation graph for %PNA vs neutrophils expressing TF (pro-coagulant neutrophils) (MFI: Neuts/CD142+) is demonstrated in B.

Table 4.8: %PNA correlation in T2DM

Parameter	r	p-value
Neuts-MFI:CD142	0.5004	**p<0.0001**
Neuts-%CD69	0.7961	**P<0.0001**
Neuts-MFI:CD69	0.4856	**0.0138**
Monos-%CD69	0.6913	**P<0.0001**
Monos-MFI:CD69	0.6638	**0.0003**
%PMAs	0.8329	**P<0.0001**
MFI:PMAs	0.6673	**P<0.0001**

Significant values (p<0.05) are in bold text

4.7 Stimulation with 20µM of ADP

4.7.1 %PMA post stimulation with ADP

Adenosine diphosphate (ADP) was used to induce irreversible platelet aggregation via the P2Y12 pathway. The control group exhibited a two-three fold increase in the level of %PMAs which was a greater increase than that in the hyperglycaemic states (pre-T2DM and T2DM). Baseline levels of %PMAs in the control group had a median of 24.02 [8.35-81.27] which was significantly increased after stimulation with ADP (median 75.90 [33.00-90.47], p<0.0001. In the pre T2DM group, the median %PMA was increased post stimulation with ADP to 83.58 [57.56-93.78] compared to the median baseline levels of 58.95 [11.74-84.97], p=0.0255 (shown in table 4.6). The T2DM group also demonstrated a significant increase in the percentage of PMAs after stimulation with 20µM ADP (mean 73.30±8.93 (SD)) as compared to baseline levels mean 47.18±9.92(SD), p=0.0256.

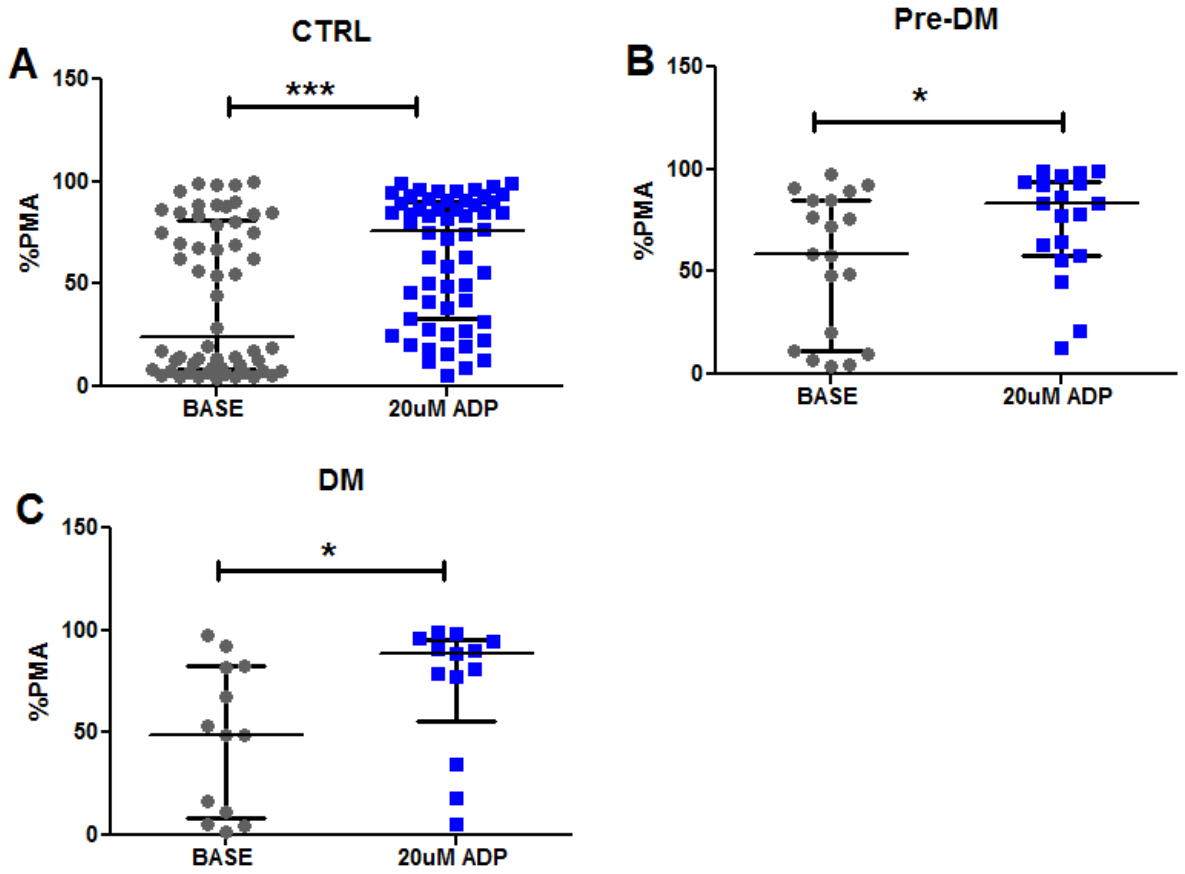

Figure 4.8: This figure illustrates the effect of stimulation by 20µM ADP on the %PMA in all three groups. A shows how the control (CTRL) group %PMA increased after stimulation with 20µM ADP. B demonstrates the increase in the pre-T2DM group while C represents the effects of ADP stimulation on the T2DM group. ns: no significance; *: p=0.009-0.05; **: p=0.0001-0.009; ***: p<0.0001. Data on plot presented as median [IQR].

4.7.2 %PNA post stimulation with ADP

After stimulation with ADP, the control group had a significant increase in the %PNA. At baseline levels the median %PNAs was 11.23 [7.01-30.86] and after stimulation with ADP the %PNA was significantly increased (median 31.31 [10.13-50.80], p<0.0001. A similar effect was observed in the %PNA of the pre-T2DM group (45.75±6.78 (SD) vs 28.09±4.99 (SD), p=0.0123) and the T2DM group (44.29 [28.66-81.27] vs 11.50 [7.48-36.60]) see figures 4.9 A, B and C.

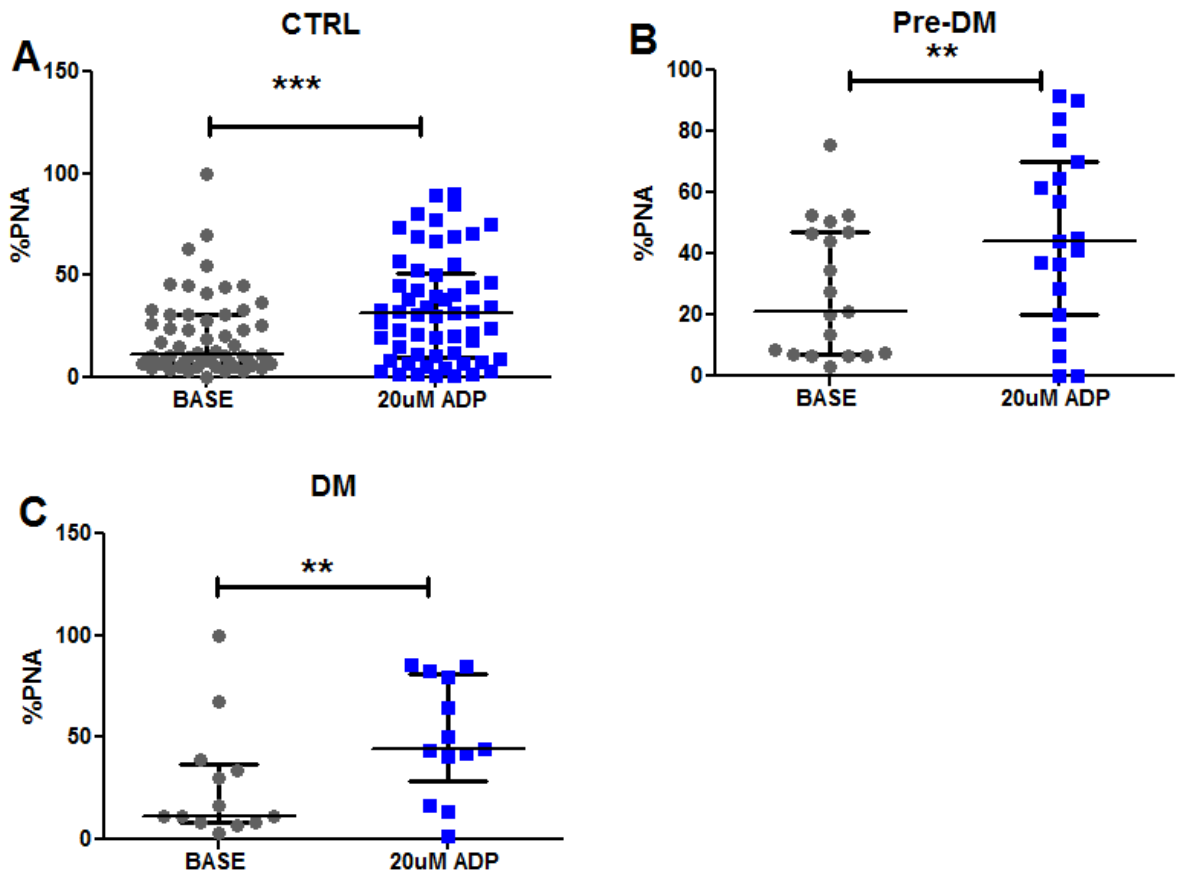

Figure 4.9: This figure illustrates the effects of 20µM ADP stimulation on %PNA formation in comparison to pre-incubation baseline levels. Plot A shows how the % PNAs within the control group responded while plot B shows the significant increase in PNA formation in the pre-T2DM group. Figure C is representative of the effects of ADP stimulation within the T2DM group. ns: no significance; *: p=0.009-0.05; **: p=0.0001-0.009; ***: p<0.0001. Data on plot presented as median [IQR].

Chapter 5

5 Discussion

The aim of this study was to investigate the relationship of platelet activation and the immune system in the process of inflammation and the consequent cardiovascular disease observed in individuals with hyperglycemia and Type 2 diabetes. A total of 124 participants were recruited from the Bellville south community and consisted of 15 diabetic and 25 pre-diabetic individuals and 84 non-diabetic controls. A previous study had revealed a high prevalence of 28.2% of type 2 diabetes in the same community in 2012 (Erasmus et al., 2012).

This study was a flow cytometry based study and pre-analytical steps were taken to ensure that no artefactual activation of platelets occurred before analysis could be done on the flow cytometer. All sample collection, preparation and analysis were done according to the British committee for standards of haematology (BSCSH). Sodium citrate was the anticoagulant of choice rather than EDTA which is known to cause platelet shape change as well as platelet aggregation (Macey et al., 2002). The samples were not centrifuged in an attempt to limit artefactual activation of platelets as recommended by the BSCSH. Phosphate buffered saline (PBS) did not contain any calcium or magnesium as these are known to induce platelet activation and aggregation via the calcium pathway. In addition, samples were analysed within 1 hour of sample collection so as to minimize artefactual activation of platelets (Harrison, 2005). The above precautions allowed flow cytometry to be reliably utilised in order to investigate whether the formation of Platelet Leukocyte Aggregates (PLA's) were increased in pre-T2DM and T2DM individuals and if any increase correlated with measured biochemical markers and other markers of inflammation .

Our results have demonstrated increased percentages of PLA's in pre-T2DM and T2DM therefore indicating increased interactions between platelets and leukocytes in the peripheral blood of individuals with hyperglycemia. Platelet monocyte aggregates were higher in both the pre-T2DM and T2DM groups when compared to the control group indicating increased interactions between platelets and monocytes. This finding supports the theory that platelets play a significant role in the process of inflammation.

Increased PMA's have been described in several conditions such as acute coronary syndromes, stroke and diabetes (Elalamy et al., 2008; van Gils et al., 2009). In diabetes, platelets are hyperactive (Kakouros et al., 2011) and release chemokines which are responsible for recruiting monocytes to the vascular endothelium (van Gils et al., 2009). P-selectin (CD62P) is expressed on platelets upon activation and binds to P-selectin

glycoprotein ligand-1 (PSGL-1) on the monocytes and thereby forming aggregates (PMAs) (Schäfer & Bauersachs, 2008). Monocytes, in addition to their inflammatory role, form heterogenous interactions with platelets which are responsible for the thrombogenic state observed in individuals with atherosclerosis (Cerletti et al., 2012).This may play a major role in the development of the cardiovascular complications characteristic of T2DM.

Activated platelets further stimulate the secretion of monocyte chemoattractant protein-1 (MCP-1) which is responsible for recruiting additional monocytes to the site of thrombus formation (Gawaz et al., 1998; Schäfer & Bauersachs, 2008). RANTES another molecule secreted by platelets also attracts monocytes to the site of inflammation where it increases the adhesiveness of these cells to the endothelium (Schober et al., 2002; Schäfer & Bauersachs, 2008). Platelet derived chemokines further activate monocytes (Jennings, 2009) leading to the release of inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-12 and IL-8 and thereby increasing inflammation (Schrottmaier et al., 2015).

In this study, increased percentages of PMAs correlated with increased levels of activated monocytes in the hyperglycaemic state (pre-diabetes and T2DM). This was demonstrated by the elevated expression of CD69 on the surface of monocytes. The expression of TF on monocytes was also investigated and it was found to be greatly elevated in both pre-diabetic and diabetic state. Elevated circulating PMA's correlated with the increase of TF expression on monocytes thereby indicating that platelet interactions with monocytes play a significant role in the phenotypic change of monocytes from the classic form to a pro-coagulant type. Platelets also stimulate the expression of vascular adhesion molecule-1 (CD11b) on the monocyte surface. This causes the monocyte to adhere to the injured endothelium and further induce an inflammatory response(Wang et al., 2014).

Tissue factor (TF) plays a major role in the initiation of the extrinsic pathway of coagulation and therefore, in T2DM, the characteristic thrombosis could be as a result of the increased TF expression on the surface of the monocytes(Bogdanov & Østerud, 2010) as well as elevated levels of factor FXa and fibrinogen which are also activated by PMA formation (Schrottmaier et al., 2015). Increased expression of TF is associated with low grade inflammation and T2DM is such a condition (Matsuda et al., 1996) hence the increase in expression of TF in individuals with diabetes in the study population.

In addition to increased percentages of platelet monocyte aggregates there was an increase in platelet neutrophil aggregates (PNAs) in both the pre-diabetes and diabetes groups which indicated that there was an increase in the interactions between platelets and neutrophils in the peripheral blood of T2DM individuals. This supports the observation made by Shantsila *et.al* who demonstrated an increase in the number of PNAs in individuals with T2DM

(Shantsila & Lip, 2009). Platelets and neutrophils interact via similar mechanisms to that of platelet and monocytes and it has been shown that platelet neutrophil aggregates are also increased in CAD. However, despite this, their role in atherosclerosis is still unclear and requires further explanation (Drechsler et al., 2011; Schrottmaier et al., 2015).

In this study we describe increased percentages of circulating activated neutrophils which directly correlate with increased PNA formation in both the pre-T2DM and T2DM group. This finding may provide an explanation for the continuing low grade inflammatory state characteristic of these groups of individuals. However, the elevated PNAs did not show a significant correlation with CRP, an acute phase protein or with increased white cell counts which are often used to determine chronic inflammation.

Activated platelets can activate neutrophils causing them to de-granulate and release pro-inflammatory cytokines (IL-1β, IL-8 and matrix metalloprotease) (Schrottmaier et al., 2015). In turn activated neutrophils can further stimulate activation and aggregation of platelets by releasing O^{2-}, platelet activating factor (PAF), neutrophil elastase and cathepsin G (Ghasemzadeh & Hosseini, 2013). Our study has shown that in individuals with diabetes, neutrophils had increased expression of TF which was similar to that observed in the monocyte population.

A study, analysing neutrophils from the blood and bone marrow using the western blot technique, demonstrated that TF was present on activated granulocytes but that this it was not present on the surface of naive neutrophils (Darbousset et al., 2012). This led to the conclusion that only activated neutrophils have the ability to express TF. (Darbousset et al, 2012). This project has demonstrated that activated platelets present in individuals with diabetes were able to stimulate neutrophils and form platelet neutrophil aggregates (PNA's). Like monocytes the binding of platelets to neutrophils is dependent on the surface expression of P-selectin which is found on the surface of activated platelets. Platelet Neutrophil Aggregates are however less stable than PMA's. This is due to the expression of PSGL-1on monocytes, which has a higher affinity for P-selectin when compared to neutrophils (Merhi et al., 1999; Rondina et al., 2013). A further *in vitro* study, examined mouse neutrophils which were incubated with activated platelets. This experiment resulted in enhanced MAC-1 expression on the neutrophils. MAC-1 promotes stable PNA and consequently resulted in neutrophil extravasation (Drechsler et al., 2011; Ghasemzadeh & Hosseini, 2013)

The increased percentage of PMA's correlated inversely with insulin in T2DM individuals. This finding suggested that decreasing levels of insulin were associated with an increase in PMA formation. Although there is insufficient research examining the direct effect of insulin

on the formation of PMAs, a few investigators have shown that platelet activation and function can be increased by insulin (Hunter & Hers, 2009; Gerrits et al., 2010). Contrary to our findings one study investigating the effects of insulin on platelet and leukocyte activity concluded that clinically significant elevated levels of insulin inhibited leukocyte-platelet interactions (Hu et al., 2002). However, despite this, both insulin and the %PMA levels were significantly increased indicating that there may be other factors initiating the formation of PMAs which are not influenced by the levels of insulin. These could include increased levels of activated platelets and cytokines from the chronic inflammatory condition in diabetes.

In addition to examining the formation of platelet leucocyte aggregates our study examined platelet function by stimulating them with a 20µM concentration of the agonist ADP. The aim was to initiate irreversible aggregation and activation of platelets. The results showed a significant increase in the formation of PLAs in both the controls as well as the hyperglycaemic group when compared to the samples which had not been incubated with ADP. Adenosine diphosphate (ADP) is an important agonist in the activation and aggregation of platelets (Kim & Kunapuli, 2011). It initiates platelet activation via the $P2Y_1$ (Gq coupled) and $P2Y_{12}$ (Gi coupled) receptors (Li et al., 2010). The overall effect of this activation is granule secretion, shape change thromboxane A_2 generation, thrombus growth and stability, increased pro-coagulant activity and increased platelet leukocyte interaction (Oury et al., 2006; Kim & Kunapuli, 2011). Activation of platelets via this pathway also leads to the expression of p-selectin (CD62P) which is required by platelets to bind to the leukocytes (Schrottmaier et al., 2015).

The results of our study imply that platelets from hyperglycaemic individuals retain their functional capacity. This was also shown in myeloproliferative disorders in which the percentage of PMAs and PNAs in ADP stimulated blood correlated positively with the expression of platelet p-selectin. As p-selectin is the main receptor initiating the formation of PLAs, this could provide an explanation (Jensen et al., 2001). Taken together this may suggest that the formation of PLAs can still be induced in hyperglycaemic state. This may mean that individuals with diabetes are at risk of even further activation of pro-inflammatory mediators and pro-coagulant factors. Therefore therapy for individuals must also focus on reducing inflammation and pro-coagulant activities.

As expected our study demonstrated a significant increase in the fasting glucose levels of the pre-T2DM and T2DM groups compared to the control groups. However, the increased fasting glucose concentration in the diabetes did not appear to influence the formation of PLAs as no correlations were identified between the two measurements. Results from our study showed a threefold increase in plasma CRP levels indicating an increased acute

inflammatory state in both hyperglycaemic groups. T2DM is considered to be a low grade inflammatory condition (Petersen & Pedersen, 2005; Xie & Du, 2011; Calle & Fernandez, 2012) which is characterised by an increase in CRP levels (Calle & Fernandez, 2012). Chronic inflammation induces endothelial dysfunction leading to decreased NO production by the EC, as well as increased production of cytokines which recruit macrophages and lymphocytes to the EC (Hess & Grant, 2011). It also leads to platelet activation and aggregation, and once activated these platelets will produce cytokines which lead to the further activation and recruitment of macrophages and lymphocytes (Hess & Grant, 2011; Cerletti et al., 2012).

From this study we have been able to provide further evidence to support the link between the immune response and the pro-thrombotic state observed in individuals with diabetes. Type 2 diabetes mellitus is a low grade chronic inflammatory condition and cells involved in normal inflammatory conditions such as leukocytes and platelets become dysfunctional (Xie & Du, 2011). Platelets from individuals with diabetes are activated and are therefore able to interact with circulating monocytes and neutrophils via the p-selectin receptor (Patkó et al., 2012)

This study focused on the effects these interactions had on monocytes and neutrophils however, the role of these interactions on the progression of the low grade inflammatory response and pro-thrombotic state in individuals with diabetes still requires investigation. Platelet–bound leukocytes undergo phenotypic changes which results in a pro-inflammatory phenotype and upon activation leukocytes produce pro-inflammatory cytokines which may drive the progression of the low grade chronic inflammatory state in diabetes. The inflammatory cells also express increasing levels of tissue factor which induces a pro-coagulant phenotype and stimulates the coagulation cascade. This on-going inflammation could promote the cardiovascular disease seen in hyperglycaemic individuals.

The percentages of PMA's and PNA's did not correlate with confounding factors such as age and BMI. All individuals in the hyperglycaemic groups were classified as obese and had median BMI scores which were greater than 30Kg/m^2 compared to the control group which were classified as overweight (25-29.9Kg/m^2). Therefore, changes observed in this study could multiplied by the presence of obesity, which is also an inflammatory condition (rephrase properly).

This study was a cross-sectional study; therefore no causative could be determined but only associations and correlations. Type 2 diabetes is considered as an inflammatory condition; however, in this study we did not account for other inflammatory states and infections such as tuberculosis (TB) and human immune virus (HIV). No viral or bacterial assays were

conducted as the participants were only screened for clinical parameters. A further limitation is that we did not account for the smoking status of the participants in all the three groups and this may influence platelet activation.

Chapter 6

6 Conclusion

In conclusion this project has demonstrated the following:

Platelets leukocyte aggregates (PLAs) are potent markers of platelet activation and in diabetes it has been previously demonstrated that platelets are highly activated (Patkó et al., 2012). Our study supports this and was able to show increased interactions between platelets and leukocytes in the pre-diabetes and diabetes groups.

In addition to forming aggregates with leukocytes, the platelets were able to initiate activation and phenotypic change to the leukocytes by increasing the expression of CD69 and TF (CD142). This finding provides further evidence that there is e a link between the inflammatory process and the prothrombotic activity evident in individuals with diabetes and pre-diabetes

Another conclusion of this work was that although platelets are already activated in individuals with diabetes they were still able to undergo further activation in the presence of an agonist (ADP) which lead to the formation of additional PLAs. Hence platelets in individuals with diabetes are still functional and therefore individuals are at risk of generating further PLA's which will contribute to the formation of a prothrombotic state.

Increases in PLA's did not correlate with the biochemical markers of diabetes such as fasting glucose and HbA1c or with markers of inflammation (CRP and white cell count) even though these were significantly increased in diabetes. This could indicate that the high levels of platelet activation found in diabetes may be able to activate immune cells and that anti-inflammatory medication does not reduce the formation of PLA's.

The increased percentages of PMA's and %PNAs did not correlate with increased HbA1c levels. This may be an important observation as it suggests that glycaemic control may not be able to reduce the levels of PLAs in individuals with diabetes. Therefore, therapeutic interventions may need to be directed towards lowering the presence of PLAs by reducing the extent of platelet function and activation using anti-thrombotic medication. Individuals with pre-diabetes (defined as having an HbA1c of between 5.7%-6.5%), had elevated levels of PLAs similar to those of diabetes. This may imply that treatment for this should begin at this stage of the disease rather than after the onset of diabetes.

Finally, this project has been able to produce further evidence that platelets are activated in individuals with hyperglycemia and that their interaction with neutrophils and monocytes could result in the escalation and the progression of a pro-thrombotic and pro-inflammatory environment which is associated with many of the complications characteristic of individuals with diabetes.

Further research should focus on control of increased expression of tissue factor on leukocytes and platelets by reducing interactions between the two cell types. Other studies could focus on the use of anti-inflammatory medication to combat the inflammatory condition in T2DM individuals.

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Appendix 1

Quality control protocol

Before any samples were analysed in the flow cytometer quality control (QC) protocol was run on the instrument. Flow check fluorophores from Beckman Coulter, Miami, Florida were run in the instrument to standardise the optical path and laminar flow of the instrument. The QC was considered to have passed if the half peak mean CV for each of the flow channels FL1, FL2, FL3, FL4, FL5, FL6, FL7 and FL8 were within the set ranges as shown in Figure 16.

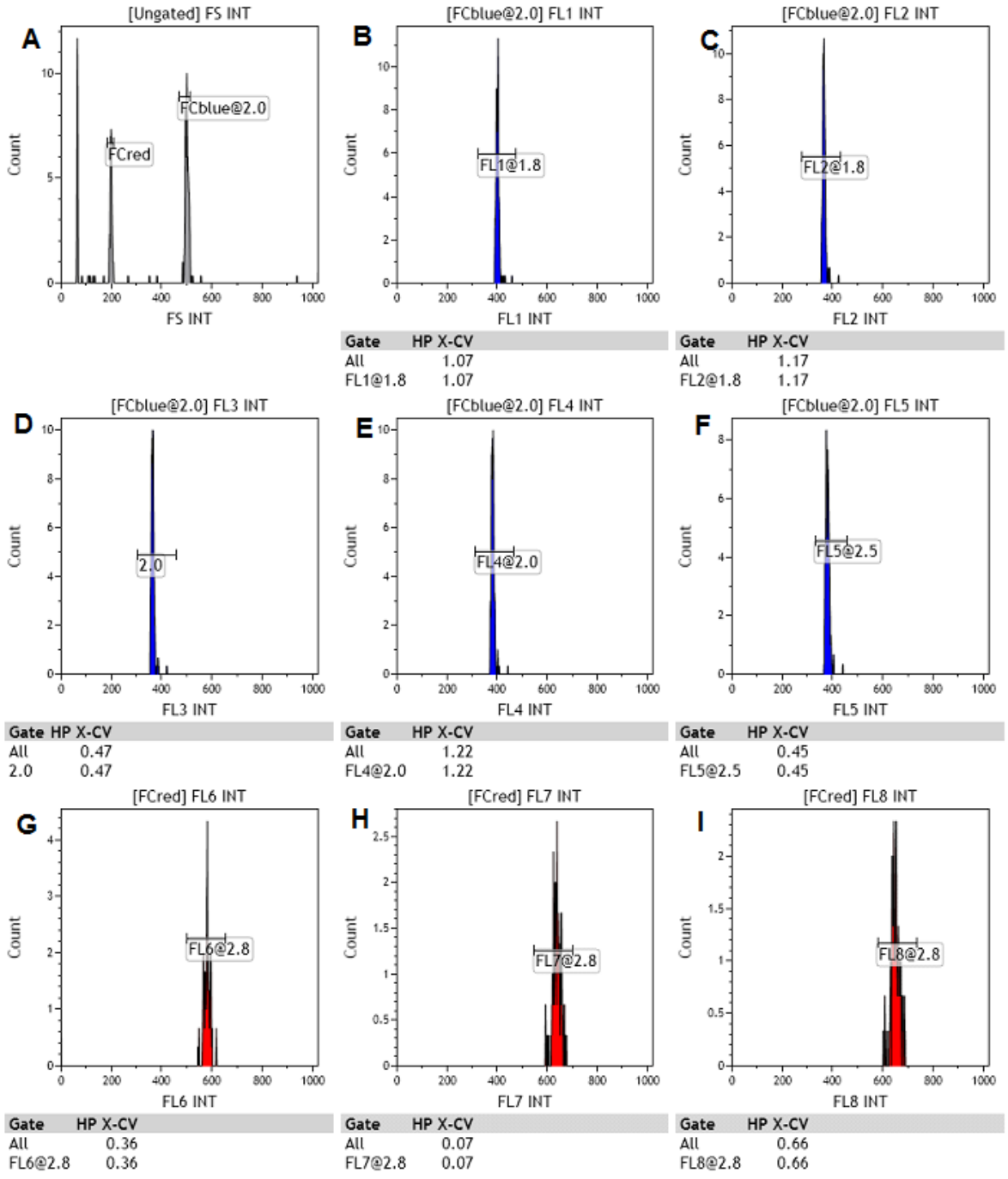


Figure 16: This figure shows QC plots which were used to determine whether the daily QC had passed. Plot A shows the primary gates on the red and blue fluorophores, plots B to F show fluorescent channels FL1 to FL5 gated on the blue fluorophores and their respective HP X-CVs which are within the set ranges by the flow check beads manufacturer. G, H and I show fluorescent channels FL6, FL7 and FL8 respectively and their HP X-CVs which are within the set ranges from the flow check beads manufacturer

Appendix 2

Fluorescence minus one (FMO)

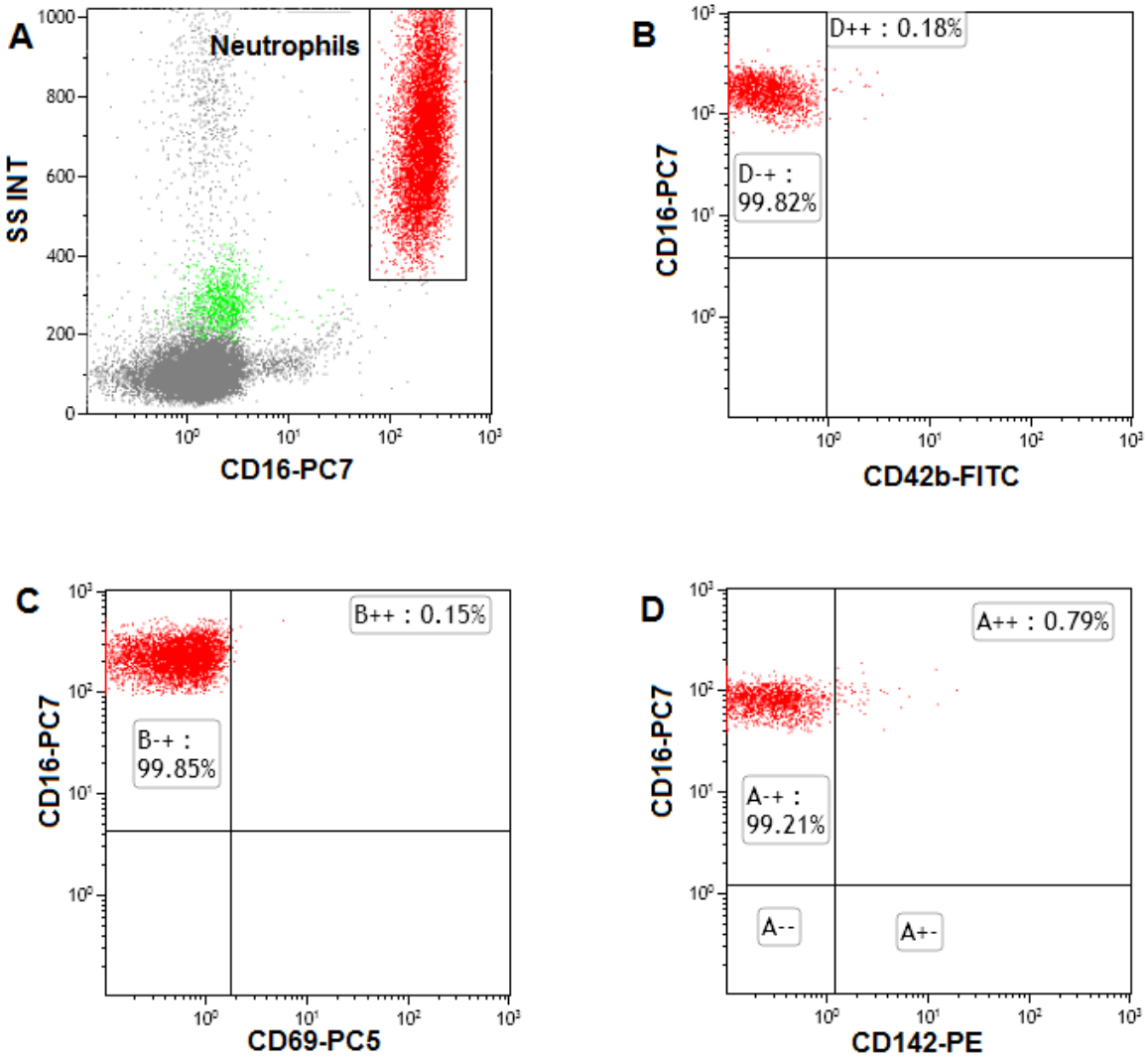
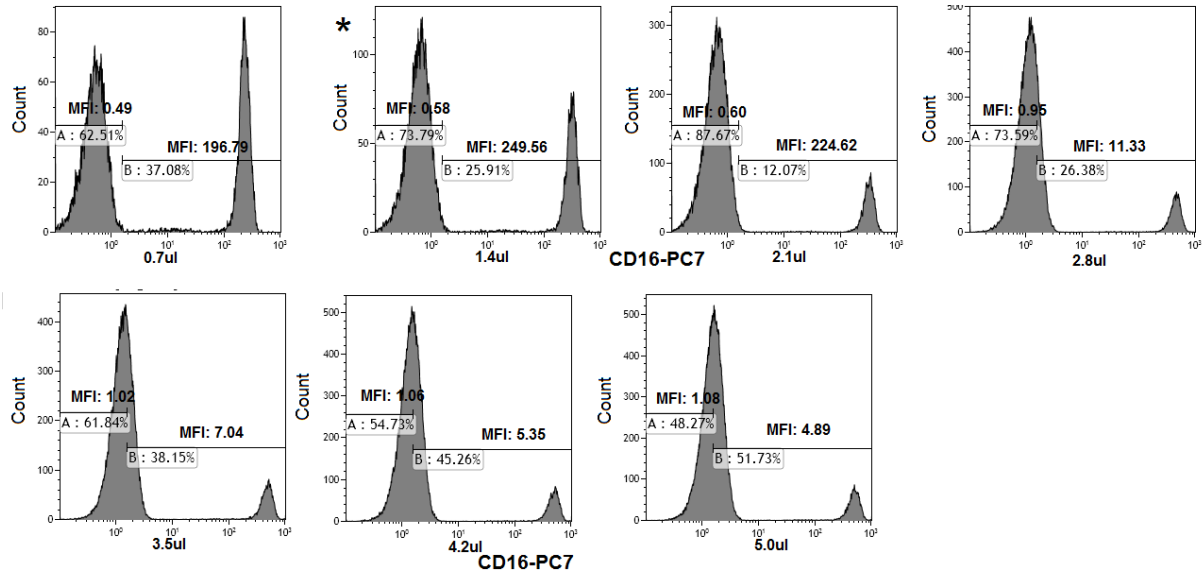


Figure 17: shows the use of FMOs for CD42b-FITC, CD69-PC5 and CD142 PE. Plot A shows the neutrophil primary gate which was used in the subsequent plots to set the FMOs for **B** (CD42b-FITC), **C** (CD69-PC5) and **D** (CD142-PE).

Appendix 3

Antibody titrations

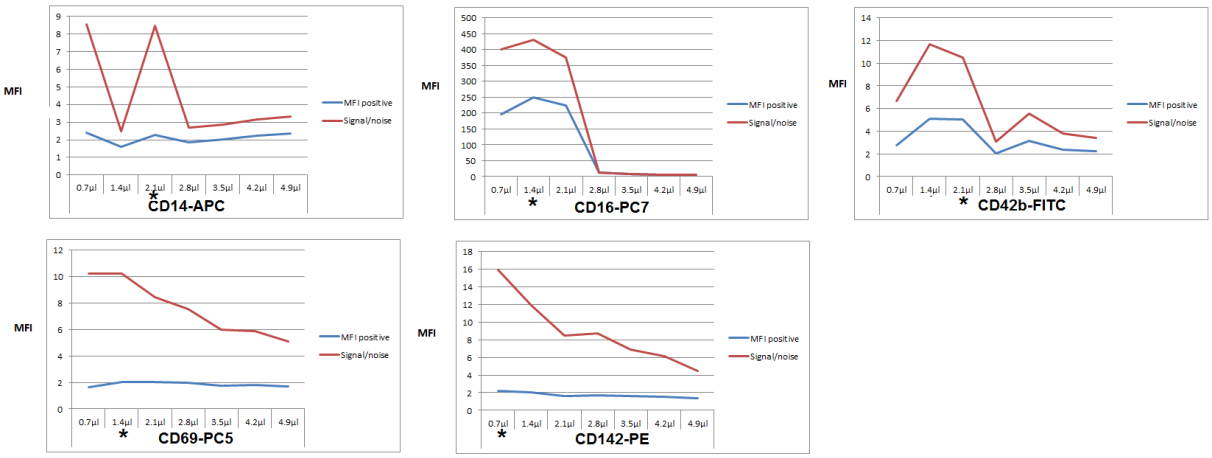
Five different volumes per antibody were each titrated into 5 different tubes with 50µl of blood, and incubated for 10 minutes in the dark. After incubation 500µl of Versalyse lysing solution was added and further incubated in the dark for 15 minutes. 500µl of Phosphate Buffered Saline (PBS) without magnesium or calcium was added and the sample was analysed on the flow cytometer. The titrated volumes for each antibody were; 0.7µl, 1.4µl, 2.1µl, 2.8µl, 3.5µl, 4.2µl, 4.9µl. To determine the signal to noise ratio, the mean fluorescence intensity (MFI) of the negative population and of the positive population where used in this equation $\frac{\text{MFI (Positive population)}}{\text{MFI (Negative population)}}$ x 100. MFIs were obtained from histograms as shown in figure 18.



*Volume which gave the highest signal to noise ratio

Figure 18: This figure shows the negative and positive populations and their respective MFIs. The MFIs were used to calculate the signal to noise ratio at each volume of antibody, the volume with the highest signal to noise ratio was used as the optimum volume for the panel.

The following graphs in figure 14 of MFI against volume were used to determine the optimum volume with the highest signal to noise ratio which was going to be used in the panel.



*Volume which gave the highest signal to noise ratio

Figure 14: This figure shows an example of the signal to noise ratio and corresponding volumes. The volumes with the highest signal to noise ratio were chosen as the optimum volumes for antibody titrations for each antibody that was used.