Investigating the amyloidogenic potential of Serum Amyloid A in Type II Diabetes Mellitus

Thesis presented in partial fulfilment of the requirements for the degree of Master of Science (Physiological Sciences) in the Faculty of Science at Stellenbosch University

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Declaration of Originality

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Abstract

Introduction: Type II Diabetes Mellitus (T2DM) is a non-communicable disease associated with chronic low-grade inflammation and persistent activation of the acute phase response (APR). Serum amyloid A (SAA) is one of the proteins of the APR and has previously been shown to induce amyloidogensis in fibrin(ogen) *ex vivo*. The impact of SAA on the haematological and its amyloidogenic potential in T2DM has however yet to be determined. Further to this, literature has noted the ability of various molecules to "mop" and reverse fibrin amyloid formation, thus identification of a molecule able to "mop" SAA's haematological impact would be highly beneficial in future.

Aim: To quantify SAA levels in T2DM before determining the impact of this molecule on the haematological system. Further to this, determining the amyloidogenic potential of SAA in this disease state. Finally, the study aims at determining whether high-density lipoprotein (HDL), lipopolysaccharide-binding protein (LBP) or the combination of these two molecules are effective SAA mopping agents.

Methods: The blood of 75 participants (n=36 control participants, n=39 T2DM participants) was collected and analysed for both quantitative and morphological markers. Quantitative markers include: inflammatory biomarker profiling and thromboelastography whereas the morphological markers include: whole blood scanning electron microscopy, fibrin clot scanning electron microscopy and fibrin clot confocal microscopy. Additionally, a control study was performed where SAA, HDL and LBP were incubated in the whole blood *ex vivo* before being analysed for these measurable and morphological markers. Lastly, an experimental study was performed whereby the efficiency of the mopping agents was tested *ex vivo* in T2DM whole blood.

Results: SAA was found to be significantly elevated ($p < 0.0001$), 10-fold, in T2DM and induced platelet hyperactivation and agglutination. Furthermore, this study confirms that SAA is indeed amyloidogenic in nature. Qualitative SEM fibrin analysis showed that SAA induced the formation of dense matted amyloid deposits in the fibrin fibres. This was confirmed quantitatively when confocal microscopy, using amyloid specific stains, showed SAA induced a significant ($p = 0.0452$ and $p = 0.0062$) increase in amyloid signal in two of the three fluorescent markers. HDL and LBP proved to be ineffective SAA mopping agents.

Conclusion: SAA is indeed amyloidogenic in nature and is contributing to the abnormal fibrin formation observed in T2DM. Despite this however, T2DM is a complex disease whereby various molecules and physiological mechanisms are altered. Thus, attributing the numerous haematological changes to a singular molecule is unfitting. Consequently,

the use of a single molecule targeting "mopping" agent to reverse or inhibit these haematological alterations seems impractical.

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List of Abbreviations:

APPs	Acute phase proteins
APR	Acute Phase Response
CRP	C- reactive protein
CVD	Cardiovascular Disease
DAMP	Danger associated molecular pattern
DM	Diabetes Mellitus
FADD	Fas-associated protein with death domain
FFAs	Free fatty acids
GLUT-4	Glucose Transporter Type 4
HDL	High-density lipoprotein
HGF	Hybridoma growth factor
HIF-1	Hypoxia-inducible Factor-1
HSF	Hepatocyte-stimulating factor
ICAM-1	Intercellular Adhesion Molecule 1
IL	Interleukin
IL-1RT	Interleukin- 1 Receptor Type
IRS1	Insulin Receptor Substrate 1
JNK	c-Jun N-terminal kinases
kDa	Kilodalton
LBP	Lipopolysaccharides binding protein
LPS	Lipopolysaccharides
MCP-1	Monocyte Chemoattractant Protein-1
MIP-1	Macrophage inflammatory protein-1
n value	Population Size
PAI-1	Plasminogen Activator Inhibitor 1
PAMPs	Pathogen associated molecular patterns
PI3K	Phosphoinositide 3-kinase

PKC- θ	Protein kinase C-theta
PRRs	Pattern recognition receptors
SAA	Serum amyloid A
SEM	Scanning electron microscopy
SODD	Silencer of death domains
sP-Selectin	Soluble P-Selectin
T1DM	Type I Diabetes Mellitus
T2DM	Type II Diabetes Mellitus
TEG	Thromboelastography
TNFR	TNF receptor
TNF-α	Tumor Necrosis Factor- α
TRADD	TNF receptor-associated death domain
VCAM-1	Vascular cell adhesion protein 1

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1.   Introduction

In recent years, a change in the global disease burden from communicable to non-communicable diseases has been observed. Cardiovascular diseases (CVD) alone accounts for up to 17.7 million deaths each year (World Health Organisation, 2018). Furthermore, the prevalence of Diabetes Mellitus, one of these non-communicable diseases, is ever growing (Mathers and Loncar, 2006). In 2015 it was estimated that over 400 million people worldwide suffered with Diabetes Mellitus, this number has been estimated to rise to approximately 650 million people by 2040 (IDF, 2018, World Health Organisation, 2017). Importantly, all non-communicable diseases are linked to chronic low-grade inflammation.

An acute inflammatory response is generally considered as a beneficial protective mechanism of the body in response to injury, infection or trauma. In contrast however, the chronic low-grade inflammation associated with non-communicable diseases produce extensive harmful effects on the body. Specifically, the haematological system is constantly exposed to the circulating inflammatory mediators which produce profound detrimental effects on all of the bloods components (Pretorius et al., 2016b). The hallmarks of this subsequent inflammatory profile in the blood, cause the blood to become more prone to clotting (hypercoagulable) which are broken down less effectively and efficiently (hypofibrinolysis) (Kell and Pretorius, 2015). Various inflammogens such as Interleukin 1β (IL-1β), IL-6, C-reactive protein (CRP) and Tumor necrosis factor-α (TNF-α) are upregulated in the various non-communicable diseases and consequently induce the hypercoagulable and hypofibrinolysis state within the haematological system (Salini et al., 2011, Pickup, 2004). In addition to these two hallmark effects, the upregulated pro-inflammatory associated cytokine-peptide hormone signals further induce the synthesis of a family of acute phase proteins, known as Serum Amyloid A (SAA), from the liver (Pickup, 2004, Badolato, 1994). Although SAA serves a natural function in the maintenance of homeostasis, the proteins' function is altered in chronic inflammation (Ye and Sun, 2015). This alteration is due to both a considerable increase in the protein concentration levels as well as the increased amount of the various other pro-inflammatory molecules which further contributes to a vicious positive feedback-loop (Uhlar and Whitehead, 1999).

An increase in concentration of circulating SAA and pro-inflammatory cytokines has also been associated with the presence of lipopolysaccharide (LPS), which is another molecule associated with chronic low-grade inflammation (Guo et al., 2013). The LPS molecules originate from cell wall components of all gram-negative bacteria which exist in the haematological system as dormant bacteria (Hurley, 1995, Kell and Pretorius, 2018,

Pretorius et al., 2017a, Pretorius et al., 2018a, Pretorius et al., 2016a), or as a constant replenishment into the blood system, possibly as a result of the individual suffering from leaky gut or gut dysbiosis(Sylvia and Demas, 2018, Saltzman et al., 2018, Kurita et al., 2017, Slyepchenko et al., 2016). This LPS is then shed from the circulating bacteria into circulation which causes various downstream impacts on the haematological system (Pretorius et al., 2018a, Pretorius et al., 2016a).

Type II Diabetes Mellitus (T2DM) is a known inflammatory disease, also associated with gut dysbiosis (Slyepchenko et al., 2016), consequently one could expect the levels of circulating SAA and LPS to be elevated. In literature, this hypothesis has been confirmed in various studies. Kumon et al. (1994) observed that individuals presenting with Non-Insulin-Dependent Diabetes Mellitus had significantly higher levels of SAA in comparison to healthy age matched controls. Additionally, Marzi et al. (2013) found further evidence to support the hypothesis of increased circulating SAA levels, in relation to T2DM, as they observed a significantly greater concentration of circulating acute-phase SAA in diabetic patients in comparison to the age matched healthy control individuals.

As previously stated, one of the hallmarks of inflammation is a hypercoagulable state as a result of pathological fibrin(ogen), caused by, amongst others, circulating SAA and LPS (Page et al., 2019, Pretorius et al., 2016a). Previous research has shown that during inflammation, fibrin(ogen) becomes amyloidogenic. This occurs through a conformation change in the protein fibrin fibre structure, from a predominately α-helix configuration into β-sheet dominant structure (Kell and Pretorius, 2016). (Pretorius et al., 2017b)(Pretorius et al., 2017b)Further studies also showed that in T2DM, a state of amyloidogenesis in fibrin(ogen) exists, which is observed via the hypercoagulable state of the blood (Pretorius et al., 2017b). Consequently, it is suggested that LPS might be the amyloidogenic inflammagen that is the causative agent in the hypercoagulability noted in T2DM. Additionally, Pretorius et al. (2017a) previously noted that this amyloid state in T2DM could be substantially removed with the use of mopping agents such as LPS-binding protein. Despite the drastic improvement when using LPS-binding protein, some amyloid signal remained. Due to this observation (Pretorius et al., 2017a), as well as the presence of other inflammatory cytokines, and inflammagens, such as SAA, one could also assume that SAA greatly influences this process of amyloidogenesis in T2DM.

Considering this knowledge, the questions then arise as to the amyloidogenic potential on fibrin(ogen) of SAA in T2DM, and whether SAA can be used as an effective (circulating) marker in T2DM. Furthermore, determining whether one could use a specific SAA mopping agents in conjunction with LPS-binding protein, for the total removal of amyloid signal in

both a healthy fibrin(ogen) plasma model incubated with SAA as well as in naïve T2DM plasma.

Consequently, the following paragraph formulates this protocol's hypothesis:

Hypothesis: Healthy plasma incubated with exogenous SAA added will results in a significant amyloid signal, which can then be removed with the addition of LPS-binding protein, HDL and a combination of the two molecules. Additionally, in T2DM we will observe increased amyloidogenic signal in plasma as well as an increased presence of circulating acute-phase SAA and circulating pro-inflammatory markers. The addition of LPS-binding protein, HDL and the combination will remove significant amyloid signal in plasma of T2DM.

Following this hypothesis, the following aim and objectives will therefore direct this thesis:

The **aim** of this study is to investigate the amyloidogenic potential of SAA and determine the effectiveness of HDL and LBP as amyloid mopping agents in the haematological system in T2DM.

The **first objective** of this MSc is to determine the levels of SAA found in T2DM individuals and determine quantifiable and morphological differences in the haematological system of age matched control and T2DM individuals.

The **second objective** of this MSc is to determine whether exogenous SAA can in fact cause amyloidogenesis when added to control whole blood and platelet poor plasma, and whether its amyloid actions can be negated using the mopping agents HDL and LPS-binding protein.

The **third objective** of this MSc is to determine whether quantifiable and morphological alterations observed in whole blood and platelet poor plasma of T2DM can be reversed using HDL and LPS-binding protein.

2. Literature Review

2.1 Epidemiology of Diabetes Mellitus

Diabetes Mellitus (DM) represents an ever-growing global health and economic burden. It was estimated that every minute that passes, six people die as a result of the disease worldwide (Wild et al., 2004). A Zheng et al. (2017) study, showed that the prevalence of DM has quadrupled over the last three decades, with almost 90% of all diabetes cases being that of Type II Diabetes Mellitus (T2DM). Furthermore, the 2017 study showed that globally, one in every eleven adults between the ages of 29 and 75, suffer from T2DM. In 2010 alone, DM was estimated to have caused approximately 3.96 million deaths in the adult population, this equates to 6.8% of the global mortality in 2010 (Roglic and Unwin, 2010). This figure rose to an estimated 5 million deaths in 2015 due to DM and the conditions associated complications, which is equivalent to a death caused by DM every six seconds (IDF, 2018).

The prevalence of T2DM is more apparent in low to middle-income countries such as "third world" countries. South Africa is no different, with T2DM placing a huge socio- and economic burden on the country. In 2009, 2 million people (representing 9% of the population) over the age of 30, suffered from DM (Bertram et al., 2013), a prevalence that has almost doubled from 5.5% in less than a decade (Bradshaw et al., 2007). The rapid rise in the prevalence of this non-communicable disease can be attributed to various factors such as the phenomenon of population ageing, economic development, urbanization, a transition to westernized dietary eating habits as well as the increase in sedentary lifestyles (Bruno et al., 2005, Holman et al., 2015, Vorster et al., 2005, Steyn et al., 1997). The combination of a sedentary lifestyle and a move towards the energy dense westernized diet results in obesity, one of the leading risk factors and comorbidities of T2DM (Eckel et al., 2011). Adiposity, with specific regard to intra-abdominal adiposity, poses the greatest risk in developing DM (Cnop et al., 2002). Importantly, in 2013 in South Africa it was estimated that approximately 38% of men and 69% of women were overweight (Ng et al., 2014), with Joubert et al. (2007) attributing 87% of all DM cases in South Africa to excessive body weight. It was estimated in the Global Burden of Disease study (2016) that the second and third leading risk factors resulting in premature death and disability in South Africa are high body mass index and hyperglycaemia respectively.

DM is associated with various microvascular and macrovascular complications (Fowler, 2008) which places a further burden on the South African health system (Pheiffer et al.,

2018). In a study by Bertram et al. (2013), it was estimated that DM annually causes approximately 8000 new cases of blindness and 2000 new cases of amputations. Additionally, Bradshaw et al. (2007) reported that DM is the root cause of approximately 14% of cases of ischaemic heart disease, 12% of hypertensive disease, 10% of stroke and 12% of renal disease.

It is evident that DM is a huge socio- and economic burden globally and more importantly in South Africa, and as a result, a new innovative way of approaching diagnosis and treatment is essential going forward.

2.2 Aetiology of T2DM

DM is one of the oldest documented diseases known to man as it was first reported in a Egyptian manuscript approximately 3000 years ago (Ahmed, 2002). The distinction between the two types of DM was first made in 1936 whereby Type I DM (TIDM) was referred to as insulin-dependent DM, whereas Type II DM was called non-insulin dependent DM. Following on from this in 1988, T2DM was then further described as a component of metabolic syndrome (Patlak, 2002). The American Diabetes Association (2009) then went on to define T2DM as a group of metabolic diseases which is characterized by the presence of hyperglycaemia, insulin resistance and relative insulin deficiency. Furthermore, the symptoms present polyuria, polydipsia, polyphagia and potentially blurred vision. It is now known that T2DM occurs as a result of an interaction between genetic, environmental and behavioural risk factors (Olokoba et al., 2012, Chen et al., 2011).

TIDM's insulin deficiency is a direct consequence of the destruction of insulin-producing β-cells, found in the islets of Langerhans within the pancreas, due to an autoimmune response (Notkins and Lernmark, 2001). In contrast, T2DM begins with initial defective insulin functioning which then develops from the subclinical impaired glucose intolerance to insulin resistance, until finally overt diabetes (Olokoba et al., 2012). The exact mechanism behind this phenomenon is not fully elucidated yet, however various theories exist.

Randle et al. (1963) studies introduced the "Lipid Theory" of insulin resistance whereby the hyperplasia and hypertrophy of adipocytes occurring in obesity results in an increased circulating concentration of free fatty acids (FFAs) within the bloodstream which goes on to impair insulin-stimulated glucose oxidation in muscle. The FFAs accumulate within the cytosol of striated muscle. This forces the mitochondria to increase fat oxidation which in

turn increases the acetyl coenzyme A:coenzyme A and NADH:NAD+ ratios in the mitochondria. This alteration inactivates pyruvate dehydrogenase which causes an accumulation of citrate in the mitochondria. This, in turn, inhibits phosphofructokinase producing a net increase in intramitochondrial concentrations of Glucose-6-Phosphate, thus promoting glycogen synthesis and inhibits hexokinase. This cascade effect produces high concentrations of intracellular glucose thus preventing any further glucose entry via facilitated diffusion through the Glucose Transporter Type 4 (GLUT-4) membrane protein (Randle et al., 1963, Samuel et al., 2010).

The "Lipid Theory" was further supported when it was discovered that diacylglycerol, a by-product of FFA oxidation, activates protein kinase C-theta (PKC- θ) (Schmitz-Peiffer et al., 1997, Griffin et al., 1999) which in turn causes the Insulin Receptor Substrate 1 (IRS1) signalling protein to be phosphorylated on the serine binding site and not the tyrosine binding site (Saad et al., 1993). This altered phosphorylation site inactivates the IRS1 protein effectively inhibiting the activation of Phosphoinositide 3-kinase (PI3K), thus negating the normal GLUT-4 translocation to the sarcolemma (Griffin et al., 1999). Consequently, fewer GLUT-4 carrier proteins are available on the sarcolemma causing reduced glucose uptake by the muscle cells, consequently causing sustained hyperglycaemia.

Further studies have been completed which implicate inflammation with hyperglycaemia and insulin resistance in the development of T2DM. Shoelson et al. (2006) historical review of inflammation and insulin resistance traced this relationship back as far as the 1800s where a study showed that a high-dose of salicylates, a group of chemicals with anti-inflammatory effects found in various foods, appeared to decrease glycosuria in patients with DM. This effect was further validated when Reid et al. (1957) showed that a high-dose of aspirin, salicylate being the active compound, for individuals presenting with diabetes produced improvements in glycemia and, in one of the individuals tested, the discontinuation of insulin treatment (Shoelson et al., 2006).

In the early 1990s, research into inflammation and insulin resistance gained traction when it was suggested that Tumor Necrosis Factor-α (TNF-α), a cytokine produced by adipocytes and therefore overexpressed in obesity, can attenuate local and systemic metabolism (Feinstein et al., 1993, Hotamisligil et al., 1993). The effects were attributed to the catabolic effect of TNF-α whereby increasing β-oxidation of FFAs, resulting in the accumulation of diacylglycerol and other metabolic intermediates, thus tying in with the "Lipid Theory". Furthermore, the increased circulating TNF-α causes the phosphorylation and activation of the protein Tyrosine Phosphatase (SH-PTPase), which causes the rapid

removal of the tyrosine phosphate group from IRS-1. This process essentially terminates the effect of insulin, resulting in a reduction of GLUT-4 translocation to the muscle sarcolemma consequently reducing glucose uptake (Engelman et al., 2000). T2DM research focusing on adipokines (Monocyte Chemoattractant Protein-1 (MCP-1), interleukin-6 (IL-6), resistin, adiponectin, Plasminogen Activator Inhibitor 1 (PAI-1) and angiotensinogen), inflammation and insulin resistance then accelerated rapidly in the science field (Cefalu, 2009).

The exact physiological event that leads to the initiation of inflammation in obesity is not fully elucidated, however, Regazzetti et al. (2009) have recently presented the 'Hypoxia Hypothesis' that occurs in obesity which eventually results in insulin resistance. Their study suggests that the hypertrophied adipocytes, found in obesity, become so large resulting in these adipocytes becoming hypoperfused. Consequently, small regional areas become hypoxic leading to the increased production and expression of Hypoxia-Inducible Factor-1 (HIF-1) (Regazzetti et al., 2009, Cefalu, 2009). The increase in circulating HIF-1 from these hypoxic regions activates the c-Jun N-terminal kinases (JNK1) and IKK/NFkB pathways as well as causing the increased expressions of various genes involved in inflammation and endoplasmic reticulum stress. Furthermore, it is suggested that this microhypoxia in the adipocytes causes the cytokines (TNF-α and IL-6) as well chemokines (Monocyte Chemoattractant Protein 1 (MCP-1)) to initiate the recruitment of macrophages into the adipose tissue. These infiltrated macrophages begin the formation of crown-like structures which further exacerbates the inflammatory response (Wang et al., 2007, Hosogai et al., 2007).

The perpetual hyperglycaemia and impaired insulin signalling previously discussed produce an initial phase of hyperinsulinemia whereby the β-cells hypertrophy and increase insulin production and synthesis (Regazzi et al., 2014). If this state of continuous hyperglycaemia remains long term, β-cell dysfunction occurs resulting in the progressive decline in the functioning of these cells leading to β-cell exhaustion whereby the cells are unable to produce the required levels of insulin (Cerf, 2013). β-cell dysfunction and death are caused by elevated levels of circulating proinflammatory cytokines inducing mitochondrial stress (Cnop et al., 2005, Eizirik and Cnop, 2010, Gurgul-Convey et al., 2011) which then alters the regulation of gene expression involved in impaired insulin secretion and increased apoptosis (Gilbert and Liu, 2012).

2.3 T2DM and Inflammation

In addition to being involved in the aetiology of the disease, low-grade inflammation persists chronically in T2DM (Xu et al., 2003). A study by Menkin (1941) was one of the first of its kind through which a firm link between inflammation and diabetes was established. The study made use of healthy dogs as well as dogs that had undergone a pancreatectomy surgery. An irritant was then injected into the pleural cavity of the lungs where after various physiological responses were monitored and studied. No changes were observed in the healthy "non-diabetic" control dog group. In contrast, the "diabetic" dogs presented with an approximate 85% increase in blood glucose in addition to proteolysis. Furthermore, enhanced rates of gluconeogenesis were observed with increased infiltration with vacuolized polymorphonuclear cells. The study went one step further when Menkin was able to block this inflammatory response via the administration of insulin. The study was crucial as it was able to illustrate that inflammation augments the degree of diabetes in addition to diabetes enhancing inflammation (Guest et al., 2008).

A Pickup and Crook (1998) study suggested that T2DM was a pro-inflammatory disease and involved the activation of the innate immune system. Activation of innate immunity induces various systemic inflammatory responses that provide the body's first line of defence against any microbial, physical or chemical insults (Beutler, 2004). The responses allow for damage repair, isolation of any microbial infectious threats as well as the restoration of tissue homeostasis (Takeda and Akira, 2004). Following the Pickup and Crook (1998) study, various studies went on to show that T2DM is in fact accompanied by innate immune system activation which induces alterations in the cytokine profile producing a shift to a pro-inflammatory state (Medzhitov and Janeway, 2000). Selected pro-inflammatory cytokines whose circulating levels are altered in T2DM are discussed in the following paragraphs.

Interleukin-1β:

Interleukin-1β (IL-1β) is a potent pro-inflammatory cytokine which is crucial for host-defence in response to any infections and/or injuries (Dinarello, 1996). IL-1β is the most prominently studied molecule of the 11 IL-1 family members (Lopez-Castejon and Brough, 2011). IL-1β is produced and secreted by various cell types although the majority of the circulating IL-1β originates from monocytes and macrophages as a result of the activation of the innate immune system (Madej et al., 2017).

Briefly, when macrophages or monocytes are exposed to any circulating repetitive molecular motifs, named 'pathogen associated molecular patterns' (PAMPs), they respond

via pattern recognition receptors (PRR's). These regulate gene expression (Takeuchi and Akira, 2010) thus producing an inactive 31 kilodalton(kDa) precursor molecule, termed pro-IL-1β (Lopez-Castejon and Brough, 2011). Importantly, pro-IL-1β can also be produced in response to factors such as activated complement components as well as the presence of other inflammatory cytokines such as TNF-α (Madej et al., 2017). These cells are now "primed" with pro-IL-1β, so that when they encounter a further PAMP or DAMP (danger associated molecular pattern) the inactive pro-IL-1β is cleaved and thus activated by the pro-inflammatory protease caspase-1 (Thornberry et al., 1992) via the recruitment of a multi-protein complex referred to as the inflammasome (Schroder and Tschopp, 2010). This mature activated IL-1β is then able to be rapidly secreted into circulation.

Once secreted, activated IL-1β acts as a ligand and binds to one of two receptors; Interleukin- 1 Receptor Type I (IL-1RTI) and Interleukin- 1 Receptor Type II (IL-1RTII) (Auron and Webb, 1994). The type I receptors are associated with various cell types such as T cells, hepatocytes, fibroblasts and endothelial cells (Essayan et al., 1998), thus when ligand binding occurs, these type I receptors transduce IL-1β most extensive biological effects (Sims et al., 1993). In contrast, the type II receptor, located on B cells and neutrophils, and act as decoy receptors as they are inactive (Peters et al., 2013). The binding of IL-1β to these receptors inhibit the transduction of the molecules' effects, thus the IL-1RTII may serve as an anti-inflammatory response system.

Due to the wide range of cell types that IL-1β interacts with, the effects of this cytokine are vast. Briefly, IL-1β, when secreted, upregulates intercellular adhesion molecule, such as ICAM-1 and VCAM-1, which cause increased endothelial cell adherence of leukocytes (Hawrylowicz et al., 1991). Additionally, IL-1β causes the induction of arachidonate metabolism which acts as a second messenger in order to induce the synthesis of various other cytokines, including TNF and IL-6 as well as acting in a positive feedback mechanism to further induce IL-1 release (Vannier and Dinarello, 1994). This cytokine also acts as a lymphocyte activating factor whereby IL-1β is required for optimal T-cell activation as well as proliferation (Shirakawa et al., 1989). Importantly, the interaction and binding of IL-1β with hepatocytes causes the inhibition in the production of various housekeeping proteins (eg, albumin) as well as stimulating the synthesis of acute phase response proteins (Essayan et al., 1998). This will be discussed in depth further into the literature review.

In T2DM, IL-1β has commonly been reported as one of the main causes of β-cell failure (Maedler et al., 2002, Ehses et al., 2007) as the β-cells themselves begin to secrete IL-1β when stimulated by increased glucose levels. This increased hepatic IL-1β concentration causes increased macrophage accumulation as the cytokine acts as a chemoattractant

molecule (Ehses et al., 2007). Eventually, β-cells in the Islets of Langerhans reduce in size and mass before undergoing cell death as infiltrated macrophages and IL-1β promote various mechanisms that induce necrosis and islet inflammation (Steer et al., 2006) as well as β-cell "autocrine apoptosis" (Donath et al., 2003).

<u>Interleukin 6:</u>

Human Interleukin-6 (IL-6) is formed from 212 amino acids, which includes a 28-amino-acid signal peptide, with its gene which has been mapped to chromosome 7p21 (Tanaka et al., 2014). The majority of IL-6 is synthesized by resident macrophages in the local area of damage or microbial insult and acts as a pro-inflammatory cytokine during the initial stage of inflammation (Heinrich et al., 1990). Additionally, pro-inflammatory IL-6 can be secreted as an adipokine by adipose tissue (Lutosławska, 2012) whereas IL-6 with anti-inflammatory properties is produced by skeletal muscle as a myokine (Muñoz-Cánoves et al., 2013).

Previously in literature, IL-6 had been termed with diverse names as the molecule has pleiotropic effects throughout the body, influencing various cell types and bodily systems, with each name reflecting the effect of the molecule (Simpson et al., 1997). IL-6 was termed interferon (IFN)-β2 due to the molecules IFN antiviral activity (Weissenbach et al., 1980). Additionally, the molecule was also named B-cell stimulatory factor 2 (BSF-2) as the molecule has the ability to induce the process of differentiation of activated B cells into antibody (Ab)-producing cells (Hirano et al., 1986). Furthermore, IL-6 was termed hybridoma growth factor (HGF) based on the ability to enhance the growth of fusion cells between plasma cells and myeloma cells (Van Damme et al., 1988, Simpson et al., 1997). Importantly for this study, however, is the term hepatocyte-stimulating factor (HSF) given by Gauldie et al. (1987) based on IL-6's ability to induce acute phase protein synthesis from hepatocytes. Due to the wide array of effects, it is evident that IL-6 plays a vital role in host defence mechanisms such as haematopoiesis, the immune response as well as in the acute-phase reaction.

In line with this molecule's functional pleiotropy, IL-6 has been implicated in the pathology of various diseases with T2DM being no different as elevated circulating IL-6 being present in this condition (Akbari and Hassan-Zadeh, 2018, Pickup et al., 2000). Although some debate in literature exists, it is believed that IL-6 alters insulin signalling via decreasing IRS-1 protein expression, as well as required insulin-stimulated tyrosine phosphorylation and finally reduction in insulin-stimulated glucose transport (Rotter et al., 2003, Kristiansen and Mandrup-Poulsen, 2005). Furthermore, IL-6 is associated with the increased β-cell

apoptosis causing reduced β-cell mass and eventually insulin deficiency (Kamimura et al., 2003).

Interleukin 8:

Interleukin-8 (IL-8), otherwise known as chemokine (C-X-C motif) ligand 8 (CXCL8), is a small chemoattractant protein, 72 amino acids peptides in length (Remick, 2005). Majority of all nucleated cells are potential sources of IL-8 however, the primary cellular sources of circulating IL-8 are monocytes and macrophages which secrete IL-8 in response to any antigens present and bound to the cells toll-like receptors (Standiford et al., 1990, Waugh and Wilson, 2008). IL-8 is an essential cytokine in the early phases of acute inflammation as it is produced in the initial phases of the inflammatory response. In contrast to most other cytokines, IL-8 remains active for a prolonged period post inflammation ensuring effective clearance of debris/bacteria at the site of interest (Vogiatzi et al., 2009).

IL-8s primary role is as a neutrophil chemotactic factor whereby monocytes and neutrophils, cells of the acute inflammatory response, are recruited and attracted to the site of injury/infection/inflammation (Vogiatzi et al., 2009). This cellular recruitment transpires via the chemotactic gradient development, which ultimately causes the monocytes and neutrophils to travel towards the area of increased chemokine (IL-8) concentration ensuring the correct cells are recruited to the site of inflammation as well as ensuring these cells remain in the affected area (Gimbrone et al., 1989). In addition to this cellular recruitment, IL-8 is essential in the activation and promotion of phagocytotic processes of monocytes and neutrophils (Standiford et al., 1990).

The Cimini et al. (2017) study showed that individuals presenting with T2DM have significantly elevated circulating IL-8 levels in comparison to that of healthy age matched controls. This elevated IL-8 levels further exacerbate muscle glucose uptake via the reduction in GLUT4 translocation which may also lead to sustained bouts of hyperglycaemia (Amir Levy et al., 2015). Further research into the impact of IL-8 in T2DM is limited.

TNF- α:

Tumor Necrosis Factor-α (TNF- α) is a pleiotropic cytokine with a wide array of effects throughout the body. In addition to being an adipokine secreted by adipose tissue, TNF- α is also produced by various cells in the body with monocytic cells, including macrophages, microglia, Langerhans cells, astroglia and Kupffer cells generally being the primary synthesizers of this cytokine (Pfeffer et al., 1993, Flynn et al., 1995). The TNF-α gene has been found to be present as a single copy gene found on human chromosome 6 with the

gene consisting of three introns and four exons (Spriggs et al., 1992). This Human TNF-α protein is then expressed as a 27-kDa (233 amino acid) protein which is proteolytically cleaved to form a mature and active 17-kDa (157 amino acid) soluble TNF-α (sTNF-α) molecule (Black et al., 1997, Parameswaran and Patial, 2010).

When stimulated to be released in response to trauma, infection, or when exposed to bacterial-derived LPS (Feldmann et al., 1994), TNF-α acts via binding to two transmembrane receptors: TNF receptor1 (TNFR1), (also referred to as p55 or p60), constitutively expressed in the majority of mammalian tissues; and TNF receptor 2 (TNFR2), (also referred to as p75 or p80), a more highly regulated receptor typically only found in the cells of the immune system (Banner et al., 1993). The literature on the effects of sTNF-α binding to TNFR2 is limited, with the majority of TNF-α inflammatory effects being attributed to TNFR1 receptor binding (Parameswaran and Patial, 2010).

The binding of TNF-α to the extracellular domain of TNFR1, causes the receptor to undergo a change in conformation resulting in the release of the inhibitory protein, silencer of death domains (SODD), which ultimately allows for the adapter protein TNF receptor-associated death domain (TRADD) to bind to the death domain of the receptor (Hsu et al., 1995). The TRADD binding induces the following three pathways:- Activation of NF-κB where free NFκB subunits bound to IκBα translocates to the nucleus and induces gene transcription of proteins such as anti-apoptotic factors, proteins involved in cell survival and proliferation, as well as the induction of proteins and cytokines involved in the inflammatory response (Vallabhapurapu and Karin, 2009). Activation of the MAPK pathways ends in an activated c-Jun N-terminal kinases (JNK) being translocated to the nucleus whereby genes involved in cell proliferation, differentiation and pro-apoptotic in nature are transcribed (Micheau and Tschopp, 2003, Rousseau et al., 2008). Finally although a minor role, the binding causes the induction of death signalling, whereby TRADD binds to Fas-associated protein with death domain (FADD) which in turn recruits procaspase 8 which is then cleaved leading to apoptosis (Gaur and Aggarwal, 2003).

Further functions of TNF-α also include being a potent chemoattractant molecule for neutrophils, causing neutrophil migration to areas of injury/infection (Smart and Casale, 1994) as well as promoting the expression of adhesion molecules, such as Intercellular Adhesion Molecule 1 (ICAM-1) and Vascular Cell Adhesion Protein 1 (VCAM-1) on endothelial cells (Mattila et al., 1992). Additionally, TNF-α activates macrophages and enhances phagocytic processes (Parameswaran and Patial, 2010). Importantly for this study, TNF-α is also a potent inducer of the liver to secrete and produce acute phase proteins (Thorn et al., 2004).

In T2DM, circulating TNF-α levels have been seen to be dysregulated with the Swaroop et al. (2012) study showing significantly elevated TNF-α levels in the disease. As previously discussed, this cytokine is associated with the aetiology of T2DM as it disrupts insulin signalling as well as influencing glucose metabolism (Aguirre et al., 2000, Zou and Shao, 2008).

MCP-1:

Monocyte Chemoattractant Protein 1 (MCP-1), also known as chemokine (C-C motif) ligand 2 (CCL2), is a small protein 76 amino acids in length and 13 kDa in size located on chromosome 17 (Deshmane et al., 2009). It is primarily secreted by adipose tissue, macrophages, monocytes, Kupffer cells and dendritic cells generally in response to the presence of oxidative stress, circulating cytokines, or growth factors (Beall et al., 1996).

MCP-1s main function is to act as a chemoattractant thus regulating the recruitment, migration as well as the infiltration of monocytes, natural killer (NK) cells, and memory T lymphocytes to the site of inflammation (Panee, 2012).

In literature, MCP-1 is strongly associated with obesity as the circulating plasma levels of MCP-1 are significantly elevated in obesity, with the degree of obesity directly correlating with the degree of cytokine elevation (Catalan et al., 2007, Harman-Boehm et al., 2007, Cox et al., 2011). The elevated MCP-1 levels induce macrophage infiltration into the adipocytes ultimately leading in crown structure formations and mass pro-inflammatory cytokine release. This is possibly one of the key role players in the chronic low-grade inflammation associated with obesity (Kanda et al., 2006).

MCP-1 is also strongly linked with T2DM, with significantly elevated MCP-1 levels being observed in various T2DM studies (Simeoni et al., 2004, Blaha et al., 2006, Kanda et al., 2006). Literature suggests that MCP-1 contributes to T2DM via various pathways including contributing to the "Inflammation Theory", previously discussed; through macrophage infiltration and crown structure cytokine signalling (Chacon et al., 2007); the induction of amylin secretion which causes increased circulating amylin levels (which disguises the actual physiological circulating glucose levels) leading to improper insulin secretion and hence insulin resistance (Cai et al., 2011); MCP-1 causes ERK1/2 activation in skeletal muscle resulting in impaired insulin signalling as well as reducing the glucose uptake by myocytes through reduced GLUT-4 translocation via the NF-κB pathway.

MIP-1β:

Macrophage inflammatory protein-1 (MIP-1), occurs in two different isoforms, MIP-1α and MIP-1β which are also commonly referred to in the literature as CC Motif Chemokine Ligand 3 (CCL3) and CCL4, respectively, and are located on chromosome 17 (Wolpe et al., 1988). Both MIP-1 isoforms are chemokines and act as strong chemotactic cytokines and are secreted by various cell types such as macrophages, dendritic cells, and lymphocytes (Maurer and von Stebut, 2004).

MIP-1β when secreted, functions to activate human granulocytes leading to acute neutrophilic inflammation, as well as inducing the synthesis and release of various pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 from macrophages (Maurer and von Stebut, 2004). MIP-1β also acts to maintain tissue homeostasis.

Research with regards to MIP-1β in T2DM is limited but it is thought that the chemotactic cytokine may indirectly facilitate β-cell death through the recruitment of macrophages (Benoist and Mathis, 1997).

sP-Selectin:

P-Selectin, otherwise referred to as CD62P, is a member of the selectin family of cell adhesion molecules, is 50 kb in size and contains 17 exons in humans and is mapped on chromosome 1 (Nicaud et al., 1998). P-selectin is found in platelets and endothelial cells where the adhesion molecule is stored in α-granules and Weibel-Palade bodies respectively (Woltmann et al., 2000). The P-Selectin is then translocated to the cell membrane of endothelial cells and platelets in response to the presence of pro-inflammatory cytokines, such as IL-4, as well as the presence of thrombin (Wasiluk, 2004, Kamath et al., 2002).

This externalised P-Selectin then plays a pivotal role in haemostasis as it mediates the adhesion of activated platelets to neutrophils and ultimately activating the innate immune response (Ebeid et al., 2014). Furthermore, the P-Selectin induces platelet-to-platelet binding and aggregation further ensuring haemostasis in the presence of inflammation.

Additionally, P-Selectin can be secreted into circulation, with this now being termed soluble P-Selectin (sP-selectin), as a part of platelet-derived microparticles or as free spliced versions of the protein. This sP-Selectin plays a key role in immune system-mediated inflammation as it promotes leukocyte migration, the adherence of leukocytes to activated platelets and endothelium as well as the production and release of key cytokines and growth factors at the site of injury (Ebeid et al., 2014).

sP-Selectin has been shown to be significantly elevated in T2DM (Gokulakrishnan et al., 2006, Woollard et al., 2014); however the role of sP-Selectin the pathogenesis of T2DM isn't yet fully elucidated.

ICAM-1:

Intercellular Adhesion Molecule 1 (ICAM-1), which is also referred to as Cluster of Differentiation 54(CD54), is a protein encoded by the ICAM1 gene which encodes for a glycoprotein located on the cell surface of endothelial cells as well as cells of the immune system, such as leukocytes (Katz et al., 1985, Hubbard and Rothlein, 2000). These cells continuously present with ICAM-1 at low levels on their cell membranes, however, when stimulated via TNF-α (Fingar et al., 1997, Javaid et al., 2003), IL-1β, Lipopolysaccharide (LPS) (Myers et al., 1992) and the presence of reactive oxygen species (ROS) (Chiu et al., 1997), ICAM-1 concentrations greatly increase.

The increased presence of ICAM-1 on the cell membranes of endothelial cells, as well as lymphocytes and monocytes, causes increased binding to leukocytes as ICAM-1 acts as a ligand LFA-1, an integrin, found on the cell membrane of these leukocytes (Rothlein et al., 1986). This cell to cell binding of endothelial cells and leukocytes then causes the leukocytes to transmigrate into tissues of inflammation or damage (Yang et al., 2005). In addition to the cell to cell binding, ICAM-1 has been observed to have signal-transducing functions which is associated with pro-inflammatory pathways, further shifting the cytokine profile towards a proinflammatory state (Etienne-Manneville et al., 1999).

ICAM-1 levels have been found to be significantly elevated in T2DM (Karimi et al., 2018) however the impact ICAM-1 has on the disease isn't fully elucidated. Elevated ICAM-1 may be as a result of elevated TNF-α and IL-1β found in T2DM, however the increased ICAM-1 may influence T2DM pathogenesis via the binding of monocytes/leukocytes to activated vascular endothelium preceding macrophage and foam cell development (Price and Loscalzo, 1999), This is a crucial event potentially resulting in T2DM.

VCAM-1:

Vascular Cell Adhesion Molecule 1 (VCAM-1), otherwise known as cluster of differentiation 106 (CD106), is a 90-kDa glycoprotein encoded by the VCAM1 gene (Osborn et al., 1989). VCAM-1 expression, like ICAM-1, is activated and expressed on the cell membrane of endothelial cells (Rice and Bevilacqua, 1989) in the presence of various pro-inflammatory cytokines such as TNF-α as well as the presence of ROS (Cook-Mills et al., 2011). Additionally, under conditions of extensive inflammation, VCAM-1 can also be expressed

on the surface of various other cells types, which includes macrophages, myoblasts, dendritic cells, oocytes, bone marrow fibroblasts as well as Kupffer cells (Sharma et al., 2017).

When stimulated by pro-inflammatory cytokines, various ligands bind to the externalised VCAM-1 causing leukocyte to endothelial cell binding which starts a cascade effect which concludes in VCAM-1–dependent leukocyte trans-endothelial migration (Wittchen, 2009).

In T2DM, literature shows that VCAM-1 is significantly elevated in T2DM (Liu et al., 2015, Braatvedt et al., 2001) and may be one of the leading causes of the abnormal endothelial function and activation observed in T2DM (De Vriese et al., 2000, Devaraj and Jialal, 2000).

2.4 T2DM and the Acute Phase Response

The acute phase response (APR) is a further process chronically activated in T2DM (Festa et al., 2002). The APR is a prominent systemic reaction that occurs in humans in response to any local or systemic disturbances in the body's homeostasis, which can be caused by tissue injury, infection, trauma, or in the case of T2DM, chronic low-grade systemic inflammation (Gruys et al., 2005).

Circulating TNF-α and IL-1β, mainly stimulate the resident Kupffer cells in the liver to begin producing and secreting IL-6, which acts as the main mediator for mass hepatocytic secretion of acute phase proteins (APPs), proteins of the APR (Jain et al., 2011).

APPs are a class of proteins whose circulating levels are significantly altered in response to inflammation. The circulating protein levels can be increased, with these proteins being referred to as positive APP, or decreased, with these proteins being referred to as negative APP (Jain et al., 2011). These proteins have a wide variety of functions, however the end goal of the APR and the APP is to re-establish homeostasis while promoting system healing (Cray et al., 2009). Table 2.1 below gives a list of all the positive and negative APPs.

Table 2.1. Table displaying the acute phase proteins.

Acute Phase Proteins	
Positive	Negative
C-reactive protein	Albumin
Serum amyloid P component	Transferrin
Serum amyloid A	Transthyretin
Complement factors	Retinol-binding protein
Mannan-binding lectin	Antithrombin
Fibrinogen, prothrombin, factor VIII, von Willebrand factor	Transcortin
Plasminogen activator inhibitor-1	
Alpha 2-macroglobulin	
Ferritin	
Hepcidin	
Ceruloplasmin	
Haptoglobin	
Orosomucoid	
Alpha-1-acid glycoprotein	
Alpha 1-antitrypsin	
Alpha 1-antichymotrypsin	

Of these APP, fibrinogen, prothrombin, factor VIII, von Willebrand factor and Plasminogen Activator Inhibitor-1, are important as they are all proteins involved in coagulation and fibrinolysis (Heinrich et al., 1995). Furthermore, the Horadagoda et al. (1999) study claims that the two most important APPs are C-reactive protein (CRP) and Serum Amyloid A (SAA).

CRP:

C-Reactive Protein is a protein that is 224 amino acids in length with a molecular mass of 25,106 Da and its gene mapped onto chromosome 1 (Thompson et al., 1999). CRP is a member of the pentraxin family which are pattern recognition proteins that play an integral role in the innate immune system (Bray et al., 2016). When secreted from the liver, in response to IL-6, CRP forms an annular pentameric protein shape which then circulates throughout the body in blood plasma (Pepys and Hirschfield, 2003).

CRP was first discovered by Tillett and Francis (1930) whose study initially hypothesised that CRP was a pathogenic secretion as it was significantly elevated in various illnesses, including cancer. This notion was disproved however when CRP was discovered to be

hepatically synthesised. CRP was later named after the molecules' capacity to precipitate the somatic C-polysaccharide of *Streptococcus pneumoniae* (Pepys and Hirschfield, 2003) with the molecule becoming the first acute-phase protein to be described as a highly sensitive systemic marker for inflammation and tissue damage (Pepys and Baltz, 1983).

When secreted, CRP acts by binding to the phosphocholine found on the surface of necrotic and apoptotic cells as well as on some bacteria (Gershov et al., 2000, Xia and Samols, 1997). The binding then allows for the activation of the complement system, thus enhancing and promoting phagocytosis by macrophages via opsonin-mediated phagocytosis (Bray et al., 2016).

CRP levels have great clinical significance as the APP has been commonly used as a diagnostic marker for disease and systemic inflammation (Allin and Nordestgaard, 2011, Ligtenberg et al., 1991, Pradhan et al., 2016, Vadakayil et al., 2015). CRP levels in healthy individuals vary, with concentrations ranging between 0.8 mg/L to 3.0 mg/L, and sometimes circulating at up to 10 mg/L (Volanakis, 2001). Importantly, these circulating levels can increase up to 1000 times during disease or inflammation (Pradhan et al., 2016), which can then be clinically measured using methods such as ELISA kits, immunoturbidimetry, rapid immunodiffusion and nephelometry. The sensitivity of CRP in response to trauma and inflammation makes this APP an effective and accurate marker for determining disease progress as well as the effectiveness of treatments.

Wang et al. (2013), among various others, showed that in T2DM, CRP levels are significantly elevated and can act as a strong biomarker for T2DM disease progression and management.

SAA:

Serum Amyloid A (SAA) is a generic term for a highly conserved family of acute-phase apolipoproteins synthesised by the liver (Eklund et al., 2012, Ye and Sun, 2015). The human SAA gene codes for a 122 amino acid polypeptide, which contains an 18 amino acid N-terminal signal sequence. Humans have four SAA genes; saa1 and saa2 which encode for acute-phase isoforms of SAA, saa3 which is an apparent pseudogene, and finally saa4 which encodes for a constitutively expressed isoform (Frame and Gursky, 2016, de Beer et al., 1995).

The SAA molecules can be divided into two groups, firstly, the acute phase SAAs that associate with HDL during inflammation and secondly, the constitutive SAAs, mouse SAA5 and human SAA4 (de Beer et al., 1995). Human apo-SAA is a 104 amino acid polypeptide

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that circulates in plasma bound to high-density lipoprotein-3 (HDL3) (Cabana et al., 1999). However, SAA has an important effect on HDL structure and function during inflammation, as the majority of SAA is an apolipoprotein of high-density lipoprotein HDL (Kisilevsky and Manley, 2012, Hua et al., 2009). During acute inflammation, SAA secreted from the liver displaces apolipoprotein A-I bound to HDL, with each HDL particle being able to bind and carry several copies of SAA (Jayaraman et al., 2015), thus becoming the major apolipoprotein of circulating HDL3 (Eklund et al., 2012). Acute-phase SAA also modifies the biological effects of HDL-C in several conditions (Zewinger et al., 2015). For the current study, we will focus on the acute-phase SAA as our molecule of choice.

Circulating SAA has various functions including influencing cholesterol and lipid metabolism (Faty et al., 2012), induction of mast cell adhesion to the extracellular matrix (Nicholson-Weller et al., 1985), recruitment and adhesion of T cells (Xu et al., 1995), inducing the migration, tissue infiltration and adhesion of monocytes and polymorphonuclear leukocytes (Badolato, 1994) as well as the induction of various pro-inflammatory cytokines (Eklund et al., 2012).

Importantly, depending on the extent of inflammation, it has been seen that SAA may increase up to 1000-fold, compared to those in the non-inflammatory state (Eklund et al., 2012). Consequently, SAA is a well-established (and potent) biomarker for infection and sepsis (Malle and De Beer, 1996, Bozinovski et al., 2008, Cicarelli et al., 2008). Furthermore, increased SAA is also an important plasma biomarker for predicting future cardiovascular events and is associated with an increase in thrombotic risk (Delanghe et al., 2002, Johnson et al., 2004) as it is an active participant in the early atherogenic process (Getz et al., 2016). Furthermore, SAA's presence is associated with the pathogenesis of chronic inflammatory diseases, such as T2DM as well as atherosclerosis (Eklund et al., 2012, Thompson et al., 2015). The table below displays relative circulating SAA levels in various diseases.

Table 2.2: Literature review of the observed concentration of SAA during various diseases.

Serum SAA concentrations (µg·mL⁻¹)	References
The median of SAA concentration in patients with non-neoplastic lesions: 6.02 µg·mL⁻¹	(Ren et al., 2014)
The median of SAA concentration in patients with cervical intraepithelial neoplasia: 10.98 µg·mL⁻¹	
The median of SAA concentration in patients with cervical carcinoma: 23.7 µg·mL⁻¹	
SAA concentration in acute coronary syndrome associated with cardiovascular risk factors: 57.1 (50.0–64.3) mg·L⁻¹	(Zewinger et al., 2015)
SAA concentration in diabetes associated with cardiovascular risk factors 36.1 (28.7–43.4) mg·L⁻¹	
SAA4 levels in controls: 55 ± 13 mg·mL⁻¹	(Malle and De Beer, 1996)
SAA levels during pneumonia: 10 to 1700 mg·mL⁻¹	
SAA4 levels during pneumonia: 6 to 150 mg·mL⁻¹	
80 µg·mL⁻¹ SAA showed regulation of apoptotic targets and a dose-dependent reduction in cell viability, with 69% cell viability observed following exposure to 80 µg/mL of SAA for 24 hours to cells in culture.	(Tan et al., 2014)

2.5 SAA and the circulating inflammagen LPS

Bacterial lipopolysaccharides (LPS), also referred to as lipoglycans or endotoxins, are essential components of the outer membrane of gram-negative bacteria and is associated with low-grade chronic inflammation (Guo et al., 2013). LPS's role is to provide structural integrity to the bacteria as well as providing the cell membrane protection from various types of chemical attack (Zhang et al., 2013). This LPS however is shed in to the haematological system via dormant bacteria (Hurley, 1995, Pretorius et al., 2018a) or is constantly entering the blood system, possibly as a result of the individual suffering from leaky gut or gut dysbiosis (Sylvia and Demas, 2018, Saltzman et al., 2018).

When LPS is found in the haematological system, it acts as a classical endotoxin, thus binding to the CD14/TLR4/MD2 receptor complexes found on various cell types including; macrophages, monocytes, dendritic cells and B cells, inducing the promotion and secretion

of pro-inflammatory cytokines and nitric oxide (Frost et al., 2002, Qureshi et al., 2012). Furthermore, LPS induces the production of superoxide, making it a major source of reactive oxygen species (Hsu and Wen, 2002, Park et al., 2015).

Crucial for this study is that a link exists between LPS and SAA with the Migita et al. (2004) study showing that LPS signalling induces hepatocytes to secrete SAA. Furthermore, the Fukushima et al. (2000) study showed that SAA is an essential molecule in LPS induced inflammation. Importantly, both SAA (Eklund et al., 2012, Marzi et al., 2013) and LPS (Creely et al., 2007, Khondkaryan et al., 2018) are upregulated in T2DM.

2.6 T2DM, Inflammation and the Coagulation System

Coagulation is the physiological process which regulates haemostasis, the arrest of bleeding, and is the initial stage of wound healing (Palta et al., 2014). The haemostasis process can be subdivided into two phases, namely primary and secondary haemostasis.

Primary haemostasis occurs due to complex interactions between platelets, damaged vessel walls as well as various local adhesive proteins which ultimately leads to the formation of a 'platelet plug' at the site of damage (Lasne et al., 2006). Platelets are "egg" shaped, anucleate cellular fragments derived from megakaryocytes (Palta et al., 2014) whose membrane consists of various integrins, glycoproteins, phospholipids and various receptors (Ibrahim and Kleiman, 2017). The various receptors, including fibrinogen, vitronectin, collagen, fibronectin and laminin receptors in circulating platelets are kept at a "resting" low-affinity state, however, these receptor types are transformed into high affinity "activated" receptors when the platelets are activated when bound and adhered to the damaged endothelial collagen in a von Willebrand Factor (vWF) mediated binding process (Heemskerk et al., 2002, Li et al., 2010). This conversion from low affinity to high conversion receptor types is called "inside-out signalling" (Li et al., 2010) and mediates platelet adhesion and aggregation at the site of endothelial damage as well as initiating thrombus formation (Coller and Shattil, 2008). For this aggregation and adhesion to occur, the newly activated platelets begin to undergo conformational changes whereby the platelets form cytoplasmic foot-like extensions referred to as pseudopodia, causing the membrane to flatten in an attempt to cover an increased surface area at the site of damage (Pretorius et al., 2018c). The binding of external fibrinogen to the fibrinogen integrin receptors on the activated platelets then initiates a series of intracellular events, referred to as "outside-in signalling", which results in platelets undergoing a degranulation process, whereby cytokines and proteins such as P-selectin, fibronectin, fibrinogen, factor VIII, factor V, platelet factor IV, platelet-derived growth factor, calcium and thromboxane A2

(TxA2) are secreted into circulation (Heemskerk et al., 2002, Li et al., 2010). The TxA2 secreted then acts as a stimulus inducing further circulating platelets to bind and enlarge the platelet aggregation leading to the formation of the platelet plug, which temporarily seals the damaged site (Palta et al., 2014).

The secondary phase of haemostasis consists of the coagulation cascade which culminates in the formation of insoluble, crosslinked fibrin meshes at that site of injury/damage (Gale, 2011). Although named the secondary phase, fibrin formation has been seen to occur simultaneously to the process of platelet aggregation (Falati et al., 2002). As seen in figure 2.1 below, the coagulation cascade can be activated through two pathways; the intrinsic and extrinsic pathway.

The intrinsic/contact activation pathway is initiated when there is damage to blood vessels and endothelial collagen is exposed which causes the activation of clotting factor XII, otherwise referred to as Hageman Factor (Renne et al., 2012). This activation begins a cascade effect resulting in the calcium-dependent activation of clotting factor X (Smith et al., 2015). The activation of factor X is where the intrinsic and extrinsic pathways merge with the extrinsic/tissue factor pathway being initiated through tissue damage which initiates the secretion of tissue thromboplastin (Scarpati et al., 1987). This thromboplastin, known as clotting factor III, then activates factor X in a process mediated by calcium.

Activated factor X then converts circulating prothrombin, secreted by the liver, into thrombin which is an essential molecule in the conversion of fibrinogen into insoluble thin "spaghetti like" fibrin fibres (Palta et al., 2014, Pretorius et al., 2017c). Thrombin also crucially activates factor XIII, a molecule which functions to covalently crosslink fibrin polymers vital in the formation of a stable secondary haemostatic plug (Ariens et al., 2002). Additionally, thrombin induces the production of the molecule thrombin activatable fibrinolysis inhibitor (TAFI) which protects the newly formed fibrin clot from undergoing fibrinolysis (Bombeli and Spahn, 2004).

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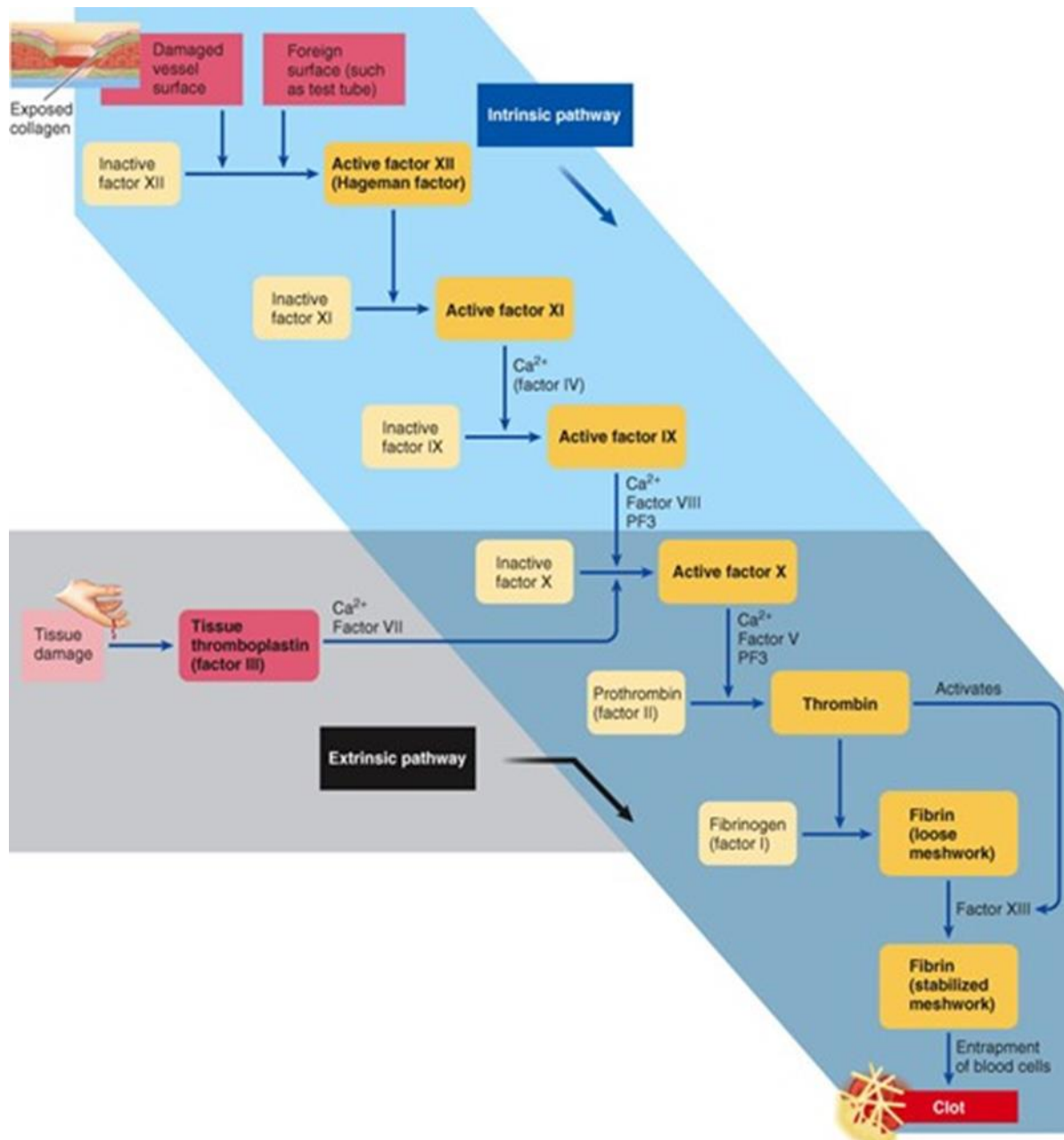
Figure 2.1. Summary of the coagulation cascade occurring during secondary haemostasis adopted from Sherwood (2013).

Literature shows that inflammation and the clotting cascade are two systems that are heavily linked to one another with excessive cross talk occurring between the two systems (Esmon, 2005, Foley and Conway, 2016, Levi and van der Poll, 2010). Kell and Pretorius (2015) went on to show that there are two key hallmark features in the blood of systemic inflammation; hypercoagulation, the increased propensity for clot formation and hypofibrinolysis, the increased resistance of the formed clots to undergo lysis.

Table 2.3. Table displaying selected biomarkers and how they influence the haematological system and cause hypercoagulation and hypofibrinolysis. Adapted from Randeria et al. (2019) (unpublished).

Name of Biomarker	Role on the Haematological System and Coagulation
IL-1β	- Induction in the synthesis of clotting factor VII within monocytes (Carlsen et al., 1988) - Enhancing expression of tissue factor and activity from endothelial cells and monocytes (Herbert et al., 1992) - Induction of IL-6 signalling pathways producing pro-coagulant fibrinogen synthesis (Yang et al., 2013) - Induction of platelet hyperactivation (Bester and Pretorius, 2016) - Downregulation of thrombomodulin thus reducing anticoagulation capacity (Bester and Pretorius, 2016)
IL-6	- Promotion of fibrinogen gene expression through stat3 phosphorylation (Duan et al., 2010) - Induction of platelet hyperactivation (Bester and Pretorius, 2016) - Upregulating circulating tissue factor levels (Bester and Pretorius, 2016)
IL-8	- Induction of platelet hyperactivation and spreading (Bester and Pretorius, 2016) - Induction of eryptosis in erythrocytes (Bester and Pretorius, 2016) - Induction of aberrant fibrin formation (Bester et al., 2018) - Reduction is fibrin lysis rate (Bester et al., 2018)
TNF-α	- Induction of platelet clumping and activation and the development of spontaneous plasma protein dense matted deposits (Page et al., 2018) - Induction of plasminogen activator inhibitor (PAI-1) thus reducing fibrinolytic capacity (Pandey et al., 2005) - Downregulates thrombomodulin thus increasing levels of tissue factor (Scarpati and Sadler, 1989)
MCP-1	- Induction of tissue factor expression in monocytes (Ernofsson and Siegbahn, 1996)
sP-Selectin	- Induces the expression of tissue factor by monocytes (Celi et al., 1994) - Induces platelet to platelet binding and platelet aggregation (Pretorius et al., 2018c) - Facilitates leukocytes recruitment thus promoting leukocyte-mediated fibrin deposition (Palabrica et al., 1992)

In addition to the biomarkers/inflammatory cytokines inducing haematological and hypercoagulation effects, the APP's induced in inflammation also influence these systems. Positive APPs includes fibrinogen, factor VIII, von Willebrand factor, PAI-1 and thrombopoietin all of which induce a pro-coagulant state leading to hypercoagulation (Patalakh and Kudinov, 2008, Spencer et al., 2007). Furthermore, the negative APPs include antithrombin and thrombomodulin which further contributes to the pro-coagulant state found in inflammation.

With an increased circulating fibrinogen level complemented with increased clotting factor availability, all diseases associated with chronic low-grade inflammation should be accompanied by a state of hypercoagulation. This notion has widely studied in literature with the hypercoagulable and hypofibrinolysis state being observed in Alzheimer's disease (Gupta and Pansari, 2003, Gupta et al., 2005), cancer (Francis et al., 1994, Rickles and Falanga, 2001, Rickles and Levine, 2001, Viale, 2005), cardiovascular disease (Borissoff et al., 2011, Tantry et al., 2010), sepsis (Hesselvik et al., 1989, Bernard et al., 2001), Parkinson's disease (Pretorius et al., 2014), Human Immunodeficiency Virus (Shen and Frenkel, 2004) and T2DM (Alzahrani and Ajjan, 2010, Beijers et al., 2012, Carr, 2001, Pretorius et al., 2015, Pretorius et al., 2017b, Pretorius et al., 2018c, Thor et al., 2002). Furthermore, literature has begun to recognise that inflammatory diseases are associated with, and may be exacerbated and even caused by, amyloid fibril formation (Chiti and Dobson, 2006, Herczenik and Gebbink, 2008, Rambaran and Serpell, 2008). These amyloid fibrils are defined as: "a protein that is deposited as insoluble fibrils, mainly in the extracellular spaces of organs and tissues as a result of sequential changes in protein folding that result in a condition known as amyloidosis" (Sipe et al., 2014).

The interest in this notion, with respect to haematology, was peaked when Pretorius et al. (2016a) proved that the insoluble fibrin(ogen) formed during thrombosis could be amyloid in nature in the presence substoichiometric levels of bacterial lipopolysaccharide. Fibrin is normally produced via the thrombin-mediated polymerisation of the circulating protein fibrinogen, whereby two fibrinopeptides are removed, which allows the fibrinogen to self-assemble via a 'knobs and holes' mechanism into thin spaghetti-like fibres (Weisel, 2005, Wolberg, 2007). The fibrin in the Pretorius et al. (2016a) study however was misfolded and amyloidogenic, with the fibrin's normally alpha-helical dominate structures being changed into fibrin with regions rich in β-sheets. Pretorius et al. (2017b) went on further to show, using novel amyloid-selective fluorescent stains, that the fibrin clots formed in T2DM were also amyloidogenic in nature. This finding potentially implicated a bacterial component in the development of T2DM as the T2DM fibrin exhibited similar structural alterations to that of the fibrin incubated with LPS.

More recently, Page et al. (2019) implicated SAA in the amyloidogenic fibrin(ogen). The study was based on the premise that elevated plasma concentrations of SAA would result in aggregation as amyloid in β-sheet fibrillar deposits and that SAA and fibrinogen could be co-deposited in the amyloid fibrils. The study displayed that the incubation of *ex vivo* SAA in platelet-poor plasma induced a significant amyloid signal, using amyloid-selective fluorescent stains, of fibrin clots thus proving that SAA does, in fact, induce β-sheet rich fibrin fibrils. This study demonstrations that LPS may not be the only molecule causing the aberrant amyloidogenic fibrin clots that occur in T2DM; however, the amyloidogenic potential of SAA in the context of T2DM needs further investigating.

2.8 Mopping agents

When it was determined that fibrin(ogen) exposed to LPS could in fact be amyloid in nature, Pretorius et al. (2017a) showed that Lipopolysaccharide binding protein (LBP) has the potential to significantly reduce, but not completely remove, the amyloid signal found in T2DM. Furthermore, Pretorius et al. (2018b) went on to show that *ex vivo* LBP incubation into PPP from Parkinson's disease patients also showed a significant reduction in fibrin clot amyloid signal. These two studies demonstrate that the addition of "mopping agents" can remove and reduce the majority of the LPS derived amyloid signal. Despite this, residual amyloid signal always remained thus hinting towards the requirement of multiple mopping agents to completely abolish amyloid structure formation.

Since Page et al. (2019) discover of SAA amyloidogenic effects on fibrin(ogen) clots, one could hypothesize that an effective SAA mopping agent in combination with LBP will potentially remove amyloid signal completely. An effective mopping agent would be a molecule that effectively binds to SAA and thus completely sequesters its effects on fibrinogen. One potential SAA mopping agent is high-density lipoprotein (HDL), a molecule SAA naturally binds with, causing the displacement of Apolipoprotein A-I (de Beer et al., 2010). Furthermore, a single HDL molecule can bind multiple SAA molecules (Frame and Gursky, 2016), thus potentially making it an extremely effective mopping agent. Additionally, the use of SAA anti-bodies may be a further potential avenue for the binding and mopping of SAA's effects as these antibodies would bind with extreme specificity thus targeting and inhibiting SAA's effects.

In theory, the use of SAA mopping agents is a viable option to inhibit the molecules' amyloidogenic effects on fibrin(ogen), however further in-depth experimental research is required to determine the viability of this notion.

2.9 Concluding Remarks

In this literature review, T2DM was shown to be associated with chronic low-grade inflammation, an altered inflammatory profile and a chronically activated APR system. This altered profile was shown to influence the haematological system as it influences not only blood components but the coagulation cascade causing a hypercoagulable state. This inflammatory state and the presence of SAA is associated with aberrant fibrin clot formation whereby the fibrin becomes amyloidogenic in nature with a change in protein structure from alpha-helical dominated fibres becoming beta-rich sheet dominant in structure. The use of LBP as a mopping agent was shown to reverse this state with SAA mopping agents potentially being able to facilitate LBP in completely abolishing amyloid signal in fibrin. The figure below displays a general overview of the literature review.
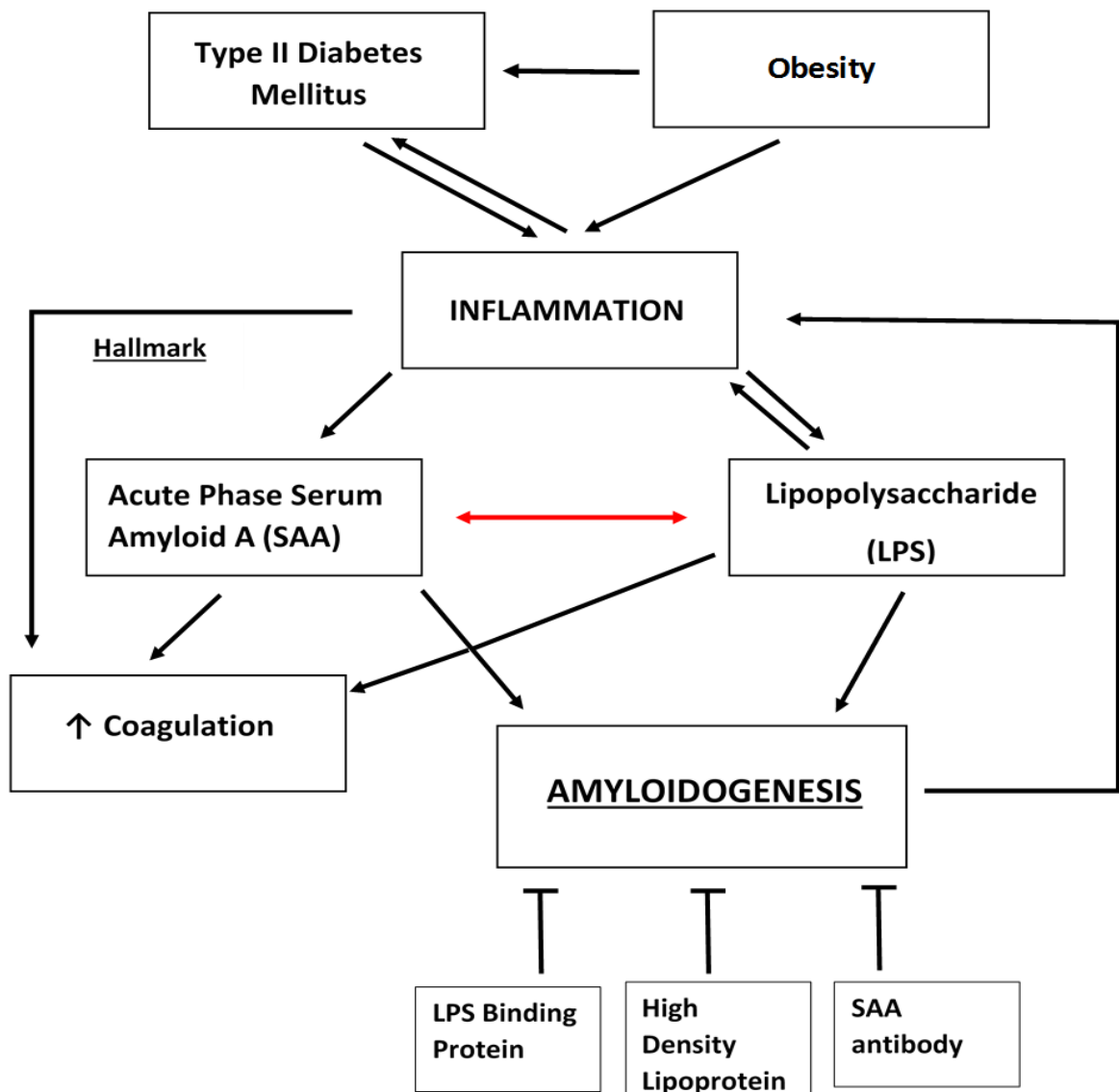
Figure 2.2. Summary diagram of topics covered in the above literature review.

3. Study Design

3.1 Ethical clearance and considerations

Ethical clearance for the study was obtained from the Health Research Ethics Committee I (HREC I) from University of Stellenbosch (South Africa) (HREC Project Reference #6399 S18/02/036: G Thomson) (Appendix A). Prior to any involvement in the study both the "seemingly healthy" control (without T2DM), as well as clinically diagnosed T2DM participants, received an in-depth verbal as well as written explanations of the study before participants signed a compulsory consent form (Appendix B) (available on request) acknowledging their participation within the study. All participant information, both T2DM and controls, was kept strictly confidential with the principle investigator being the only person able to access the data. Importantly all investigators involved in the study were certified in Good Clinical Practice. Strict ethical codes of conduct were followed during blood sample collection, in addition to all methodologies being carried out in line with the relevant guidelines of the ethics committee (HREC Reference # S18/02/036: G Thomson). Lastly the Declaration of Helsinki was strictly adhered to by all investigators.

3.2 Study Layout

A cross-sectional study design was performed in collaboration with Dr Laubscher from the Mediclinic in Stellenbosch who provided the whole blood (WB) from T2DM study participants. WB samples from healthy control participants were collected at the Department of Physiological Sciences at the University of Stellenbosch by the qualified on-site phlebotomist after a consensual recruitment process.

3.3 Study Population

The current study was performed at Department of Physiological Sciences at the University of Stellenbosch in the Clinical Hemorheology and Coagulation research group. The study involved the recruitment of n=75 (n=36 "seemingly healthy" control participants, n=39 T2DM participants) male and females over the age of 30 (Table below). Individuals were classified as "seemingly healthy" controls if they were observably healthy and not presenting any symptoms of disease, not on chronic medication, having no prior history of thrombotic disease or inflammatory conditions as well as being non-smokers. Furthermore, females were excluded upon the use of contraception in addition to being pregnant and/or lactating at the time of sample collection. Participants unable to provide written consent were further excluded from the study. Finally, post sample collection

exclusion occurred, such as samples CC28 and CW19 from table 3.2 below, if the "seemingly healthy" control individuals' displayed Pathcare inflammatory profile tests values above the recommended/healthy baseline values.

Inclusion criteria for T2DM patients included both male and female participants that had been diagnosed with T2DM at least 3 months prior to the study. The Diabetic individuals were diagnosed according to the Society for Endocrinology, Metabolism and Diabetes of South Africa (SEMSDA) guidelines which follow the American Diabetes Association (ADA) criteria to define T2DM. The Diabetics individuals also did not present with any signs of infection at the time of sample collection. Congruent with healthy controls, smokers and pregnant and/or lactating women were excluded at the time of sample collection.

To limit and exclude any possible confounding factors, study participants from both the control and diabetic groups were only included if they did not have HIV, tuberculosis or any malignancies.

Table 3.1. Table displaying the demographics of the study participants.

	Healthy Individuals (n =36)	Diabetic Individuals (n=39)	P-values
Gender	Male (n=16) Female (n=20)	Male (n=20) Female (n=19)	
Age (years)	58.11 ± 1.86	64.44 ± 2.00	
HbA1c (%)	5.25 ± 0.09	8.96 ± 0.45	<0.0001
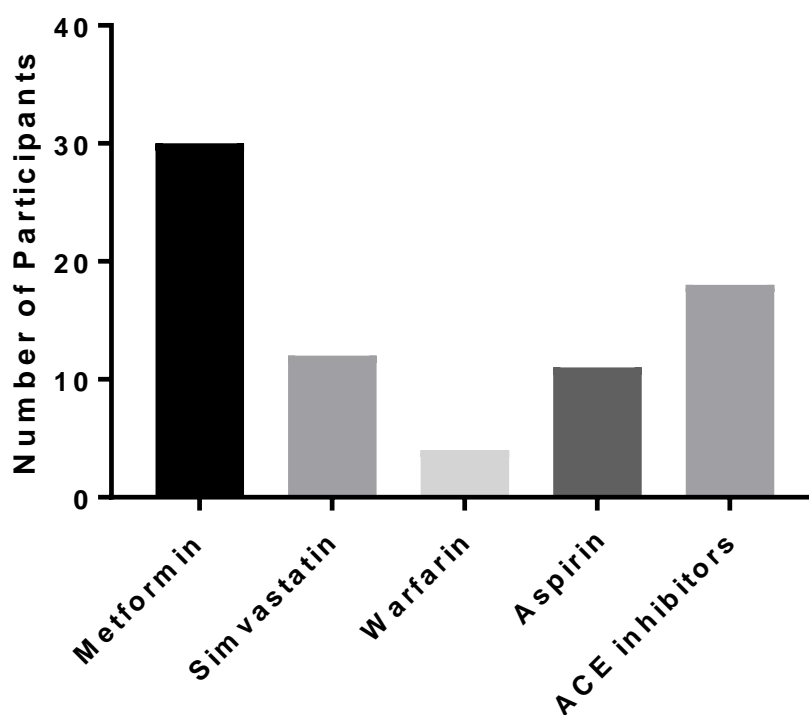
Figure 3.1. Bar graph representing the most commonly prescribed chronic medication used by the T2DM study participants at the time of involvement of the study.

Table 3.2. Table displaying the Pathcare analysis of control participants of the study, with HbA1c having reference range of between 3,9-6,1%, Total Cholesterol (S-cholesterol) < 5,0 mmol/L , Total HDL (S-HDL) >1,2 mmol/L, Total LDL (S-LDL) <3,0 mmol/L, Total Triglycerides (S-Triglycerides) < 1,70 mmol/L, Total Non-HDL cholesterol (S- Non-HDL) <3,8 mmol/L, Cholesterol:HDL ratio <4,0 and finally ultrasensitive CRP levels <1mg/L

Sample	Age	Gender	HbA1c (%)	S-Cholestrol	S-HDL Cholesterol	S-LDL Cholestrol	S-Triglyceride	S-Non-HDL Cholestrol	CHOL:HDL ratio	CRP ultrasentitive
CC1	57	F	5.7	6.3	1.3	3.7	5.53	5	4.8	2.93
CC2	56	F	4.8	6.2	1.6	3.9	1.93	4.6	3.9	3.89
CC4	50	F	4.9	7.6	1.6	5.1	1.7	6	4.8	1.68
CC10	48	F	5	5.1	1.6	3.1	1.03	3.5	3.2	0.35
CC11	58	F	4.8	6.6	2.2	3.9	1.74	4.4	3	2.37
CC12	49	F	4.4	4.7	1.1	3	1.74	3.6	4.3	4.84
CC13	34	F	4.1	5.5	1.3	3.6	0.79	4.2	4.2	1.6
CC18	53	F	4.6	4.8	2.9	2.9	0.65	3.3	3.2	0.4
CC19	38	F	4.5	5.3	1.5	3.2	0.86	3.8	3.5	0.31
CC21	55	F	4.4	4.2	1.5	2.2	0.88	2.7	2.8	2.87
CC22	41	F	4.9	4.9	1.8	2.7	0.79	3.1	2.7	0.2
CC25	43	M		4	1	2.5	1.37	3	4	
CC28	33	F	5	5.2	1.6	3.1	1.15	3.6	3.3	24.82
Cw4	51	M	5.5	6.6	1.4	4.8	0.86	5.2	4.7	0.87
CW5	49	M	5.4	7	1.6	4.7	1.95	5.4	4.4	0.63
Cw6	68	M	5.2	6	1.4	3.9	2.11	4.6	4.3	0.93
CW9	35	M	5	5.1	1.2	3.2	2.13	3.9	4.3	0.63
Cw12	50	M	5.6	4.1	0.9	2.8	2.1	3.2	4.6	3.59
CW14	55	M	4.9	4.7	0.9	3.1	2.8	3.8	5.2	2.03
CW31	50	M	5.4	5.6	1	3.8	0.95	4.6	5.6	1.07
Cw20	51	M	5.4	4.7	1	2	3.84	3.7	4.7	0.94
Cw33	77	M	5.4	3.8	1.3	1.9	1.6	2.5	2.9	2.47
Cw34	81	M	5.3	5.8	1	3.9	1.49	4.8	5	1.59
Cw207	60	M	5.6	5.7	1.9	3.2	0.79	3.8	3	0.43
Cw211	76	M	6.4	3.3	0.9	1.7	3.3	2.4	3.7	2.49
Cw208	77	M	5.1	5.6	1.5	3.6	1.49	4.1	3.7	1.02
CW3	54	M								
CW19	72	F	5.3	5.7	1.4	3.5	1.27	4.3	4.1	14.65
Cw22	61	F	5.2	5.5	2.5	2.5	0.93	3	2.2	2.53
Cw15	51	F	5.3	6	2.6	3.1	0.52	3.4	2.3	1.89
Cw28	64	F	5.1	5.1	1.2	3.5	1.36	3.9	4.3	0.76
Cw32	72	F	6.5	6.5	1.7	4.2	1.27	4.8	3.8	1.33
Cw27	56	F	5.9	5.8	1.2	4.1	1.75	4.6	4.8	1.09
Cw18	69	F	5.6	5	1.8	2.7	1.89	3.2	2.8	1.93
CW26	79	F	6	4.7	1.8	2.2	1.05	2.9	2.6	3.61
CW30	49	F	5.3	6.1	1.8	3.7	1.12	4.3	3.4	0.42

3.4 Blood Sample Collection

Blood samples were obtained from fasted participants, for accurate HbA1c and cholesterol analyses, via venepuncture of the median cubital vein with whole blood samples being collected in one 4.0 mL ethylenediaminetetra-acetic acid (EDTA) tube (BD Vacutainer®, 367861), two 4.5 mL sodium citrate (3.2%) tubes (BD Vacutainer®, 369714) and one 8.5 mL serum separating tube (SST) (BD Vacutainer®, 367958).

3.5 Blood Sample Preparation

Samples collected in the SST and EDTA tubes of the healthy control participants were sent to Pathcare Laboratory, Stellenbosch for the analyses (Table below) of blood parameters such as ultra-sensitive C-Reactive Protein (us-CRP), glycosylated haemoglobin (HbA1C) and cholesterol levels. Whole blood samples collected in the two Citrate tubes were inverted approximately 10 times directly after blood sample collection before being left for a minimum of 30 minutes at room (±22 °C) temperature to allow the sodium citrate to remove all available calcium from the whole blood, inhibiting thrombosis. The whole blood was then used to make whole blood smears for electron microscopy as well as being used in the thromboelastography (TEG) methodology. Residual whole blood in the Citrate tubes were centrifuged at 3000 g for 15 minutes at room temperature (±22 °C) to separate blood components whereby the platelet poor plasma (PPP) was collected using a Pasteur pipette and aliquoted into labelled 2 mL Eppendorf tubes before being stored at -80°C.

3.6 Blood Sample Exposure

In addition to analysis of naïve whole blood, samples were exposed to various molecules and mopping agents. The SAA and HDL used in this study was obtained as a donation from Prof F de Beer, Kentucky University USA.

To determine the appropriate concentration for the test, previous literature was assessed, and it showed that physiological SAA levels vary (refer to table 2.2 in chapter 2). Following on from Page et al. (2019), it was determined that a final concentration of 30 µg.ml^{-1} SAA would be used in the study as it represents a relevant physiological level of the molecule. Previous studies performed by Pretorius et al., 2017 observed a significant reduction in amyloid signal when using 2 ng.L^{-1}, despite this, not all signal was abolished therefore the LPS-binding protein concentration used in this study will be at 5 ng.L^{-1}. After consulting

the literature of relevant physiological levels, a final exposure concentration of 30 µg.ml^{-1} HDL will be used in the study.

Once added, all molecules and mopping agents were given a minimum of 10 minutes to allow for reaction and binding prior to analysis/addition of further added molecules. Importantly, SAA was always incubated in the sample last allowing for the mopping agents to have the greatest chance to bind and mop the SAA effects. The following table describes the exposure details used within the study.

Table 3.3. Table displaying the varying sample exposure schedules used during the study.

CONTROL STUDY	EXPERIMENTAL STUDY
NAIVE CONTROL BLOOD	**Naïve T2DM blood**
+ SAA (30 µg.mL^{-1})	**+ HDL (30 µg.mL^{-1})**
+ HDL (30 µg.mL^{-1})	**+ LBP (5 ngL^{-1})**
+ LBP (5 ngL^{-1})	**+ HDL (30 µg.mL^{-1}) + LBP(5 ng.L^{-1})**
+ HDL (30 µg.mL^{-1}) + SAA (30 µg.mL^{-1})	
+ HDL (30 µg.mL^{-1}) + LBP (5 ng.L^{-1})	
+ LBP (5 ngL^{-1}) + SAA (30 µg.mL^{-1})	
+ HDL (30 µg.mL^{-1}) + LBP (5 ngL^{-1})+ SAA (30 µg.mL^{-1})	

3.7 Statistical Analysis

All statistical analyses were performed using GraphPad/Prism version 7.04 (GraphPad Software, San Diego, USA). Each parameter was analyzed using descriptive statistics for the entire study, whereby normality was assessed using the Shapiro-Wilks normality test. On the premise that data was distributed normally, paired as well as unpaired T-tests were performed to determine between group analysis. When the standard deviations of the groups compared differed, a Welch's correction was implemented. This parametric data is expressed as the mean ± standard error of the mean (SEM). In contrast, non-parametric data was analyzed using the Mann–Whitney test to report differences between appropriate groups. This non-parametric data is expressed as box and whisker plots. Where necessary, outliers were identified and removed using the ROUT method whereby the Q

value was set at 1%. Additionally, mixed model ANOVA was implemented where relevant with a Dunnett's multiple comparisons pos hoc test being used to determine where differences between groups exist. Finally, statistical significance was accepted at $p < 0.05$.

4. Control vs Type II Diabetes Mellitus

4.1 Introduction

In this chapter, blood from individuals presenting with Type II Diabetes Mellitus was compared to that of age matched control samples. The comparison was completed using both quantitative and morphological markers. The quantitative markers include biomarker analyses via the analysis of cytokine profiles using a 20-plex inflammatory cytokine assay as well as a V-plex inflammatory and tissue injury assay. In addition to this, the efficiency of coagulation was analysed using thromboelastography. The morphological markers include the use of scanning electron microscopy for the analysis of whole blood and fibrin clots, as well as the use of confocal microscopy to analyse amyloid signal in fibrin clots. This chapter was aimed at determining if an altered circulating cytokine profile is present in T2DM, whether elevated levels of SAA occurs in the disease, and how this altered cytokine and APP profile may affect the haematological system.

The following paragraphs give brief introductions to each technique as well as the application in which they can be used in this study.

Biomarker analysis:

As the prevalence of non-communicable diseases increase, the need for new methodologies and markers for the early diagnosis, management of disease progression and pharmacological responses to therapeutic intervention are essential in managing this ever-growing socio- and economic burden. Consequently, biomarkers have become a growing field of interest in the science field. Biological markers otherwise referred to as biomarkers was defined by Hulka and Wilcosky (1988) as biochemical, cellular or molecular alterations that are measurable in biological media such as human cells, fluids or tissues. This definition however has extended with time to include any biological characteristics which can be measured and evaluated as an indicator of any normal or abnormal physiological processes which includes pathogenic and therapeutic responses (Naylor, 2003).

Two main categories for biomarkers exist, namely: biomarkers of exposure and biomarkers of disease (Mayeux, 2004, Perera and Weinstein, 2000). Biomarkers of exposure are used when an individual is suspected of suffering from a condition or disease due to exposure to a toxin, these biomarkers when analysed would allow for risk prediction

of the exposure/disease. An example of this would be the analysis of hair or nail samples to determine lead levels in an individual who had previously been exposed to lead which would allow a practitioner to determine the patients risk of lead poisoning.

Of more interest to this study is that of the biomarkers of disease, as these markers allow for disease prognosis, the early screening of diseases as well as the development of diagnostic tests (Selleck et al., 2017, Burke, 2016). The latter two allow for the detection of the disease at a more primitive stage in the disease progression which would generally allow for a more favourable treatment outcome. Biomarkers for various diseases exist in literature already such as the BRCA1/2 gene for breast and ovarian cancer and HbA1c levels in Diabetes Mellitus (Savolainen et al., 2017, Selleck et al., 2017).

Biomarkers can vary greatly as the markers can be genomic markers, whereby DNA analysis occurs with specific respect to single nuclear polymorphisms (SNPs), transcriptomic biomarkers which uses the analysis of RNA profiles, protein markers which analyses protein expression levels and finally metabolic markers analysing metabolites and intermediates of metabolism (Hwang et al., 2018). Due to the varying types of biomarkers, methods to quantify the marker levels vary greatly.

For this study, the biomarkers analysed was that of protein expression. Consequently, a multiplex cytokine analyses were performed using the Invitrogen's Inflammation 20-Plex Human ProcartaPlex™ Panel (catalogue number: EPX200-12185-901) to determine the cytokine profile (TNF- alpha; IL-1 beta; IL-6; IL-8; MCP-1; MIP-1 beta and sP-Selectin) of both the control as well as T2DM participants. Additionally, a Meso Scale Discovery V-Plex Vascular Injury Panel 2 Human Kit (catalogue number: K15198D-1) was performed to determine levels of biomarkers linked with inflammation and tissue damage (CRP, SAA, ICAM-1 and VCAM-1).

Thromboelastography (TEG):

Previous research has shown that a hallmark of systemic inflammation is that of a hypercoagulable and hypofibrinolytic state in the coagulation system (Kell and Pretorius, 2015) which one would be able to determine using Thromboelastography, otherwise known as TEG. TEG is a point of care method commonly used in hospitals for surgeries, anaesthesiology and the assessment of coagulopathies (Bose and Hravnak, 2015, Howley et al., 2018, Pretorius et al., 2017c). TEG assesses the coagulation system and determines overall haematological health by providing information about various clotting parameters,

kinetics as well as providing an indication of the fibrin clot structure and strength (Nielsen et al., 2007a, Nielsen et al., 2007b, Swanepoel et al., 2015, Kell and Pretorius, 2015, Pretorius et al., 2017c). These clotting parameters can then be used as a measure of the degree of pathology of the coagulation status.

Table 4.1.1 Table defining the 7 viscoelastic parameters generated by the TEG. Table adopted from (Pretorius et al., 2017c)

PARMETERS	EXPLANATION
R value: reaction time measured in minutes	Time of latency from start of test to initial fibrin formation (amplitude of 2 mm); i.e. initiation time
K: kinetics measured in minutes	Time taken to achieve a certain level of clot strength (amplitude of 20 mm); i.e. amplification
A (Alpha) Angle: slope between the traces represented by R and K. Angle is measured in degrees	The angle measures the speed at which fibrin build up and cross linking takes place, hence assesses the rate of clot formation; i.e. thrombin burst
MA: Maximal Amplitude measured in mm	Maximum strength/stiffness of clot. Reflects the ultimate strength of the fibrin clot, i.e. overall stability of the clot
Maximum rate of thrombus generation (MRTG) measured in Dyn.cm^{-2}.s^{-1}	The maximum velocity of clot growth observed or maximum rate of thrombus generation using G, where G is the elastic modulus strength of the thrombus in dynes per cm^{-2}
Time to maximum rate of thrombus generation (TMRTG) measured in minutes	The time interval observed before the maximum speed of the clot growth
Total thrombus generation (TTG) measured in Dyn.cm^{-2}	The clot strength: the amount of total resistance (to movement of the cup and pin) generated during clot formation. This is the total area under the velocity curve during clot growth, representing the amount of clot strength generated during clot growth

Table 4.1.2 Table showing how the viscoelastic parameters will change in hypercoagulable and hypocoagulable states. Table adopted from (Pretorius et al., 2017c) .

		HYPERCOAGULABLE		HYPOCOAGULABLE
R **min**	↓	Clot forms faster	↑	Clot forms slower
K **min**	↓	Clot reaches a set (20 mm) strength quicker	↑	Clot reaches a set (20 mm) strength slower
A **Alpha**	↑	An increased thrombin burst resulting in more cross-linking of fibrin fibres	↓	A decreased thrombin burst resulting in less cross-linking of fibrin fibres
MA **mm**	↑	Increased platelet* and/or fibrin(ogen) interaction resulting in a denser clot that is more rigid	↓	Decreased platelet* and/or fibrin(ogen) interaction resulting in a denser clot that is less rigid
MRTG** **Dyn.cm^{-2}.s^{-1}**	↑	Increased clot growth	↓	Decreased clot growth
TMRTG** **min**	↓	Decreased time from clot initiation to maximum clot formation	↑	Increased time from clot initiation to maximum clot formation
TTG** **Dyn.cm^{-2}**	↑	Increased clot strength	↓	Decreased clot strength
*platelet activity only in whole blood not PPP **In whole blood MRTG, TMRTG and TTG focuses on the fibrin in the sample				

The TEG machine uses cup and pin mechanics where citrated WB or PPP is added into the cup before $CaCl_2$ is added to initiate fibrin clot formation. The cup is then rotated at a 4° 45′ arc, six times a minute, at a temperature of 37 °C mimicking the conditions of slow blood flow in the venous circulation. A stationary pin attached to a torsion wire is then inserted into the cup, whereby newly formed fibrin attaches. This binding causes the pin to begin to drag forming mechanical energy, in the form of resistance, on the wire which is then transduced into an electrical current which is analysed by the computer software. The computer then uses various algorithms to produce a split symmetrical reaction curve. The magnitude of this curve is then directly proportional to the strength of the clot formed over time (Bose and Hravnak, 2015). Table 4.1.1 and table 4.1.2 above describes the parameters generated using the TEG in addition to how these parameters will alter when in a hypercoagulable or hypocoagulable state.

Scanning Electron Microscopy:

A dysregulated circulating cytokine profile has previously been shown to cause changes in the haematological system (Esmon, 2005, Choi et al., 2006, Conway, 2012, Kell and Pretorius, 2015, Swanepoel et al., 2015) as these cytokines are constantly interacting with erythrocytes, platelets, circulating fibrin(ogen) as well as other various proteins involved in coagulation. These alterations may be seen indirectly via a hypercoagulable state observed using TEG, however direct imaging of the alterations is an extremely valuable way in determining the exact impact that the dysregulated cytokine profile is having on the haematological system.

The method of Scanning Electron Microscopy (SEM) allows scientist the ability to analyse whole blood smears for red blood cell morphology, platelet morphology and activation status as well as factors such as erythrocyte to erythrocyte interaction, agglutination status and finally platelet to platelet interaction (Pretorius et al., 2018c, Pretorius et al., 2017c). Additionally, SEM allows for the analysis of fibrin fibre morphology when imaging fibrin clots. Using this method, one can directly visualise fibrin fibre diameter as well as the presence of any aberrant fibrin fibres. Additionally, visualisation of proteins interacting with the fibrin clots formed is another power of this technique (Pretorius et al., 2016a).

Confocal Microscopy:

Confocal microscopy is an optical imaging technique which uses fluorescent markers to image samples. Confocal microscopy is a technique that allows for great optical resolution as the microscope captures multiple two-dimensional images at various depths of the sample before images are analysed and processed to form three-dimensional structures (Paddock, 2000). Furthermore, confocal microscopy provides micrographs with excellent contrast as the technique makes use of a spatial pinhole which blocks out any background light (Paddock, 2000, Thorn, 2016).

Due to the increased resolution and contrast, confocal microscopy is a technique widely used throughout science, however in the context of this study, the technique was used to determine amyloid signal found in fibrin clots. Various fluorescent markers and dyes exist but for this study we made use of three; Thioflavin T (ThT), Amytracker™ 480 and Amytracker™ 680.

ThT is a classic amyloid fluorescent marker (Biancalana and Koide, 2010, Biancalana et al., 2009, Naiki et al., 1989) as it has a strong affinity and thus binds to β-sheet rich areas

which are found in high concentrations within amyloid regions. The ThT emits a strong fluorescent signal at 482 nm when excited at 450 nm (Naiki et al., 1989). Additionally, two proven fluorescent amyloidogenic markers (Magnusson et al., 2014), which are luminescent conjugated oligothiophenes (LCOs) dyes, marketed as Amytracker™ 480 and Amytracker™ 680, were used for this study. Previous work completed by the Clinical Hemorheology and Coagulation research group has proven the viability of these two Amytrackers in the context of amyloid formation in fibrin clots (Pretorius et al., 2017b, Pretorius et al., 2018a) as it was shown that the two markers stain for similar but somewhat different areas in comparison to that of ThT.

4.2 Materials and Methods

Material and methods for quantitative markers:

<u>20-Plex Cytokine Analysis:</u>

On the morning of the experiment, stored PPP from the -80°C freezer was thawed on ice while the Invitrogen's Inflammation 20-Plex Human ProcartaPlex™ Panel (catalogue number: EPX200-12185-901) was removed from the 4 °C refrigerator before both were allowed to reach room temperature (±22 °C). PPP samples were then vortexed before 25 µL of each sample was added to 25 µL of internal controls. This sample dilution was then pipetted into each sample's designated well in the 96-well plate, which had been pre-incubated with washed magnetic beads. The plate was then sealed before being placed on a shaking bed for 90 minutes at 500 rpm at room temperature (±22 °C). 25 µL of the 1X detection antibody was then added to each well before again being sealed and incubated on the plate shaker for 30 minutes at room temperature (±22 °C). The detection antibody was then removed, and each well was washed twice using the 1X wash buffer for 30 seconds per wash. 50 µL of the Streptavidin, R-Phycoerythrin Conjugate (SAPE) solution was added per well before the plate was covered and allowed to incubate of the plate shaker for 30 minutes at 500 rpm. Following this, a series of two wash steps occurred before 120 µL of the reading buffer was added to each well. The 96-well plate was covered and placed on the plate shaker to incubate for 5 minutes while being agitated at 500 rpm. The plate was then analysed using a Bio-Plex® 200 (BioRad Luminex) system with all inflammatory markers being presented in pg.mL^{-1}. The protocol was completed and analysed at the Department of Biochemistry at Tygerberg Medical campus.

V-Plex Cytokine Analysis:

Similar to that of the Inflammation 20-Plex Human ProcartaPlex™ Panel, on the day of the experiment, stored control and T2DM PPP were removed from the -80 °C and thawed on ice before being allowed to reach room temperature (±22 °C). While PPP samples thawed, the Meso Scale Discover Vascular Injury Panel 2 (human) Kit (catalogue number: K15198D-1) was removed from the 4 °C refrigerator and allowed to reach room temperature. The 96-well plate was then washed 3 times for 30 seconds using 150 µL per well of the 1X wash buffer provided. 25 µL of diluted (1:1000 in diluent 101) plasma samples, in addition to internal controls, were then pipetted into assigned wells. The plate was sealed and allowed to incubate at room temperature (±22 °C) for 2 hours while on a plate shaker rotating at 500 rpm. The plate was washed again 3X 30 seconds using the wash buffer before 25 µL of a master-mix of the 4 detection antibodies (SULFO-TAG Anti-human SAA Antibody; SULFO-TAG Anti-human CRP Antibody, SULFO-TAG Anti-human VCAM-1 Antibody and SULFO-TAG Anti-human ICAM-1 Antibody) was added to each well. The plate was sealed and covered before being incubated on the plate shaker for an hour at room temperature. The plates were wash a further 3 times using the 1X wash buffer before 150 µL of the 1X read buffer provided was added to each well before the plate was immediately analysed using the MSD Discovery Workbench 4 machine. Data received was measured in pg.mL^{-1} before being multiplied by 1000 to account for the dilution used. The values were then converted and presented in µg.mL^{-1}. The protocol was completed and analysed at the Department of Biochemistry at Tygerberg Medical campus.

Thromboelastography:

TEG analysis was performed on naïve (untreated) control and T2DM whole blood samples drawn in the sodium citrate (3.2%) tubes (BD Vacutainer®, 369714). Briefly 20 mL of 0.2 M calcium chloride ($CaCl_2$) (Haemoneticsâ, 7003) was pipetted into a disposable TEG cup (Haemoneticsâ, HAEM 6211) before 340 mL of the whole blood was then added to the cup. The calcium chloride reverses the effects of the sodium citrate thus initiating fibrin(ogen) clot formation which is then analysed using a Haemoscope **TEG**® 5000 Haemostasis Analyser. Importantly, before each day of use, a *e* calibration test was performed on the TEG machine to ensure the two channels were in equilibrium. Furthermore, internal quality control checks were performed every 50 runs of the TEG to ensure the accuracy of the readings generated.

Materials and Methods for morphological markers:

<u>Scanning Electron Microscopy:</u>

For whole blood analysis: 10 µL of WB from control and T2DM patients was used to prepare scanning electron microscopy smears. The sample was pipetted onto the 10 mm round glass slide before gently being smeared over the surface of the slide, this was left for ± 3 minutes to allow for attachment to the slide. The slides were then transferred into a 24 well plate before being washed with 1X Gibco™ PBS (phosphate-buffered saline), pH 7.4 (ThermoFisher Scientific, 11594516). The smears were fixed with 4% paraformaldehyde in PBS for at least 30 min, before three 1X PBS washes. The samples were then fixed with 1% osmium tetroxide (Sigma-Aldrich, 75632) in double distilled H_2O for an additional 30 min in the fume hood. Following this, the samples were washed three times in 1X PBS before a series of ethanol dehydration steps were performed in which samples were washed in increasing concentrations (30%, 50%, 70%, 90% and 100%) of ethanol for 3 minutes in each concentration with the 100% step being repeated three times to ensure full dehydration. A final dehydration step was then performed in 99.9% hexamethyldisilazane *ReagentPlus*® (Sigma-Aldrich, 379212) for 30 minutes in the fume hood before being left to air dry in a fume hood overnight (± 16 h). The dried samples were then mounted on glass microscope slides which had been covered in double-sided carbon tape. These slides were then carbon coated immediately before scanning electron microscopy ultrastructural analyses of the whole blood could be performed. All imaging was performed on the Zeiss MERLIN™ field emission scanning microscope located in the Central Analytical Facility (CAF) Electron Microbeam Unit, Stellenbosch University. All micrographs were captured using the high resolution InLens capabilities at 1 kV.

For fibrin analysis: 10 µL of Platelet Poor Plasma from control and T2DM participants were pipetted onto the 10 mm round glass slide before 5 µL of Thrombostin Human Thrombin (lot number: 090118T) was added and mixed into the sample. The glass slides were immediately transferred to the 24 well plate before the rest of the protocol was followed as stated above.

Confocal Microscopy:

On the day of imaging, stored PPP of control as well as T2DM samples were removed from the -80 °C freezer before being allowed to thaw and reach room temperature (±22 °C). 95 µL of each sample was pipetted into a labelled 2 mL Eppendorf tube before 2 µL of each Amytracker™ (final exposure concentration of 0.1 µL stock solution into 100 µL PPP of Amytracker™ 480 and 680) and 1 µL of ThT (final concentration 5 µM) was added and allowed to incubate in the dark for at least 30 minutes. Once incubation had finished, 10 µL of the PPP + fluorescent marker mixture was pipetted onto a labelled microscope slide before 5 µL of thrombin (provided by the South African National Blood Service) was added and mixed into the PPP to form an extensive fibrin network. This was left for 30 seconds before a cover slip was placed onto of the newly formed fibrin clot before the samples were imaged immediately using a Zeiss LSM 780 with ELYRA PS1 confocal microscope with a Plan-Apochromat 63x/1.4 Oil DIC objective at the CAF Fluorescent Microscope Unit in Stellenbosch University campus. For this study; Amytracker™ 480 was excited using the 405 nm excitation laser whereby the emission was measured at 478–539 nm; Amytracker™ 680 was excited using the 561 nm excitation laser before the emission was measured at 597–695 nm; and ThT was excited using the 488 nm excitation laser whereas the emission was measured at 508–570 nm. Images were then processed using Image J where images were split into each channel before histograms were created with the mean fluorescent intensity and standard deviation being recorded. The coefficient of variation was then calculated using the following formula: $CV = \frac{Standard\ Deviation}{Mean}$.

4.3 Results

4.3.1 20-Plex Cytokine Analysis
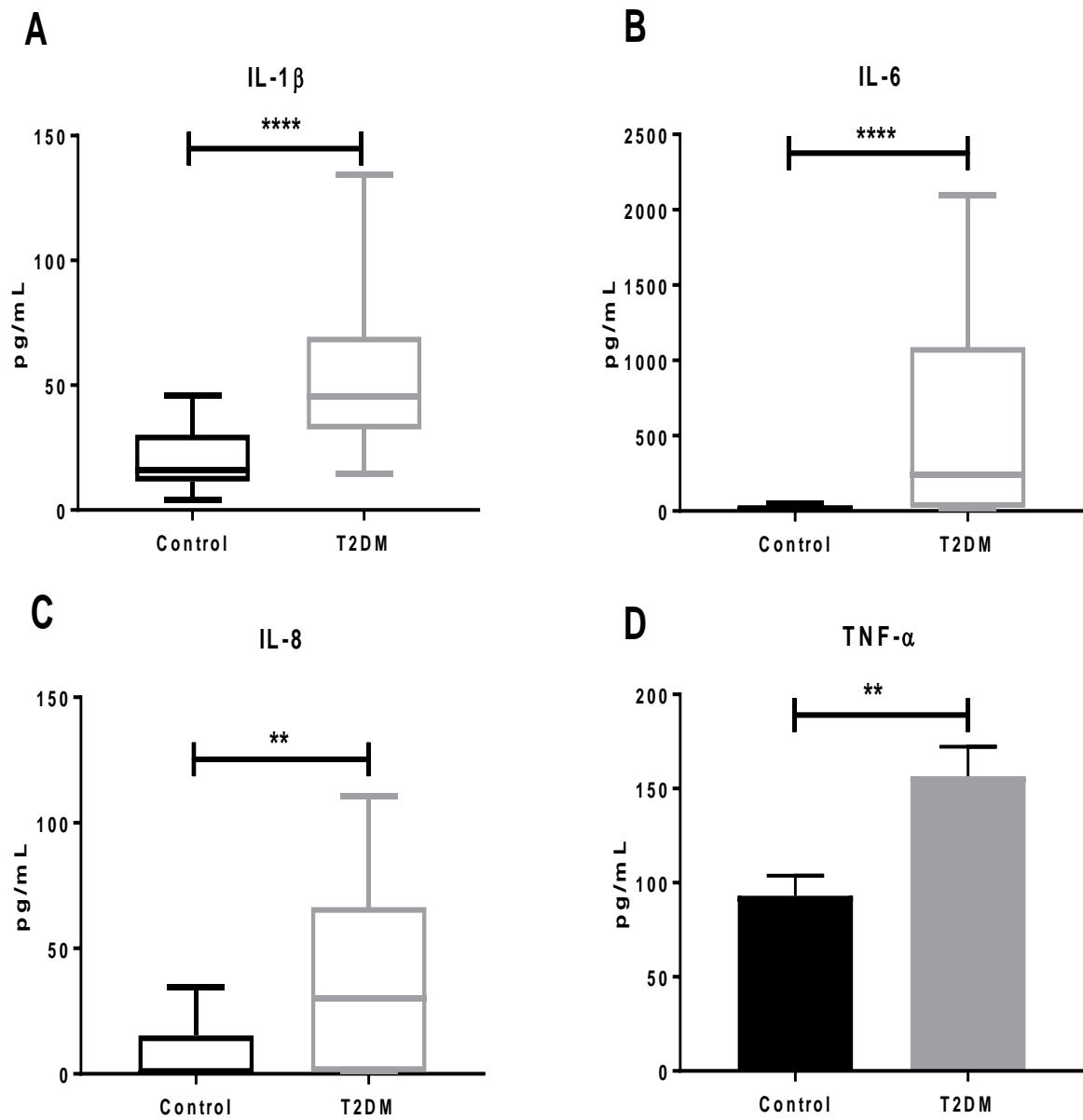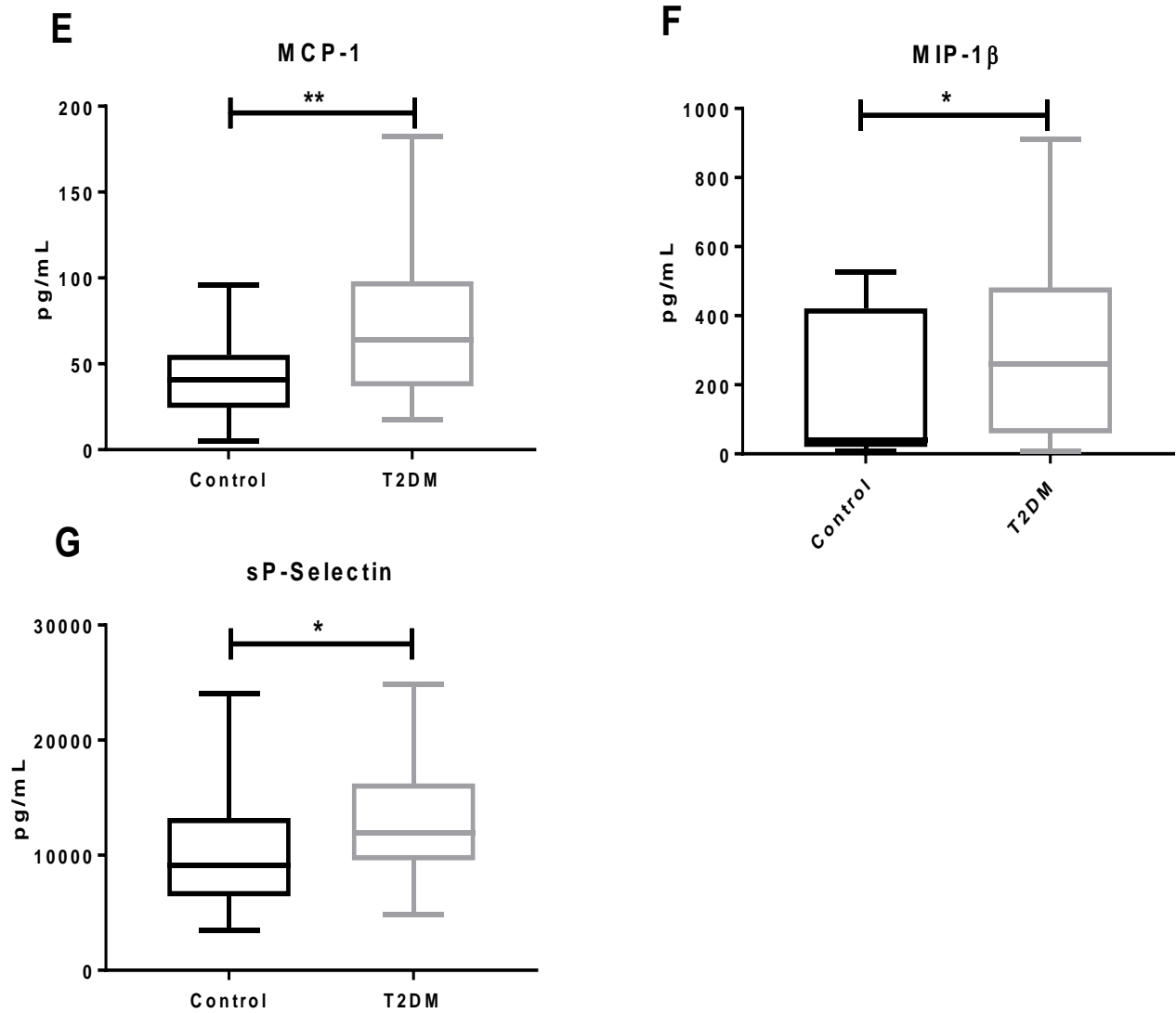
Figure 4.3.1. Graphs of measured circulating inflammatory markers: **A**- Interleukin-1 Beta (IL-1β), **B** -Interleukin-6 (IL-6), **C** - Interleukin-8 (IL-8), **D** - Tumor Necrosis Factor alpha (TNF-α), **E** - Monocyte Chemoattractant Protein-1 (MCP-1), **F** - Macrophage Inflammatory Protein-1β (MIP-1β) and **G** - soluble-P selectin measured using Invitrogen's Inflammation 20-Plex Human ProcartaPlex Pane in 25µL of platelet-poor plasma of control (n=24) and Type II Diabetic Mellitus (n=25) individuals. Parametric data (Graph **D**) is expressed as mean ± SEM where as non-parametric data (Graphs **A**, **B, C, E**, **F** and **G**) is presented as box and whisker plots. Significance is set as $*p < 0.05$; $***p < 0.001$ and $****p < 0.0001$. All data is presented in pg. mL^{-1}. Values in controls that were lower than detectable ranges were allocated 0.001.

Upon analysis of the Inflammation 20-Plex Human ProcartaPlex™ Panel it is evident that the T2DM population used within this study had an altered inflammatory cytokine profile producing a pro-inflammatory state. This was observed with significant differences in all seven of the relevant parameters used in this study. With regards to IL-1β, graph A in figure 4.2.1, the T2DM groups average circulating levels were ±3 times greater than that of the age matched control group ($p < 0.0001$). Additionally, the circulating IL-6 levels, graph B, were significantly different ($p < 0.0001$) between the two groups with the mean value of the T2DM group being around 573 pg.mL^{-1} in comparison to that of the control groups mean value of 10.26 pg.mL^{-1}. Significant differences ($p = 0.0013$) were also observed in circulating IL-8 levels, graph C, with a ± 6 times greater mean circulating cytokine difference between the two groups. Following the trend of the interleukin pro-inflammatory cytokines, TNF-α, graph D, was also significantly elevated ($p = 0.0019$) in T2DM with a near double average circulating protein concentration found in T2DM participants in comparison to the control group.

Furthermore, the chemoattractant cytokines MCP-1 and MIP-1β, graphs E and F, were both found to be significantly altered ($p = 0.0025$ and $p = 0.0137$) in the T2DM population with a ± 1.8 and ± 2.1 times elevated circulating levels respectively compared to the control group. The final biomarker assessed in this assay was soluble P-selectin, graph G, which was also significantly dysregulated ($p = 0.021$) in the diabetic participants with these individuals having on average ± 3000 pg.mL^{-1} more circulating P- selectin molecules in circulation at any given time.

4.3.2 V-Plex Cytokine Analysis

When analysing figure 4.3.2 below, it is evident that the T2DM population used in this study has a dysregulated inflammatory profile. Graph A shows that in Diabetes Mellitus, individuals have a significantly elevated ($p < 0.0001$) circulating CRP level with the median and mean circulating concentrations being ± 4 and ± 12 times greater respectively than that of the age-matched control group. Furthermore, graph B shows a similar significant difference ($p < 0.0001$) between the two groups with regards to circulating Serum Amyloid A levels. Analysis shows that the diabetic group's average circulating concentrations were 10-fold higher than that of the control group. When analysing the adhesion markers, I-CAM-1 and VCAM-1 (graph C and D), both parameters proved to be significantly elevated in the diabetic group ($p = 0.009$ and $p = 0.023$ respectively). When assessing the figure as a whole, a distinct difference in the inflammatory profile between the two populations is

clear to see with all the T2DM individuals having a significant pro-inflammatory cytokine profile.
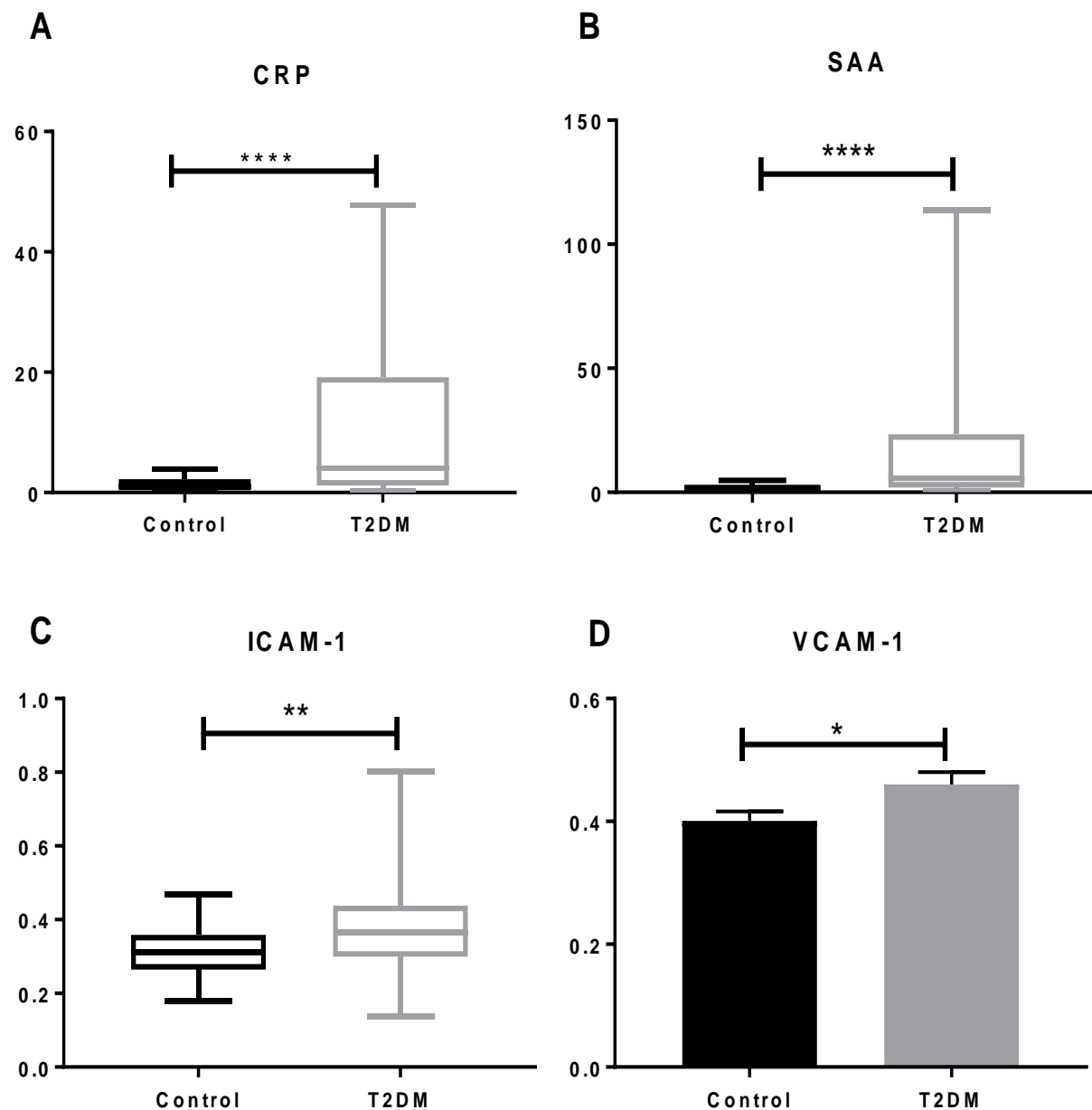
Figure 4.3.2. Graphs of measured circulating inflammatory and tissue damage markers: **A**- C-Reactive Protein (CRP), **B** – Serum Amyloid A (SAA), **C** – soluble Intercellular Adhesion Molecule 1 (ICAM-1) and **D** – soluble Vascular Cell Adhesion protein 1 (VCAM-1) measured using the Meso Scale Discover Vascular Injury Panel 2 (human) Kit using platelet-poor plasma of control (n=36) and Type II Diabetic Mellitus (n=39) individuals. Parametric data (Graph **D**) is expressed as mean ± SEM where as non-parametric data (Graphs **A**, **B** and **C**) is presented as box and whisker plots. Significance is set as *p < 0.05; ***p < 0.001 and ****p < 0.0001. All data is presented in µg.mL^{-1}.

4.3.3 Cytokine Correlations
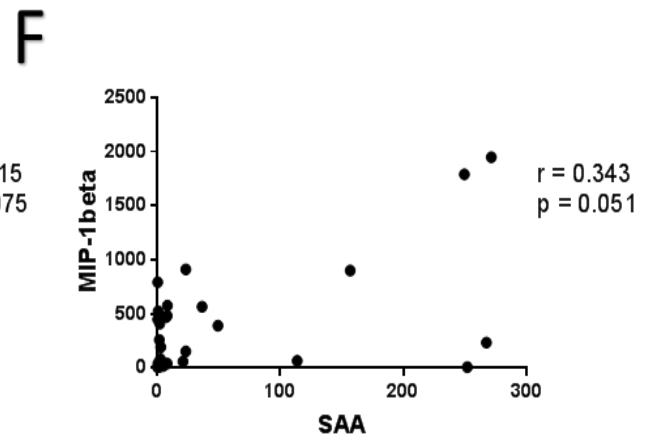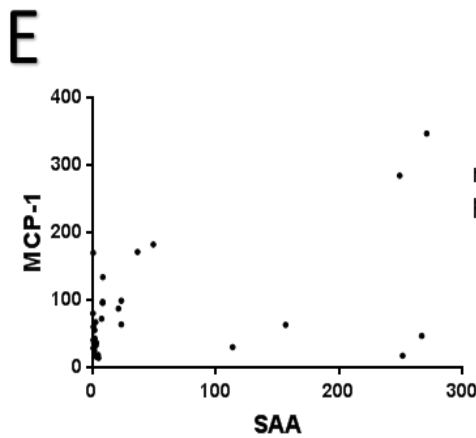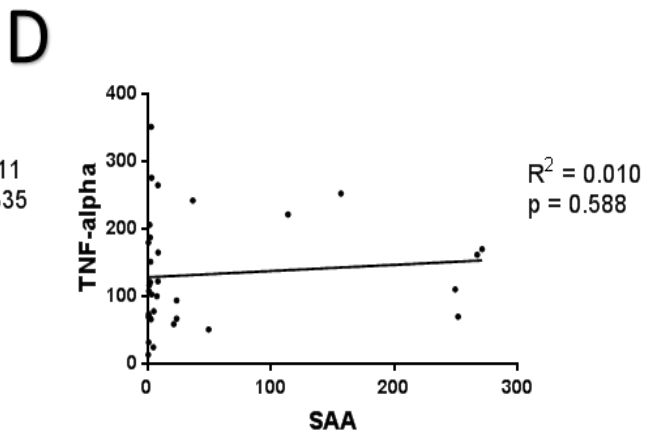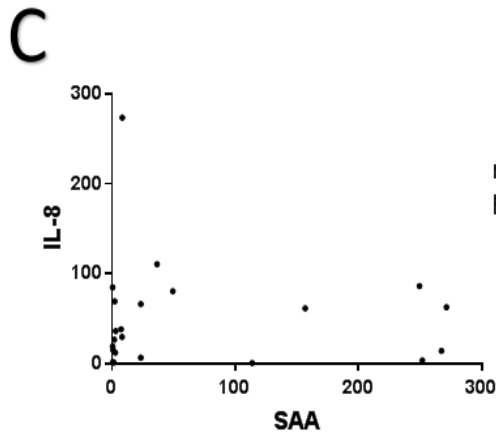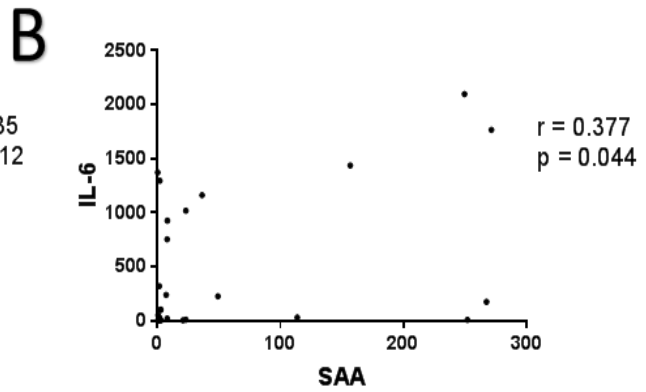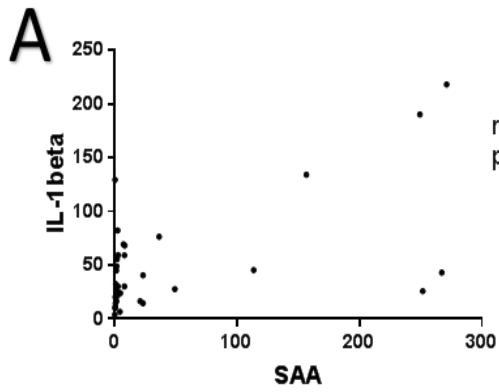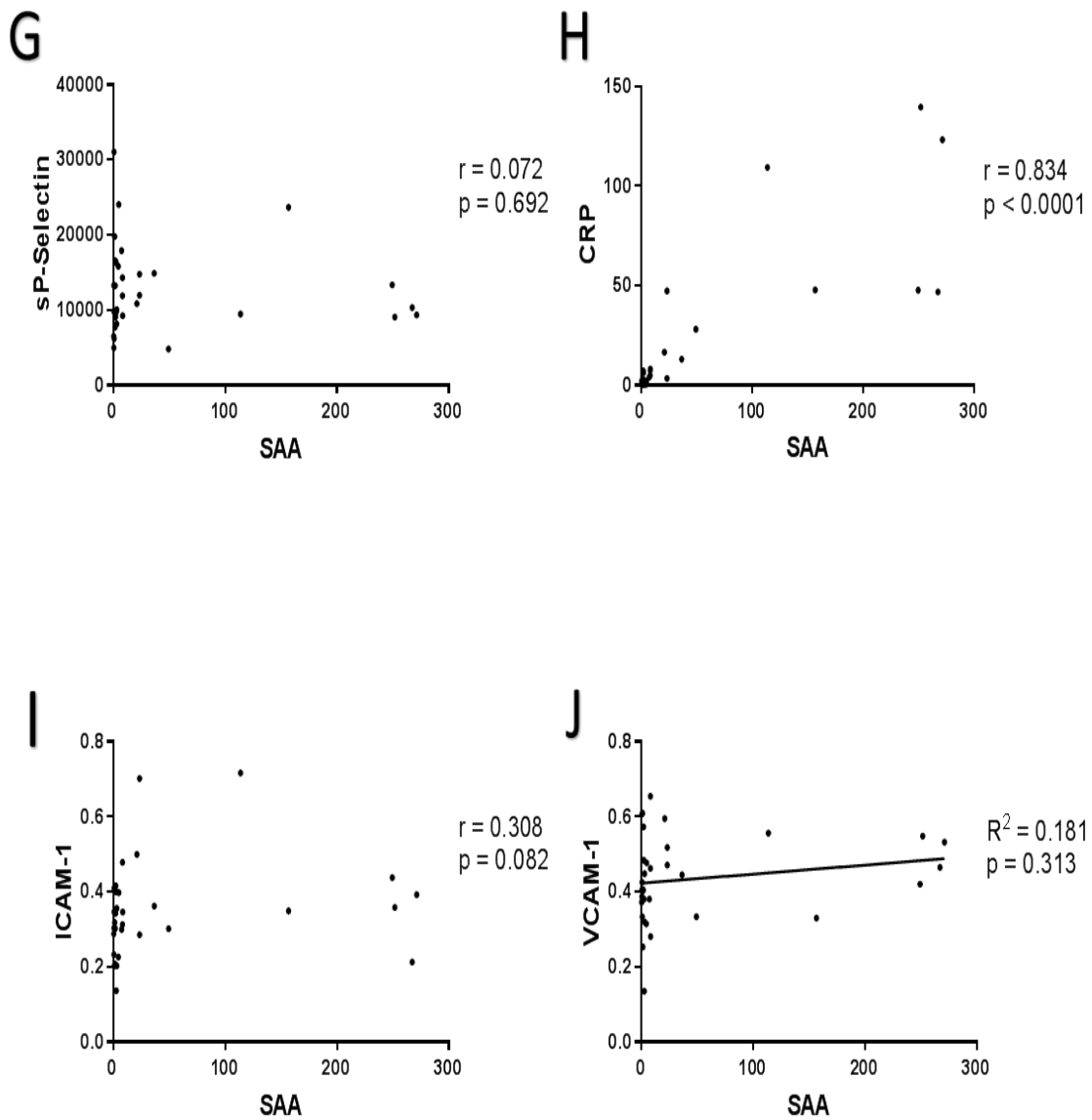
Figure 4.3.3. Correlations between Serum Amyloid A and the other 10 cytokine markers assessed. Parametric data (image D and J) were assessed using the Pearson's correlation test and is presented with a linear regression line. Non-parametric data was assessed using the Spearman's correlation test. The number of data points assessed per correlation ranged from n =23 to n=33.

Assessing the relationship between all ten measured inflammatory cytokines and Serum Amyloid A, one can start by inferring that the data indicates no correlation exists between SAA and IL-8 (r = 0.211; p =0.335), TNF-alpha (R^2 =0.010; p = 0.588), sP-Selectin (r = 0.072; p = 0.692) as well as VCAM-1 (R^2 = 0.181; p = 0.313) depicted in images C, D, G and J respectively.

In contrast however, trends towards moderately significant correlations were noted between SAA and MCP-1 (r = 0.315; p = 0.075), MIP-1β (r = 0.343; p = 0.051) and ICAM-1 (r = 0.308; p = 0.082) images E, F and I respectively. Furthermore, a significant (p = 0.044) moderate association between SAA and IL-6 (r = 0.377) was observed. Finally, a strong and very strong significant correlation was detected between SAA and IL-1β (r = 0.435; p = 0.012) as well as CRP (r = 0.834; p < 0.0001). These results indicate SAA is linked and associated to various inflammatory cytokines which are associated with diverse roles in the human body.

4.3.4 Thromboelastography
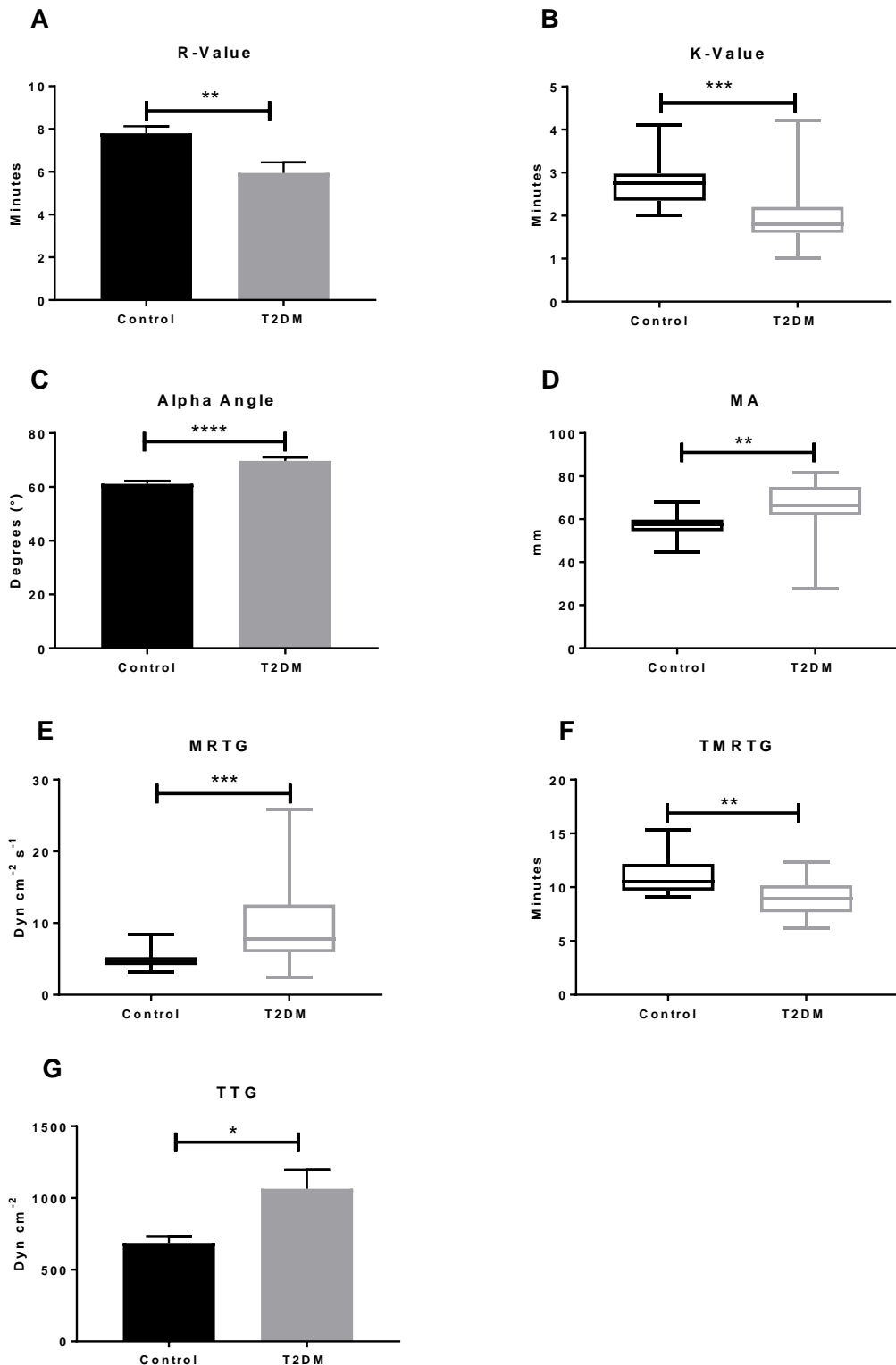
Figure 4.3.4. Bar graphs representing the 7 viscoelastic parameters assessing efficiency of coagulation in whole blood of Control (n= 16) and T2DM (n= 15) participants. Data was generated using the Haemoscope **TEG**® 5000 Haemostasis Analyser. Parametric data (Graph **A**, **C** and **G**) is represented as mean ± SEM and analysed using Welsh's corrected T-test where as non-parametric data (Graph B, D, E and F) is expressed as box and whisker plots and were analysed using a Mann-Whitney test. Significance is set as *p < 0.05; ***p < 0.001 and ****p < 0.0001.

The results displayed in figure 4.3.4 show a significant difference in all seven viscoelastic parameters assessed by the TEG. For the three parameters measured in time, reaction rate (Graph A), clot kinetics (Graph B) and the time to maximum rate of thrombus generation (Graph F), the T2DM participants had significantly reduced ($p = 0.0044$; $p = 0.0002$ and $p = 0.0024$ respectively) time periods. This difference indicates that fibrin clots of T2DM participants are being initiated and are reaching their maximal speed of thrombus generation at a more rapid rate than that of age matched controls. Moreover, the maximum rate of thrombus generation (Graph E) is significantly higher ($p = 0.0003$) in the T2DM population.

When assessing the stability and strength of the thrombus formed, again significant differences exist between the two groups assessed in the study. The alpha angle (Graph C), which represents the speed at which fibrin build up and cross linking takes place is significantly greater ($p < 0.0001$) in the T2DM individuals in comparison to that of control group. Furthermore, the maximal amplitude (Graph D) is elevated ($p = 0.0082$) in the T2DM group indicating that the clot formed was significantly stiffer and more stable. This was further confirmed in graph G whereby the total clot strength was significantly stronger ($p = 0.0140$) in the T2DM individuals.

When comparing the results presented in figure 4.3.4 with traits defined in table 4.1.2 above, the changes displayed in all 7 of the viscoelastic parameters fall in the line with changes that occur in the hypercoagulable state; thus individuals with T2DM have an increased propensity for thrombus generation.

4.3.5 Scanning Electron Microscopy

Qualitative analysis of the representative whole blood smear SEM images depicted in figure 4.3.5.1 below, reveal morphological alterations in the T2DM individuals (represented in C, D, E and F) in comparison to that of age matched control individuals. These images were selected as they are representative images that reflect a trend observed in majority of both control and T2DM samples imaged. In images A the erythrocytes are presenting with their regular biconcave shape with slight contact activation of the platelets being observed. This is confirmed in image B where similarly the erythrocyte is biconcave and the platelets attached are not overly activated. In contrast however, Images C and D show the eryptotic morphology of the erythrocytes in T2DM, additionally image C shows hyperactivation of the platelets whereas image D depicts the agglutination of erythrocytes

that occur in diabetic blood. Focusing on the platelets of T2DM individuals, image E shows platelet activation and spreading which is further shown in image F in conjunction with platelet aggregation and agglutination.
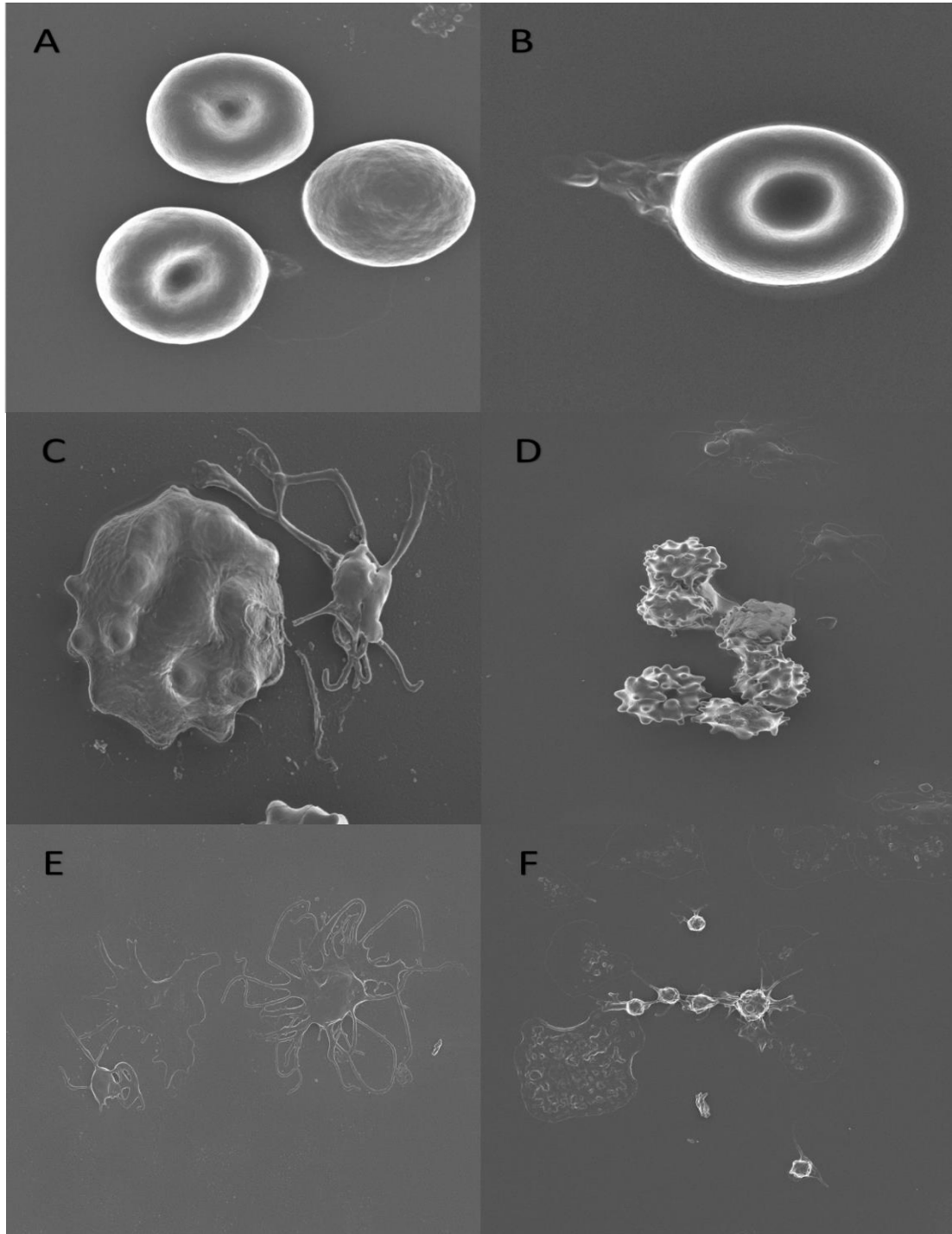
Figure 4.3.5.1. Representative micrographs taken on the Zeiss MERLIN™ field emission scanning microscope of whole blood from Control "**A** and **B**" and Type II Diabetic "**C**, **D**, **E** and **F**" participants. Images were captured at various magnifications using high resolution InLens capabilities at 1 kV.
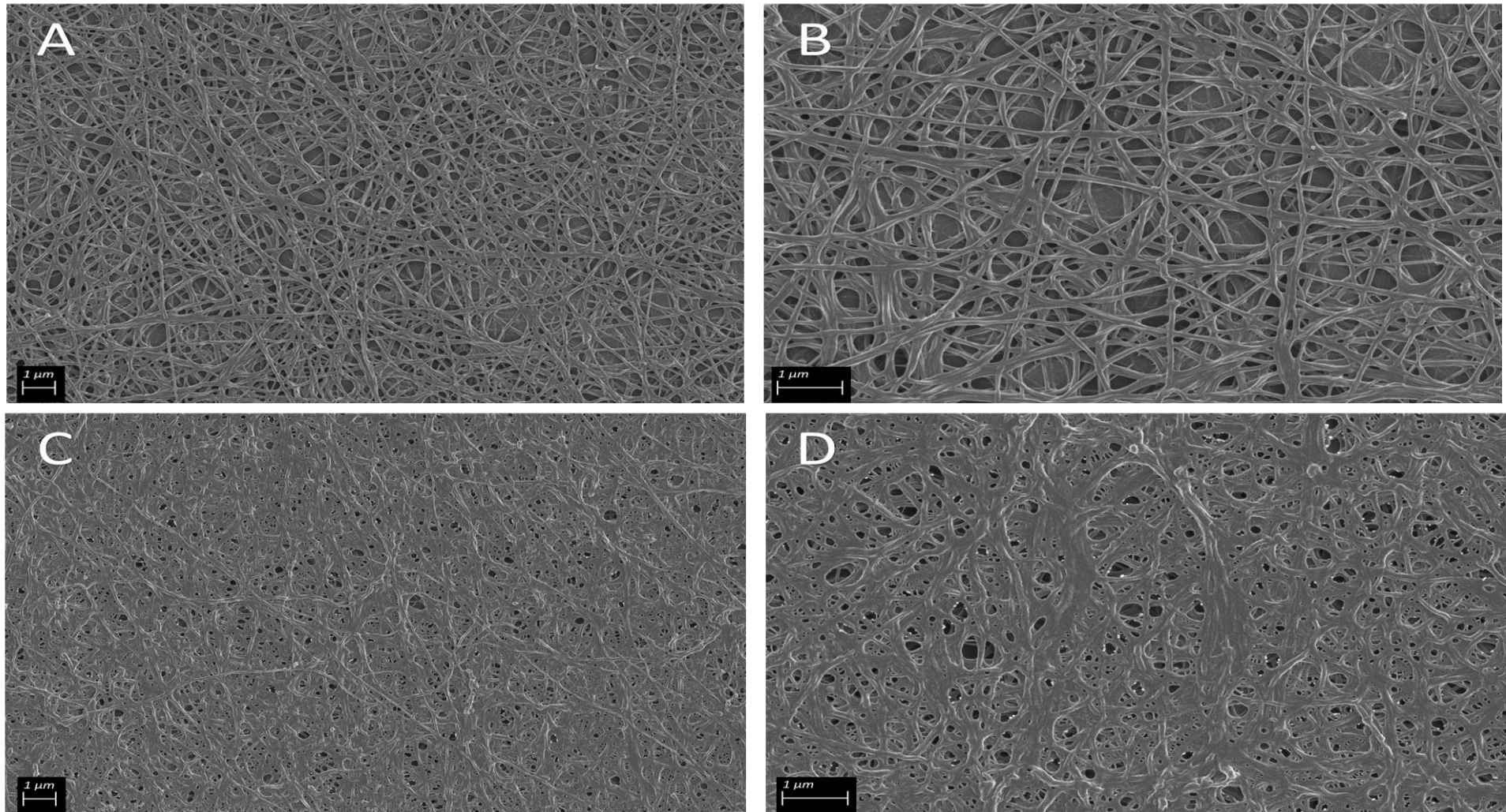
Figure 4.3.5.2. Representative micrographs taken on the Zeiss MERLIN™ field emission scanning microscope of fibrin clots formed from control (image **A** and **B**) and age matched Type II Diabetes Mellitus (image **C** and **D**) platelet poor plasma. Images were captured at 5000X (image A and C) as well as 10000X (image B and D) using the high resolution InLens capabilities at 1 kV.

When qualitatively analysing fibrin clot images, figure 4.3.5.2 above, clear morphological alterations can be seen in the clots of the T2DM individuals (image C and D) when compared to that of age matched control clots (image A and B). The control fibrin clots have thin "spaghetti like" fibrin fibres with large open areas between the various fibrin fibres which can be seen in image A and B. In contrast however, in the clots formed from the T2DM PPP, no singular fibrin fibres can be seen, instead large dense matted amyloid fibrin areas are seen with little to no open areas in the clot. Furthermore, the small white spots seen in image D are small plasma proteins than have been trapped in the thick dense matted amyloid fibrin.

4.3.6 Confocal Microscopy
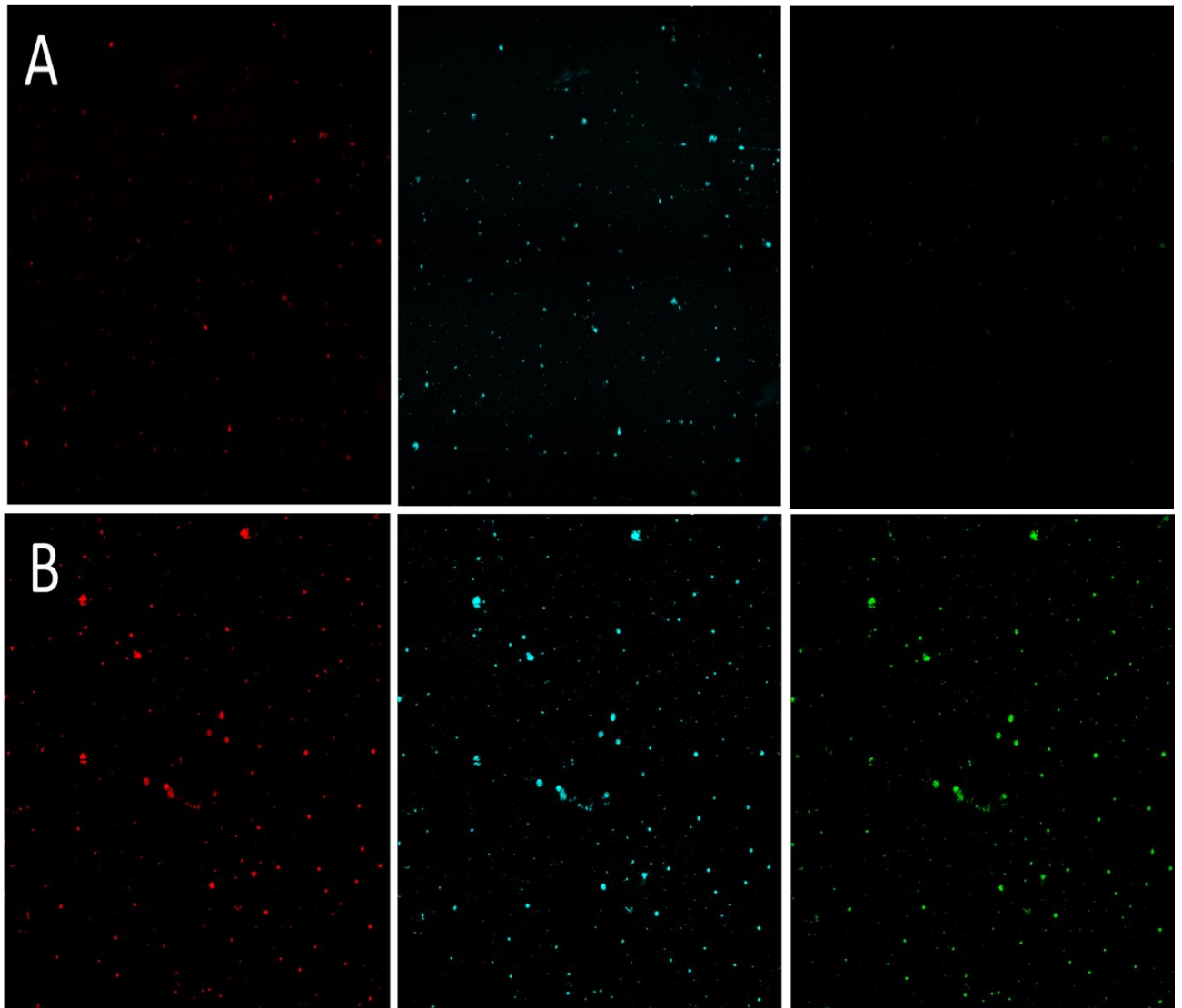
Figure 4.2.6.1. Representative fibrin clots from control PPP (image **A**) and Type II Diabetes Mellitus PPP (image **B**). Images from channel left to right represents Amytracker 680 (red), Amytracker 480 (blue) and Thioflavin T (green) respectively. All images were captured at 63X magnification using a 2X2 tile scan on the Carl Zeiss LSM 780 confocal microscope.
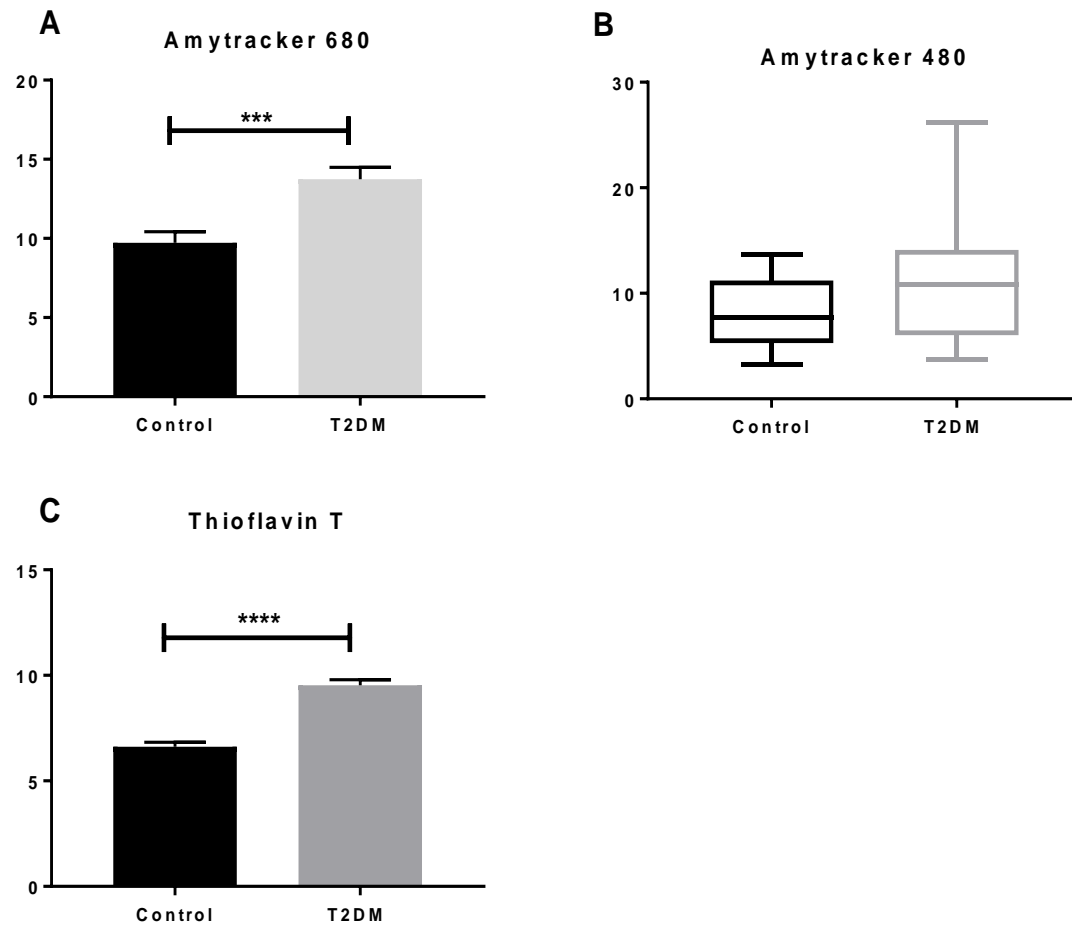
Figure 4.2.6.2. Graphs displaying coefficient of variation (CV) values extrapolated using Image J from fibrin clot confocal micrographs of control PPP (n = 5) and Type II Diabetes Mellitus PPP (n = 10). Parametric data (image **A** and **C**) was analysed using Welch's corrected t test and is represented as the mean ± SEM whereas non-parametric data (image **B**) was analysed using a Mann-Whitney analysis and is represented as box and whisker plots. Significance is set as *p < 0.05; ***p < 0.001 and ****p < 0.0001.

Analysis of figure 4.2.6.2 above shows that a significant increase (p = 0.0002) was observed in the red Amytracker 680 fluorescent channel when comparing the control fibrin clots to that of age matched T2DM fibrin clots. The mean fluorescent intensity was ± 5 arbitrary units (AU) greater in the T2DM fibrin clots. Furthermore, a significant increase (p < 0.0001) in the ThT signal was also observed with a near double in fluorescent signal being seen in the T2DM individuals. Finally, a trend (p = 0.0804) was observed in the Amytracker 480 channel when the control fibrin clots were compared to that of the age matched T2DM fibrin clots.

4.4. Discussion

The initial aim of this chapter was to determine whether individuals presenting with T2DM do in fact have an altered cytokine profile when compared to that of healthy age matched control individuals. A multiplex assay was performed with the results being displayed in Figure 4.3.1 whereby one can see a significant increase in all the circulating pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α) thus indicating a pro-inflammatory state in T2DM. This finding aligned well with literature as various studies implicate chronic low-grade inflammation with T2DM (Xu et al., 2003, Pickup, 2004, Pickup and Crook, 1998). Furthermore, the specific pro-inflammatory cytokines analysed in this study: IL-1β (Maedler et al., 2002, Ehses et al., 2007), IL-6 (Akbari and Hassan-Zadeh, 2018, Pickup et al., 2000), IL-8 (Cimini et al., 2017) and TNF-α (Swaroop et al., 2012, Zou and Shao, 2008) have been previously observed to be significantly elevated in T2DM.

The multiplex assay in addition to the VPLEX assay, figure 4.3.2, further showed that the profile of chemokines (MCP-1 and MIP-1β) and cell adhesion molecules (sP-Selectin, ICAM-1 and VCAM-1) are also altered and upregulated in T2DM. Again, the results are in line with literature as MCP-1 (Simeoni et al., 2004, Blaha et al., 2006), MIP-1β (Maier et al., 2008), sP-Selectin (Gokulakrishnan et al., 2006, Pretorius et al., 2018c), ICAM-1 (Karimi et al., 2018) and VCAM-1 (Liu et al., 2015, Braatvedt et al., 2001) have been seen to be altered in patients presenting with T2DM.

The cytokine profile analysis shows that T2DM is indeed a condition associated with chronic low-grade inflammation and an altered cytokine profile. Due to the increased presence of these proinflammatory cytokines in T2DM, it is expected to observe increased circulating levels of APPs (Festa et al., 2002), a hypercoagulable and hypofibrinolytic coagulation state (Kell and Pretorius, 2015), altered erythrocytes and platelet morphology (Bester and Pretorius, 2016, Page et al., 2018) as well as amyloidogenic fibrin clot formation (Pretorius et al., 2016a).

Following on from the first chapter aim, a VPLEX assay, displayed in figure 4.3.2, was performed to determine whether in fact elevated circulating levels of SAA and other APPs exist in T2DM. As T2DM is a condition associated with chronic inflammation, and our previous results proving there to be an altered cytokine profile, it is expected to see elevated levels of APPs such as SAA and CRP especially as it has already been observed

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that IL-6, a potent inducer of the APR (Jain et al., 2011), is significantly upregulated. This proved true as both CRP and SAA, figure 4.3.2, were upregulated 12 and 10 times respectively. Elevated CRP levels are a common trend in inflammatory diseases, as the molecule is a potent biomarker for inflammation (Allin and Nordestgaard, 2011, Vadakayil et al., 2015). Further to this, significantly elevated CRP levels have been observed in various T2DM studies (Hu et al., 2004, Soinio et al., 2006, Wang et al., 2013); thus the results obtained from the VPLEX analysis aligns well with literature surrounding T2DM and the APR. In line with CRP, SAA is another APP widely researched as a biomarker for various inflammatory conditions (Malle and De Beer, 1996, Bozinovski et al., 2008), with T2DM featuring as one of these (Marzi et al., 2013, Griffiths et al., 2017). The circulating SAA levels observed in this study were significantly elevated in T2DM in comparison to healthy age matched controls. The SAA levels in this study displayed a 10-fold significant increase, this however doesn't fully match literature as it has been seen that SAA can be upregulated up to 1000-fold (Eklund et al., 2012).

In literature, IL-1β and TNF-α are major inducers of the production of the cytokine IL-6 by hepatic Kupffer cells which then mediates the secretion of SAA and other APPs (Jain et al., 2011). When correlation analyses were performed, significant correlations were observed SAA and IL-1β, IL-6 and CRP (figure 4.3.3). This is expected as it was previously stated that IL-1β and IL-6 are inducers of the secretion of APPs while CRP is an APP itself, therefore it is expected to be secreted at the same time as SAA. Trends towards significant correlations were observed between SAA and MCP-1 which may potentially be as a result of Kupffer cells being a source of MCP-1 (Hildebrand et al., 2006) thus when stimulated to secrete APPs, the activated Kupffer cells also secreted MCP-1 as a by-product of activation. Furthermore, a correlation trend was observed between SAA and MIP-1β, which may be attributed to the fact that MIP-1β binds and activates macrophages (Maurer and von Stebut, 2004, Hsieh et al., 2008), thus with Kupffer cells being specialised resident hepatic macrophages, MIP1-β may potentially bind to these cells and be an alternate inducer of the secretion of APPs.

The fact that no correlation was observed between SAA levels and TNF-α was a surprising finding in this study as in literature TNF-α is commonly associated with this APR (Thorn et al., 2004, Jain et al., 2011). The reason for this finding is not completely understood however, it is hypothesised that due to TNF-α being a ligand that is able to bind to various cell types and having vast functions throughout the body. Consequently, the majority of the TNF-α in T2DM may be bound to the various cell types thus not being able to be effectively measured using the multiplex analysis that was performed in this study. As a

result, the correlation analysis for this cytokine and SAA would not give an accurate measurement. Additionally, it may be plausible that IL-1β is in fact a greater inducer of the IL-6 production and thus the APR than that of TNF-α.

To evaluate the third aim of this chapter of how an altered cytokine profile can influence the haematological system, viscoelastic parameters of the coagulation system, visualising morphological changes of erythrocytes, platelets and fibrin clots as well as quantifying the amount of amyloid signal found in the insoluble fibrin clots were performed. When analysing the seven viscoelastic parameters that assesses the efficiency of coagulation, figure 4.3.4, the coagulation system of the T2DM group formed fibrin clots at a more rapid rate while the clots formed were significantly stronger and more stable. These results are in line with various studies performed previously (Bester et al., 2018, Kell and Pretorius, 2015, Page et al., 2018, Pretorius and Bester, 2016, Pretorius et al., 2015, Pretorius et al., 2017c) whereby the hallmark of inflammation is hypercoagulability. This may be one of the causative factors in why individuals suffering with T2DM are highly susceptible to suffering cardiovascular complications and strokes (Low Wang et al., 2016).

Analysis of the morphology of the erythrocytes and platelets in WB of T2DM, figure 4.3.5.1, indicates that the altered cytokine profile induces erythrocyte eryptosis, platelet hyperactivation, platelet aggregation as well as agglutination. Similar results have been observed in literature (Pretorius et al., 2015, Pretorius et al., 2016b, Pretorius et al., 2018c). These whole blood alterations can be attributed to elevated IL-1β, IL-6 and IL-8 inducing platelet hyperactivation (Bester and Pretorius, 2016), TNF-α and sP-Selectin inducing platelet clumping and agglutination (Page et al., 2018) and IL-8 inducing eryptosis (Bester and Pretorius, 2016). Furthermore, the pro-inflammatory state in T2DM induces the formation of thick dense matted amyloid deposits in fibrin clots, figure 4.3.5.2. This is another phenomenon that the Clinical Hemorheology and Coagulation research group at Stellenbosch University has previously observed (Kell and Pretorius, 2016, Pretorius et al., 2016a, Pretorius et al., 2017a, Pretorius et al., 2017b, Swanepoel et al., 2015). The dense matted fibrin clots may be a further contributing factor in T2DM individuals increased susceptibility to cardiovascular diseases and complications. This phenomenon can be attributed to the altered cytokine profile inducing increased availability in various clotting factors (Carlsen et al., 1988, Bester and Pretorius, 2016, Ernofsson and Siegbahn, 1996, Celi et al., 1994), increased circulating levels of fibrinogen (Spencer et al., 2007) and down regulated levels of antithrombin and thrombomodulin (Patalakh and Kudinov, 2008).

Finally, the amyloid signal quantified in PPP clots, figure 4.2.6.1, was significantly increased in the T2DM clots compared to the control clots. This result is agreeing with previous literature whereby T2DM fibrin had dramatically higher amyloid signal (Pretorius et al., 2016a, Pretorius et al., 2017a, Pretorius et al., 2017b). It must be noted that the healthy control group did however have elevated amyloid signal which may be due to population size used in the control group being only 5 samples. Despite this, the significant amyloid signal using ThT and the two Amytrackers produce results that match the observations seen in the fibrin clot SEM analysis.

Assessing this chapters results, it is evident that an altered cytokine profile exists in T2DM, circulating SAA levels in T2DM are significantly raised and that these two alterations induce a profound effect on the haematological system by inducing hypercoagulation, eryptosis, platelet hyperactivation and agglutination as well as amyloid fibrin clot formation.

5. Control Study

5.1 Introduction

In this chapter, all the experiments were performed on control blood and PPP. Serum Amyloid A was added *in vitro* to determine the effects of this molecule on the efficiency of coagulation as well as to determine if the molecule effects whole blood and fibrin morphology. Additionally, High Density Lipoprotein was added *ex vivo* as it is known that it binds to SAA, this chapter thus aims to determine the amyloidogenic potential of SAA and whether the addition of HDL may be able to negate the effects of SAA on the haematological system. Furthermore, LPS-binding protein was added *ex vivo* as we know LPS is associated with SAA thus potentially the binding protein may be able to "mop" the effects of SAA on the haematological system.

5.2 Materials and Methods

Material and methods for quantitative markers:

Thromboelastography:

The protocol followed for the TEG is identical to that described in chapter 4. The only alteration is that in addition to analysis of naïve whole blood, control blood was exposed to LPS, HDL and LBP as well as various combinations of the afore mentioned molecules. For a detailed description of the exposures please refer to table 3.4.

Material and methods for morphological markers:

Scanning Electron Microscopy

Protocols for both whole blood and fibrin analysis followed was identical to that of chapter 4. Whole blood morphology analysis was conducted on naïve control blood as well as blood exposed to SAA, LBP and HDL as well as the combinations discussed in table 3.4. Fibrin clot morphology was assessed using naïve control PPP as well as PPP incubated with 30 µg.mL^{-1} SAA.

<u>Confocal Microscopy</u>

The protocol followed is identical to the protocol discussed in depth in chapter 4. Naïve fibrin clots as well as fibrin clots of naïve control PPP exposed to 30 µg.mL^{-1} SAA were imaged and analysed.

5.3 Results

5.3.1 Thromboelastography Results

Table 5.3.1. Table displaying the 7 viscoelastic parameters assessing efficiency of coagulation in whole blood of control participants (n=14). Data was generated using the Haemoscope **TEG**® 5000 Haemostasis Analyser. All samples were incubated with 30 µg.mL^{-1} Serum Amyloid A, 5 ng.L^{-1} Lipopolysaccharide- binding protein and HDL 30 µg.mL^{-1} as well as varying combinations of the aforementioned molecules. Incubation of each molecule was 10 minutes per molecule added with SAA always being added last. Friedman tests and one-way ANOVAs were performed with a Dunnett's Multiple comparison being used to compare the mean of each group to that of the mean of the naïve group. Data is presented as the mean and interquartile ranges with significance set as *p < 0.05; ***p < 0.001 and ****p < 0.0001.

		Viscoelastic Parameter						
		R-Value (mins)	K-Value (mins)	Alpha Angle (deg)	MA (mm)	MRTG (Dyn.cm^{-2}.s^{-1})	TMRTG (mins)	TTG (dyn.cm^{-2})
Treatment regime	Naïve	7.79 [6.95 - 8.4]	2.84 [2.45 - 3.00]	60.71 [59.50 - 64.10]	56.30 [53.88 - 59.53]	4.53 [4.03 - 5.26]	11.05 [9.73 - 12.00]	662.00 [584.10 - 735.90]
	SAA	6.97 [6.53 - 7.65]	2.96 [2.38 - 3.43]	61.01 [58.48 - 64.08]	55.18 [53.88 - 59.03]	4.36 [3.48 - 5.20]	10.51 [9.71 - 11.00]	625.70 [586.80 - 721.00]
	LBP	7.02 [6.25 - 7.55]	2.84 [2.38 - 3.20]	61.80 [59.13 - 64.00]	53.99 [51.98 - 56.50]	4.33 [3.74 - 5.05]	10.05 [8.92 - 11.02]	595.30 [544.30 - 650.70]
	HDL	7.63 [6.55 - 8.18]	3.11 [2.48 - 3.80]	59.46 [56.05 - 63.35]	55.32 [53.65 - 58.13]	4.24 [3.61 - 5.18]	11.01 [9.23 - 12.96]	628.30 [577.20 - 698.20]
	HDL + SAA	6.56 [5.68 - 7.33] *	2.79 [2.40 - 3.25]	61.87 [60.80 - 64.73]	55.06 [52.93 - 58.00]	4.64 [3.94 - 5.65]	9.64 [8.65 - 10.87]	617.20 [565.80 - 659.80]
	HDL + LBP	6.66 [6.08 - 7.33]	3.04 [2.35 - 3.73]	60.28 [57.10 - 65.28]	54.07 [51.70 - 57.28]	4.33 [3.61 - 5.05]	9.97 [8.67 - 11.02]	593.90 [536.50 - 672.60]
	LBP + SAA	6.00 [5.20 - 7.00] ***	2.94 [2.38 - 3.30]	62.93 [60.15 - 66.30]	53.36 [49.58 - 56.58] *	4.43 [3.83 - 5.12]	8.66 [7.61 - 10.10] **	580.80 [487.20 - 653.70]
	HDL + LBP + SAA	5.87 [4.95 - 7.08] ****	2.68 [2.35 - 2.93]	64.05 [61.28 - 66.75]	53.66 [51.45 - 56.98] *	4.96 [3.94 - 5.57]	8.61 [7.36 - 10.29] ***	594.40 [532.50 - 664.40]

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The control study was performed using control whole blood samples (n=14) collected in sodium citrate tubes, whereby naive samples were analysed before 7 different molecules, mopping agents as well as the combinations of the molecules was incubated in the sample prior to analysis. A summary of which can be found in table 3.4. Table 5.3.1. above shows that the Serum Amyloid A, High density lipoprotein and LPS -binding protein groups alone as well as HDL+ LBP group did not cause any significant differences in any of the seven viscoelastic parameters assessed in comparison to that of the control naïve group.

Three of the combination groups however did produce significant changes. When HDL was incubated in conjunction with SAA, a significant reduction ($p = 0.0208$) was observed in the R-value hence causing the initial thrombus generation to occur at a more rapid rate. When LBP and SAA were incubated together, the R-value and time to maximal rate of thrombus generation (TMRTG) as well as the maximal amplitude (MA) were all significantly reduced ($p = 0.0002$, $p = 0.0013$ and $p = 0.0142$ respectively). This means that LBP and SAA together were causing the rate of thrombus generation to increase while the end clot formed had a reduced clot strength potentially due to decreased platelet and/or fibrin(ogen) interaction. Finally, the treatment group with all three molecules incubated together produced a similar effect to that of the LBP+SAA group where significant reductions ($p < 0.0001$, $p = 0.0383$ and $p = 0.0008$) were again observed in the R-value, TMRTG and MA. This again indicates that the treatments caused the rate of thrombus generation to increase while the end clot formed had a reduced clot strength potentially due to decreased platelet and/or fibrin(ogen) interaction.

5.3.2 Scanning Electron Microscopy Results

Qualitative analysis of the control study whole blood SEM micrographs indicates that the LBP alone (image C below) and the HDL+LBP combination (image E below) incubation were the only two treatment groups that produced no noticeable morphological change in comparison to that of the naïve control images. In contrast, the HDL (image B) and the SAA alone incubation (image D) displayed noticeable differences in the platelet morphology and activation status, this can be seen in both images by the widespread membranes of the platelets as well as the occurrence of platelet aggregation. It must be further noted that in the SAA alone treatment (not represented in images below), the presence of eryptotic erythrocytes were observed thus indicating that SAA causes alterations in platelet and erythrocyte morphology. The HDL + SAA incubation showed signs of disrupting the membrane of erythrocytes (image F) as well as erythrocyte

aggregation and rouleaux formation. Furthermore, this combination treatment also displayed widespread platelet activation and aggregation.

Following this trend, the LBP + SAA and HDL + LBP + SAA incubations both showed altered erythrocyte and platelet morphology whereby extensive platelet activation and aggregation was observed. These two incubations qualitatively display the greatest change in morphology. When qualitatively analysing fibrin clot images, figure 5.3.4.2 below, it is evident that the exposure of PPP to 30 µg.mL^{-1} Serum Amyloid A causes clear morphological alterations when compared to that of the naive control clots (image A and B). The control fibrin clots have thin "spaghetti like" fibrin fibres with large open areas between the various fibrin fibres which can be seen in image A and B. In contrast however, when analysing the clots formed when exposed to SAA (image C and D), no singular fibrin fibres can be seen, instead large dense matted aberrant fibrin areas are seen with little to no open areas in the clot. This indicates that SAA is possibly amyloidogenic in nature when exposed to PPP.
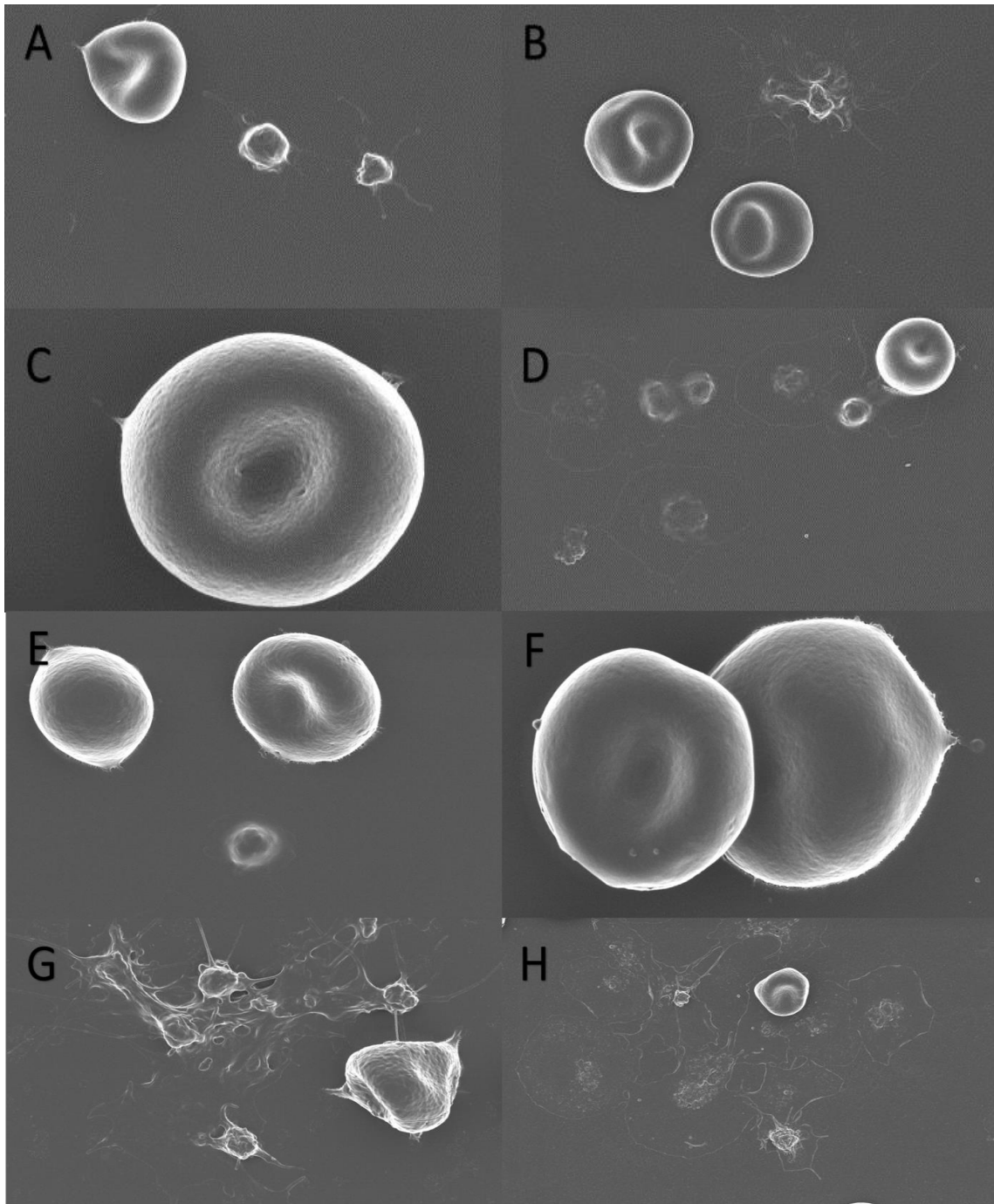
Figure 5.3.2.1. Figure showing representative micrographs taken on the Zeiss MERLIN™ field emission scanning microscope of whole blood from Control samples. Micrographs of naïve whole blood (image **A**) as well as the whole blood incubated with HDL (image **B**), LBP (image **C**), SAA (image **D**). Furthermore, treatment combinations HDL + LBP (image **E**), HDL + SAA (image **F**), LBP + SAA (image **G**) as well as HDL + LBP + SAA (image **H**). Micrographs were captured at various magnifications using high resolution InLens capabilities at 1 kV.
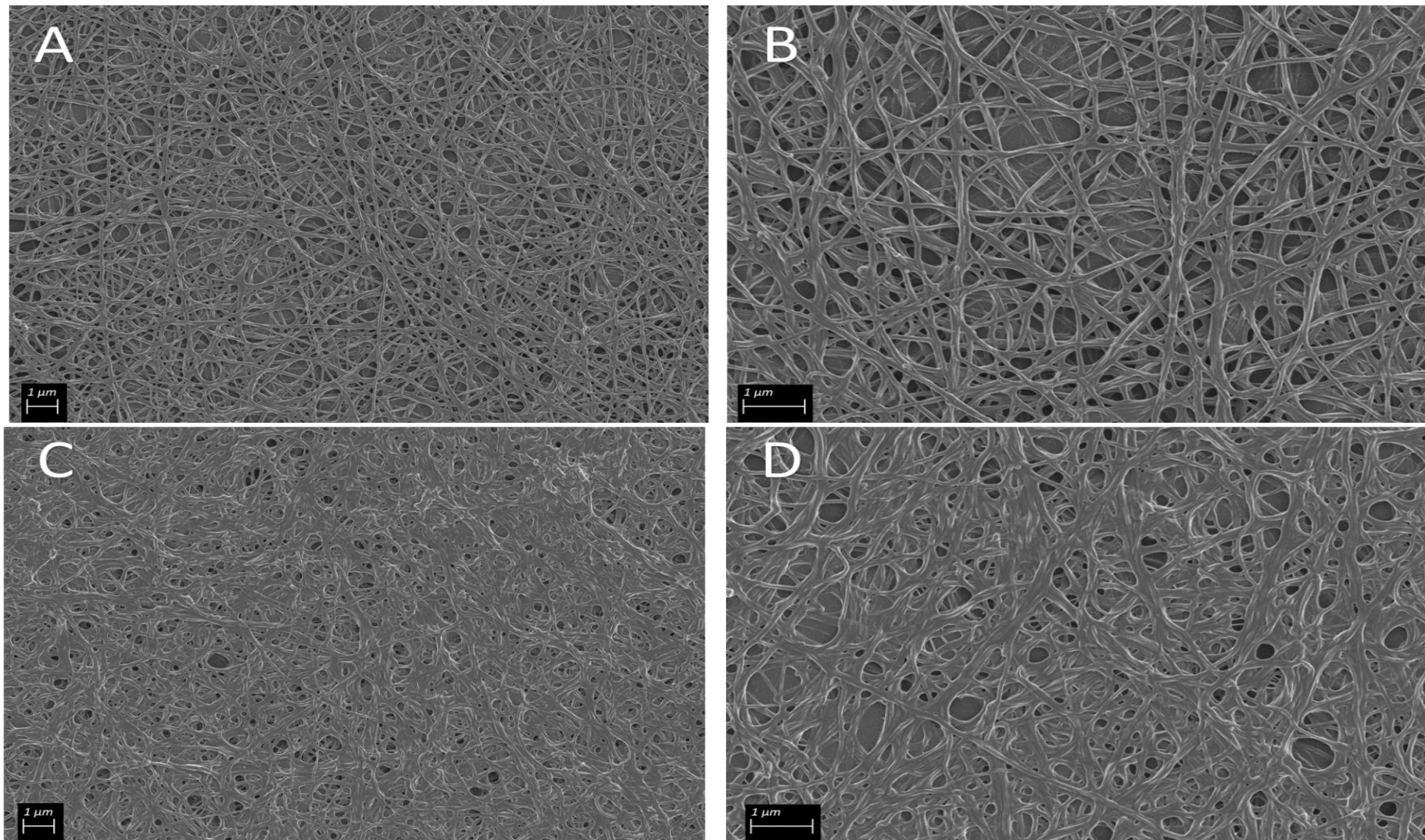
Figure 5.3.2.2. Figure showing representative micrographs taken on the Zeiss MERLIN™ field emission scanning microscope of fibrin clots formed from naïve control (image A and B) PPP and control PPP exposed to 30 µg.mL^{-1} Serum Amyloid A (image C and D). Images were captured at 5000X (image A and C) as well as 10000X (image B and D) using the high resolution InLens capabilities at 1 kV.

5.3.3. Confocal Microscopy Results
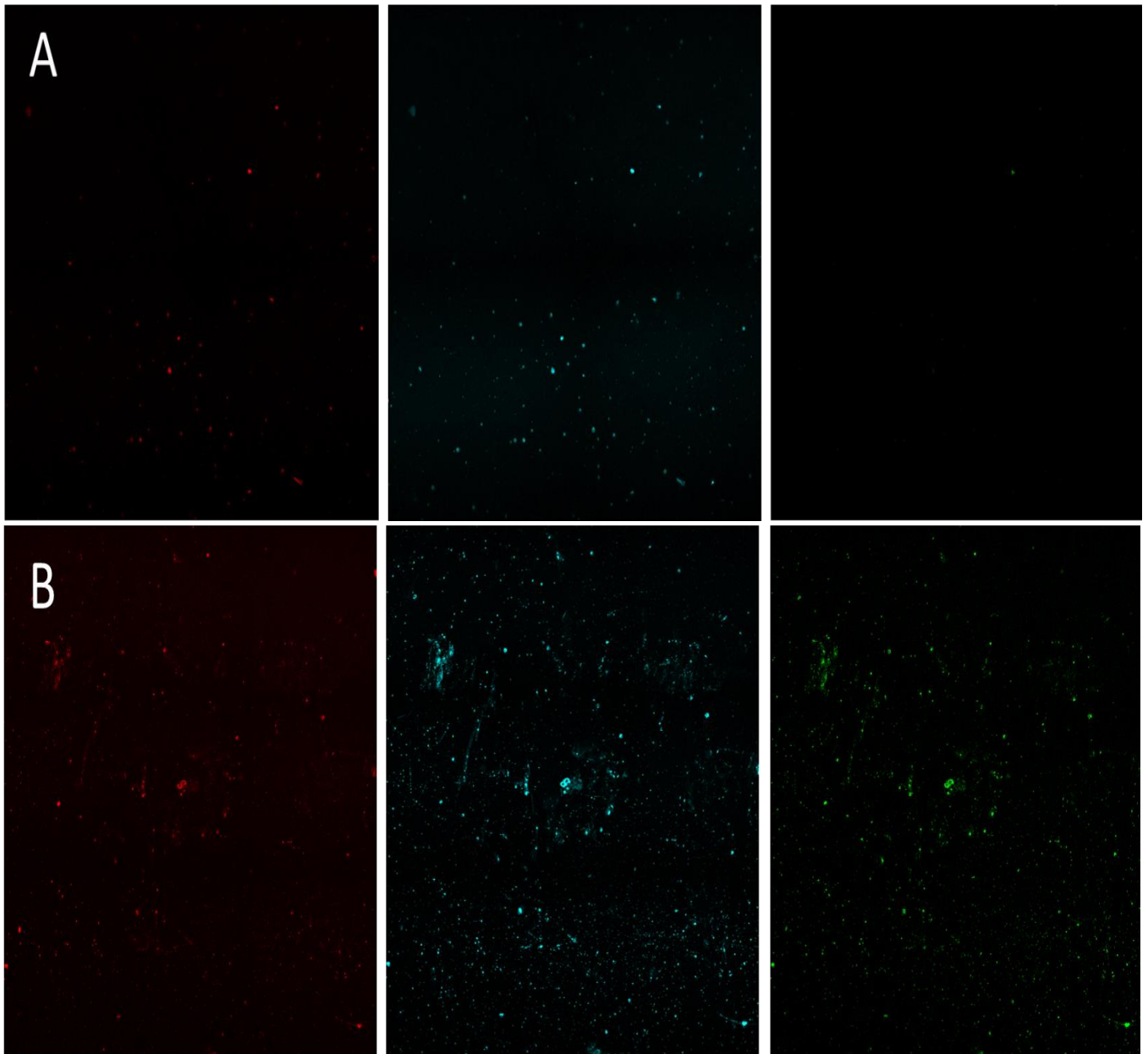
Figure 5.3.3.1. Representative fibrin clots from naïve control PPP (image **A**) and control PPP exposed to 30 µg.mL^{-1} Serum Amyloid A (image **B**). From channel left to right represents Amytracker 680 (red), Amytracker 480 (blue) and Thioflavin T (green) respectively. All images were captured at 63X magnification using a 2X2 tile scan on the Carl Zeiss LSM 780 confocal microscope.
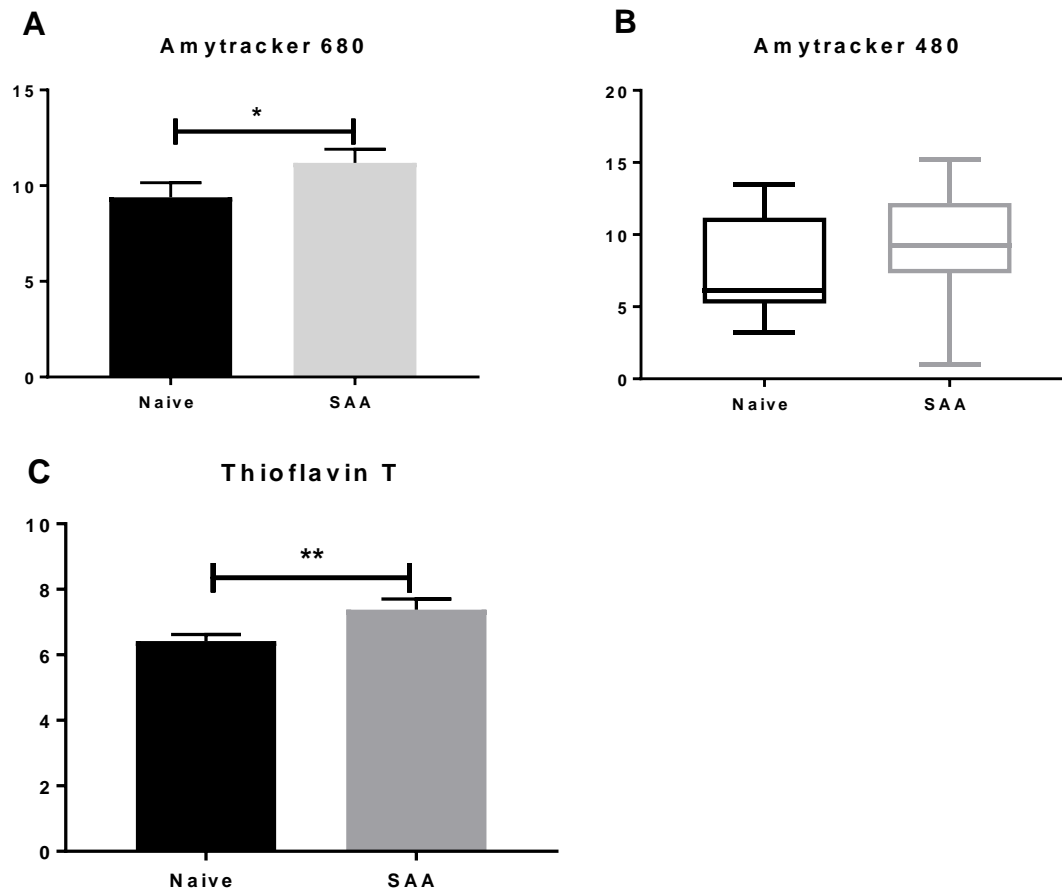
Figure 5.3.3.2. Graphs displaying coefficient of variation (CV) values extrapolated using Image J from fibrin clot confocal micrographs of naïve control PPP (n = 5) and control PPP exposed to 30 µg.mL^{-1} Serum Amyloid A. Parametric data (image **A** and **C**) was analysed using Welch's corrected t test and is represented as the mean ± SEM whereas non-parametric data (image **B**) was analysed using a Mann-Whitney analysis and is represented as box and whisker plots. Significance is set as *p < 0.05; ***p < 0.001 and ****p < 0.0001.

Figure 5.3.3.2. shows that the exposure of 30 µg.mL^{-1} Serum Amyloid A into control PPP induced a significant increase in the amyloid signal of the red Amytracker 680 (p = 0.0452) and green Thioflavin T (p = 0.0062) fluorescent channels. No significant changes were observed in the blue Amytracker fluorescent signal however a trend (p = 0.0888) was seen. Although the population size was low (n = 5), these results seem to correlate well with the SEM image observed whereby SAA does in fact induce amyloidogenesis in fibrin fibres.

5.4 Discussion

In chapter 4 it was determined that SAA, among various other cytokines, was upregulated in T2DM. The initial aim for this chapter was thus to assess the amyloidogenic potential of SAA when added to control WB and PPP *ex vivo*. SAA incubated in control blood produced no significant impact on any of the seven viscoelastic parameters, table 5.3.1, that assess the efficiency of coagulation. This result indicates that SAA potentially has no significant impact on the coagulation cascade, however, Page et al. (2019), using the same SAA concentration, 30 µg.mL^{-1}, in a younger healthy female population with a greater sample size, found that SAA caused a decrease in the MA and TGG in whole blood thus the clot formed was less stable. The same study found significantly elevated MA, MRTG and TGG values when the SAA was incubated in PPP of the same population, suggesting that SAA produces a fibrin(ogen)-mediated enhancement of the coagulation system, while simultaneously inhibiting platelet to fibrin(ogen) interaction however this was not observed in the current study.

Assessment of morphological changes induced by physiologically low levels of SAA, figure 5.3.2.1 Image D, indicates the initial stages of erythrocyte eryptosis and platelet activation and platelet to erythrocyte agglutination similarly to what is seen in literature (Page et al., 2019). In contrast to literature however, platelet hyperactivation and total spreading was observed in this study, which may be attributed to the age and health status of the control population used in the study. This phenomenon may also be due to the fact that a close association between inflammation and thrombosis exists, whereby the pro-inflammatory state promotes coagulation and activation of platelets as a protective homeostatic mechanism (Esmon, 2005). Furthermore, SAA induced the formation of dense matted amyloid deposits in the fibrin clots, figure 5.3.2.2, when compared to the PPP naïve fibrin clots. This is in line with Page et al. (2019) who used correlative light and electron microscopy (CLEM) to determine whether SAA binds and interacts with soluble and circulating plasma molecules, like fibrinogen, therefore inducing structural (amyloidogenic) changes. This amyloid fibrin formation was further seen using confocal microscopy and amyloid-specific fluorescent markers whereby the fibrin clots incubated with SAA had significantly elevated signal, figure 5.3.3.2, in comparison to that of the naïve clots. This can again be accredited to SAA binding and interacting with circulating fibrinogen thus impacting the autocatalytic formation of insoluble fibrin fibres and hence causing beta-sheet rich misfolded amyloid regions in the fibrin clots.

The second aim of this study was to determine whether HDL, LBP and a combination of the two are effective mopping agents to inhibit the effect of SAA on the haematological system. It was thought that these two markers would be effective as HDL naturally associates with and binds SAA whereas LBP has been a proven effective mopping agent in T2DM (Pretorius et al., 2017a). Furthermore, a close link between SAA and LPS exists naturally. This notion however proved false as the co-treatment of SAA and HDL, SAA and LBP as well as SAA, HDL and LBP all induced increased initiation rates of clot formation, table 5.3.1, compared to the naïve control coagulation state. Furthermore, the SAA and LBP as well as SAA, HDL and LBP combination groups also altered the clot strength with significantly reduced MA values possibly indicating decreased platelet and/or fibrin(ogen) interaction. These results were further validated as these same three treatment groups caused the greatest alterations in whole blood morphology. The SAA and LBP combination and the SAA, HDL and LBP combination groups displayed the greatest impact of platelet activity and agglutination indicating the combination groups may indeed impact the platelet to fibrin interaction. These findings may be attributed to the binding of SAA to HDL inhibiting the anti-inflammatory effects of the lipoprotein (Han et al., 2016), consequently, enhancing the adverse effects of SAA on the haematological system. Furthermore, as the structure of LBP isn't fully understood yet, the molecule may be able to bind and interact with SAA molecules potentially altering the molecules structure, function or the fibrin(ogen) binding capacity thus further enhancing the SAA molecules impact of the haematological system, this may be a further way to understand the results obtained in this chapter.

This chapter confirmed that SAA is amyloidogenic in nature and induces the formation of dense matted amyloid deposits in fibrin clots. The commonly associated molecule HDL and a previously proven mopping agent LBP were not able to effectively mop the effects of SAA, in contrast they seemed to further exacerbate the deleterious haematological effects of SAA.

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6. Experimental Study

6.1 Introduction

In this chapter we performed all the experiments on Type II Diabetes Mellitus blood. High density lipoprotein was added *in vitro* to the T2DM blood to determine if this so called "good" cholesterol can reverse the hypercoagulable and amyloidogenic state observed in T2DM. Furthermore, LPS-binding protein was added *in vitro* as we know elevated levels of circulating LPS is associated with T2DM thus potentially the binding protein may be able to "mop" and reverse the detrimental effects of T2DM on the haematological system. The aim of this chapter was thus to determine if these two mopping agents are effective in removing detrimental haematological effects seen in T2DM. The effects of the added molecules were tested via analysis of the efficiency of coagulation as well as determining whether the molecules affect whole blood and fibrin morphology.

6.2 Materials and Methods

Material and methods for quantitative markers:

Thromboelastography:

The protocol followed for the TEG is identical to that described in chapter 4. The only alteration is that in addition to analysis of naïve whole blood, T2DM blood was exposed to HDL, LBP and the combination of the afore mentioned molecules. For a detailed description of the exposures please refer to table 3.4.

Material and methods for morphological markers:

Scanning Electron Microscopy

Protocols for both whole blood and fibrin analysis followed was identical to that of chapter 4. Whole blood morphology analysis was conducted on naïve T2DM blood as well as blood exposed to HDL, LBP and the combination of the two discussed in table 3.4. Fibrin clot morphology was assessed using naïve T2DM PPP as well as PPP incubated with 30 μg. mL^{-1} HDL.

Confocal Microscopy

The protocol followed is identical to the protocol discussed in depth in chapter 4. Naïve T2DM fibrin clots as well as fibrin clots of T2DM PPP exposed to 30 µg. mL^{-1} HDL were imaged and analysed.

6.3 Results

6.3.1 Thromboelastography Results

Table 6.3.1. Table displaying the 7 viscoelastic parameters assessing efficiency of coagulation in whole blood of Type II Diabetes Mellitus participants (n=14). Data was generated using the Haemoscope **TEG**® 5000 Haemostasis Analyser. All samples were incubated with 5 ng.L^{-1} Lipopolysaccharide- binding protein (LBP) and HDL 30 µg.mL^{-1} as well as the combination of the two molecules. Incubation of each molecule was 10 minutes per molecule added. Friedman tests and one-way ANOVAs were performed with a Dunnett's Multiple comparison being used to compare the mean of each group to that of the mean of the naïve group. Data is presented as the mean and interquartile ranges with significance set as $*p < 0.05$; $***p < 0.001$ and $****p < 0.0001$.

		Viscoelastic Parameter						
		R-Value (mins)	K-Value (mins)	Alpha Angle (deg)	MA (mm)	MRTG (Dyn.cm^{-2}.s^{-1})	TMRTG (mins)	TTG (dyn.cm^{-2})
Treatment regime	T2DM Naïve	6.06 [4.43 – 6.95]	1.99 [1.58 – 2.23]	69.46 [65.45 – 72.83]	63.84 [59.28 – 75.03]	9.47 [5.89 – 10.28]	8.95 [7.34 – 10.32]	1053.00 [745.20 – 1505.00]
	HDL	5.78 [4.18 – 7.75]	2.21 [1.50 – 2.88]	68.56 [62.15 – 73.50]	63.46 [56.45 – 70.95]	7.99 [4.62 – 9.94]	8.51 [6.67 – 10.42]	972.10 [649.20- 1231.00]
	LBP	5.51 [3.75 – 7.08]*	2.29 [1.40 – 3.25]	67.15 [58.40 – 73.75]	57.67 [48.18 – 68.05]	7.65 [4.07 – 9.68]	8.33 [5.98 – 10.75]	792.60 [464.00 – 1073.00]
	HDL + LBP	5.20 [3.88 – 6.30] ***	2.43 [1.28 – 3.38]	67.94 [61.70 – 75.40]	57.86 [51.10 – 68.60]	7.87 [4.15 – 9.49]	7.63 [6.29 – 8.90]**	849.20 [530.80 – 1102.00]

The experimental study was performed using T2DM (n=14) whole blood collected in sodium citrate tubes, the naïve whole blood as well as the whole blood incubated with LBP, HDL and the combination of the two molecules was analysed using the TEG to analyse the efficiency of coagulation. Table 6.3.1. below indicates that five of the seven viscoelastic parameters (K-value, Alpha angle, MA, MRTG and TGG) had no significant changes throughout the 3 different treatment groups. Furthermore, the HDL incubated

treatment in T2DM whole blood produces no significant effect on any of the seven parameters assessed.

In contrast, LBP incubated treatment in the T2DM whole blood, produced a significant reduction (p = 0.0252) in the R-value in comparison to that of the T2DM naïve group. This reduction indicates that the LBP causes the rate of initial thrombus generation to occur at a faster rate than that of the naïve group. Moreover, the HDL+LBP group produced significant reductions in the R-Value (p = 0.0006) as well as the time to maximal rate of thrombus generation (TMRTG) (p = 0.0015), therefore the combination group causes the rate in which the thrombus generates to increase in speed in comparison to the T2DM naïve group.

6.3.2 Scanning Electron Microscopy Results

Qualitative analysis of the whole blood SEM results indicates than neither the HDL, LBP or the HDL + LBP incubations produced notable changes to the whole blood morphology in comparison to the naïve T2DM whole blood. In all four groups, eryptotic erythrocytes were images with erythrocyte agglutination. Furthermore, platelet hyperactivity and aggregation were seen throughout.

In contrast, the SEM analysis of the fibrin clots showed that the HDL may possibly reduce amyloid fibrin(ogen) when incubated in T2DM PPP. This phenomenon can be seen in image C and D from figure 6.3.2.2, where although still very dense, individual fibrin fibres are distinguishable as well as more open spaces being observed in comparison to that of the naïve T2DM fibrin clots.
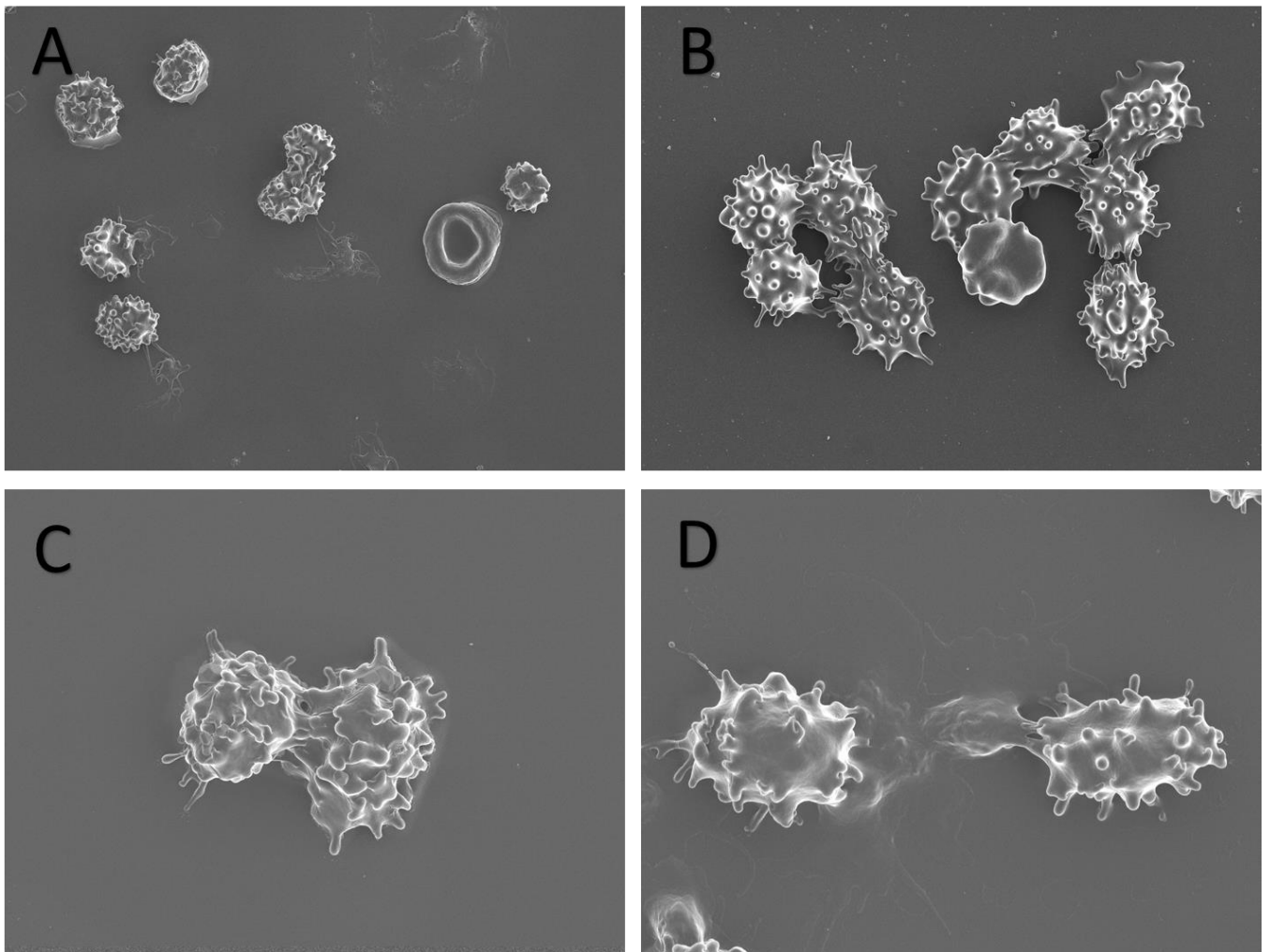
Figure 6.3.2.1. Figure showing representative micrographs taken on the Zeiss MERLIN™ field emission scanning microscope of whole blood from Type II Diabetes Mellitus samples. Micrographs of naïve whole blood (image **A**) as well as the whole blood incubated with HDL (image **B**), LBP (image **C**), as well as the combination of HDL + LBP (image **D**). Micrographs were captured at various magnifications using high resolution InLens capabilities at 1 kV.
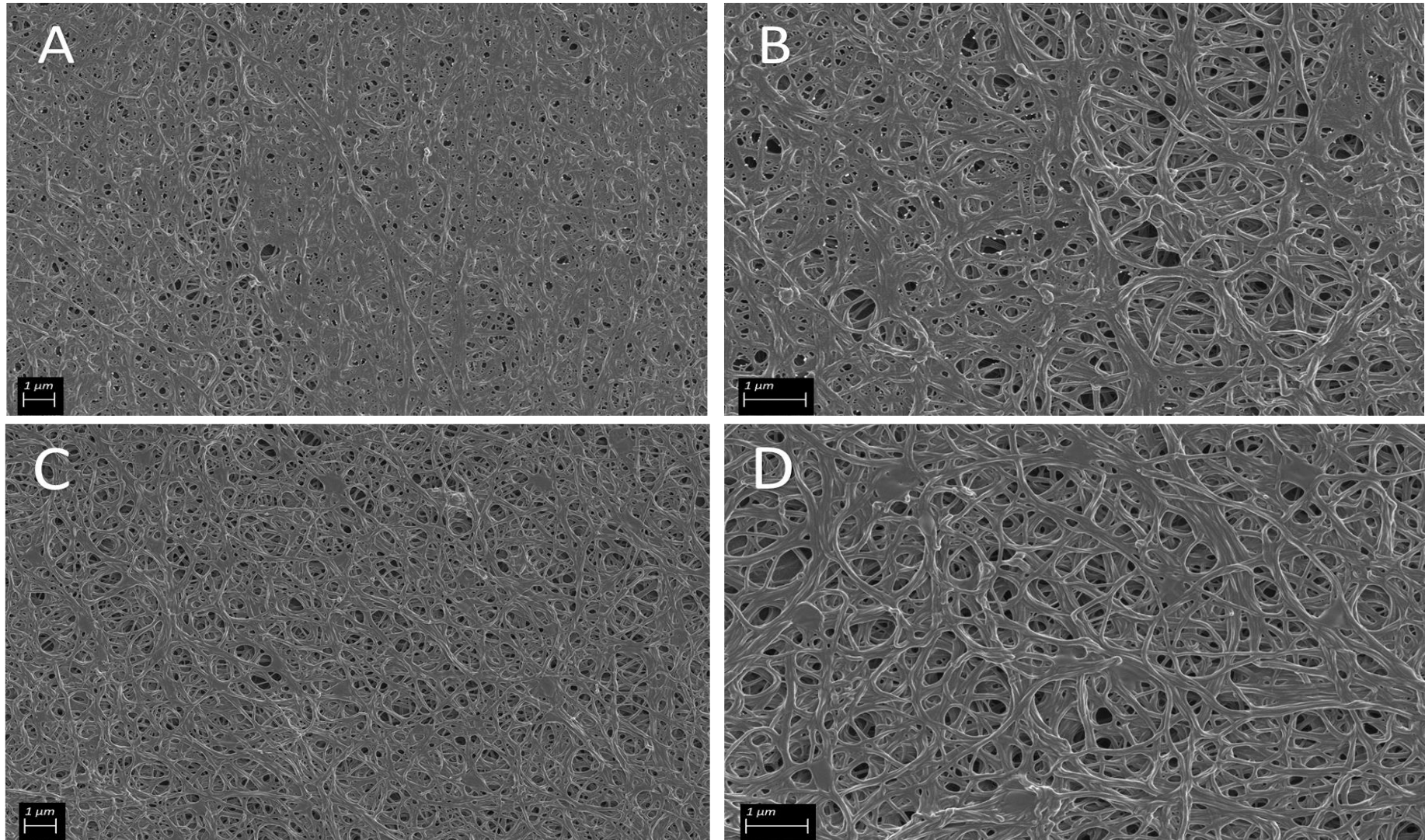
Figure 6.3.2.2. Figure showing representative micrographs taken on the Zeiss MERLIN™ field emission scanning microscope of fibrin clots formed from naïve T2DM (image **A** and **B**) PPP and T2DM PPP exposed to 30 µg.mL^{-1} High Denisty Lipoprotein (image **C** and **D**). Images were captured at 5000X (image A and C) as well as 10000X (image B and D) using the high resolution InLens capabilities at 1 kV.

6.3.3 Confocal Microscopy Results
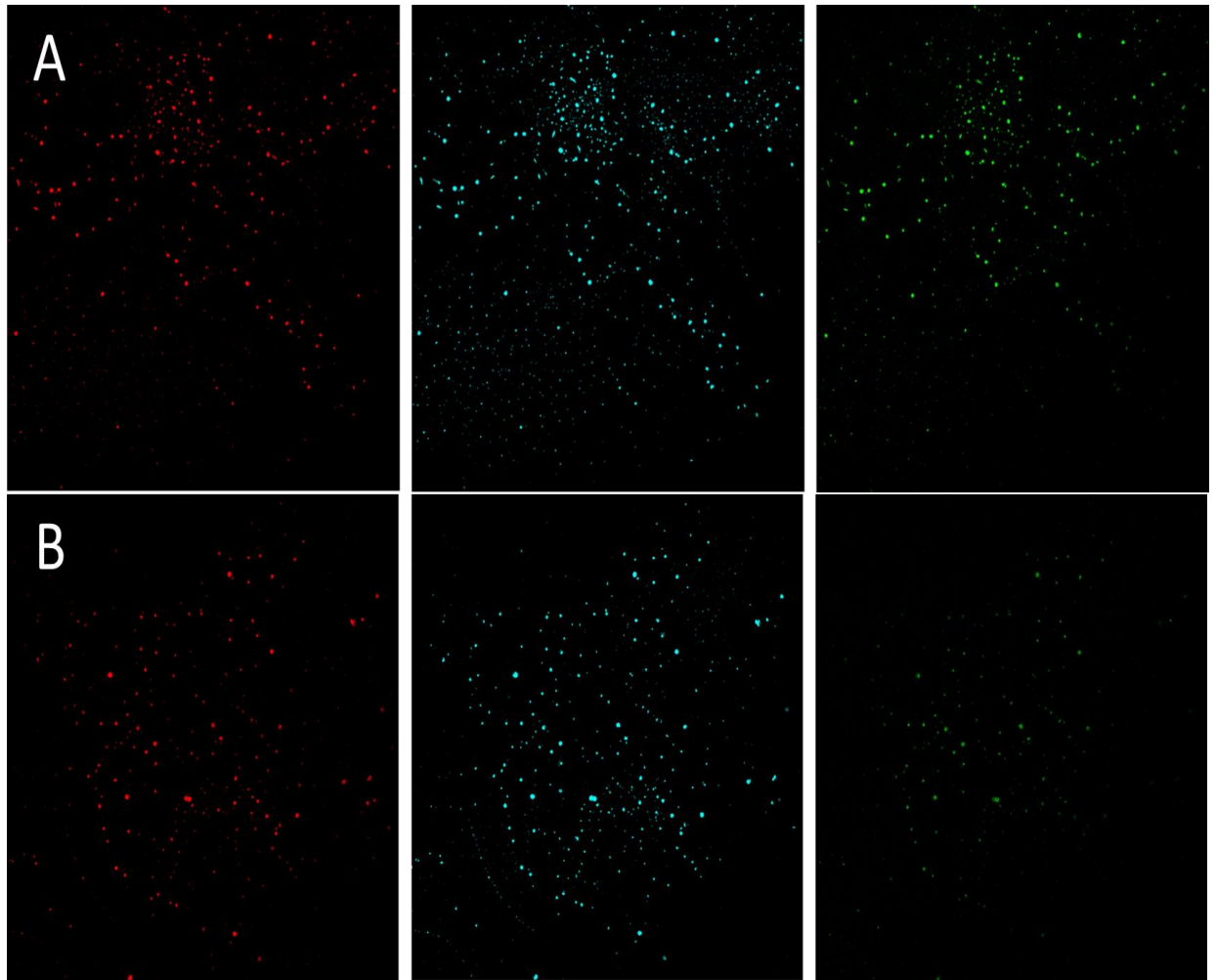
Figure 6.3.3.1. Representative fibrin clots from naïve Type II Diabetes Mellitus PPP (image **A**) and T2DM PPP exposed to 30 µg.mL^{-1} High Density Lipoprotein (image **B**). From channel left to right represents Amytracker 680 (red), Amytracker 480 (blue) and Thioflavin T (green) respectively. All images were captured at 63X magnification using a 2X2 tile scan on the Carl Zeiss LSM 780 confocal microscope.
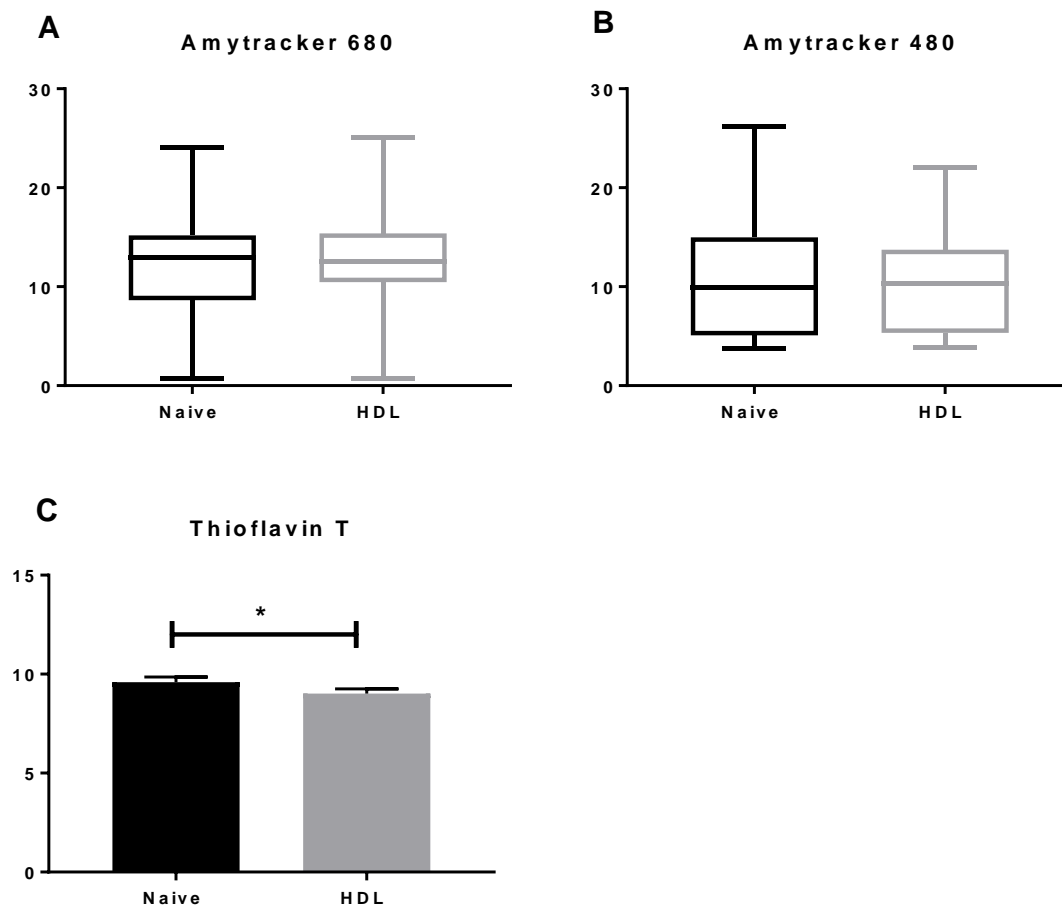
Figure 6.3.3.2. Graphs displaying coefficient of variation (CV) values extrapolated using Image J from fibrin clot confocal micrographs of naïve T2DM PPP (n = 10) and T2DM PPP exposed to 30 µg.mL^{-1} High Density Lipoprotein. Parametric data (image **A**) was analysed using Welch's corrected t test and is represented as the mean ± SEM whereas non-parametric data (image **B** and **C**) is was analysed using a Mann-Whitney analysis and is represented as box and whisker plots. Significance is set as *$p < 0.05$; ***$p < 0.001$ and ****$p < 0.0001$.

Figure 6.3.3.2 above shows that amyloid signal in the green Thioflavin T channel was significantly reduced in the HDL group, however, no differences were observed in either the red Amytracker 680 or blue Amytracker 480 fluorescent channels.

6.4. Discussion

The aim of this chapter of the study was to determine whether HDL and LBP are effective mopping agents in negating the detrimental haematological effects observed in T2DM. This study showed that HDL was able to reduce the fibrin amyloid formation whereby both the SEM and confocal analysis showed slight reductions in the amyloid present. These changes are consistent with Zabczyk et al. (2013) who showed that increased HDL was associated with improved fibrin clot permeability and lysis and speculated that HDL might possibly have an antithrombotic mechanism.

In contrast, the TEG data generated showed that LBP incubation and HDL and LBP co-incubation surprisingly induced fibrin clot formation to occur at a more rapid rate (table 6.3.1.). This result conflicts the SEM images and confocal data, possibly implying that these molecules are not effective mopping agents in T2DM. These results display opposing results obtained by Pretorius et al. (2017a) whereby the incubation of 2 ng.L^{-1} significantly reduced amyloid signal in T2DM.

Due to the complexity in the pathophysiology of T2DM, the use of a single target "mopping agent" seems to be an infeasible approach to tackle the vast impact of the disease on the haematological system. A plethora of cytokines, proteins and signalling pathways are altered in this disease (Cefalu, 2009) with various of these already being proved to influence the coagulation system and the structure of insoluble fibrin fibres (Bester et al., 2018, Bester and Pretorius, 2016, Kell and Pretorius, 2015, Page et al., 2019, Pretorius et al., 2016a, Pretorius et al., 2017b). Furthermore, the presence of raised glucose levels, one of the hallmarks of T2DM, alters the fibrin(gen) structure via glycation (Pieters et al., 2006) possibly inducing fibrin amyloidogenesis. Additionally, T2DM is a condition associated with chronically elevated reactive oxygen species (ROS) (Kaneto et al., 2010), molecules that circulating fibrinogen is extremely vulnerable to as the interaction induces an altered fibrin(ogen) structure which could further lead to fibrin(ogen) amyloidosis (Rosenfeld et al., 2016).

Various other molecules that are altered in T2DM also have the potential to induce amyloid fibrin, where the intricate mechanisms on how these various alterations cause the changes in the haematological system not being fully understood yet. Consequently, proposing a single or multi molecule targeting mopping agent that does not account for the vast systemic molecules and mechanistic alterations which may have amyloidogenic potential, thus seems an impractical option.

7. Conclusion

In this study, using both quantitative and qualitative methods, we confirmed that T2DM is associated with chronic low-grade inflammation with an altered pro-inflammatory cytokine profile. Furthermore, the APR is chronically activated in T2DM with the presence of significantly elevated circulating levels of APPs CRP and SAA. This altered profile induces hypercoagulability in the coagulation cascade, alterations in platelet and erythrocyte morphology as well causing the insoluble fibrin fibres to be amyloid in nature. These changes influence and increase the susceptibility of T2DM individuals to cardiovascular complications and stroke.

The study further went on to show that the increased circulating SAA concentrations influence the haematological system via hyperactivation of the platelets, inducing eryptosis as well as inducing the formation of dense matted amyloid deposits in the insoluble fibrin fibres. The study proved that the *ex vivo* incubation of low physiological levels of SAA in healthy control whole blood and platelet-poor plasma induces haematological alterations that mimic some of the alterations observed in T2DM. This implicates SAA to these changes observed in the disease. Furthermore, due to the alterations observed in the fibrin, we can confirm that SAA is indeed amyloidogenic in nature.

Finally, the study aimed at determining whether HDL and LBP were effective mopping agents in reversing the detrimental haematological alterations induced by SAA. These two molecules especially when co-incubated proved to further the problem whereby significantly influencing and increasing the rate of the clotting cascade. Due to the complexity of a disease like T2DM whereby the whole cytokine profile and signalling pathways are altered, the use of a single molecule mopping agent before further research is done to understand amyloid mechanisms seems like an unfeasible option. Despite this, *ex vivo* studies, like the present one, are essential to further the understanding of the coagulation system in diseased states as well as advancing the understanding in the use of mopping agents as treatment options in future.

8. Limitations and Future Recommendations

As with all studies, a greater sample size would be valuable in increasing statistical power. This would allow the researcher greater confidence in the data generated, enabling for the generation of more viable hypotheses for future studies. Additionally, the age of the population used in this study limits the study as finding suitable healthy individuals at such advanced ages proved extremely difficult. Further to this, locating and recruiting T2DM participants at such an advanced age with no comorbidities is almost impossible. Both of which would influence the data generated thus impacting the validity of the study.

A further limitation of this study was that the baseline SAA values were not accounted for when the external SAA was added *ex vivo*. Consequently, the SAA exposed to each sample was not fully standardised possibly influencing the results obtained. This limitation however was out of the control of the researcher as the exposure of SAA to WB for TEG and SEM analysis had to be performed on the day the blood was drawn. In contrast, the VPLEX analysis for SAA levels was performed at the end of the study once all blood samples had been obtained and stored as PPP.

The T2DM participants in this study were all on various medications which are known to influence the coagulation status. Although this was a factor out of the researcher's control, this is a confounding factor that may have influenced the results of the study. Furthermore, in-depth details such as the duration of T2DM and history of concomitant medical conditions was unclear for the Diabetic participants. Consequently, results obtained may be skewed as the duration of disease is an independently associated with microvascular complications.

Although limitations exist, the principle researcher is confident that the study was performed in good faith and that the results generated from this study are a true reflection *ex vivo* of the haematological system in T2DM *in vivo*.

My future recommendations to further elevate this study would include: using CLEM to determine how SAA, HDL and then binding of the two molecules influence the fibrin clot formation, using a purified fibrinogen model and repeating the same exposure with the mopping agents to negate any unwanted effects caused by the total altered cytokine profile in T2DM, performing the study with a younger healthier population as well as with a recently diagnosed unmedicated T2DM population and finally performing various methods

which include; fluorescent microscopy, atomic force microscopy, microfluidics and an animal model, which would all provide a greater mechanistic understanding of how SAA impacts coagulation during inflammation, allowing for the understanding of its value in diagnosis and even therapy. Furthermore, quantification of CV values for SEM fibrin clots would be a valuable technique which could then be correlated with SAA values to gain a greater understanding of the relationship between the two parameters.

References:

2009. Diagnosis and classification of diabetes mellitus. *Diabetes Care,* 32 Suppl 1**,** S62-7.

2016. Global, regional, and national comparative risk assessment of 79 behavioural, environmental and occupational, and metabolic risks or clusters of risks, 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet,* 388**,** 1659-1724.

AGUIRRE, V., UCHIDA, T., YENUSH, L., DAVIS, R. & WHITE, M. F. 2000. The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). *J Biol Chem,* 275**,** 9047-54.

AHMED, A. M. 2002. History of diabetes mellitus. *Saudi Med J,* 23**,** 373-8.

AKBARI, M. & HASSAN-ZADEH, V. 2018. IL-6 signalling pathways and the development of type 2 diabetes. *Inflammopharmacology,* 26**,** 685-698.

ALLIN, K. H. & NORDESTGAARD, B. G. 2011. Elevated C-reactive protein in the diagnosis, prognosis, and cause of cancer. *Crit Rev Clin Lab Sci,* 48**,** 155-70.

ALZAHRANI, S. H. & AJJAN, R. A. 2010. Coagulation and fibrinolysis in diabetes. *Diab Vasc Dis Res,* 7**,** 260-73.

AMIR LEVY, Y., CIARALDI, T. P., MUDALIAR, S. R., PHILLIPS, S. A. & HENRY, R. R. 2015. Excessive secretion of IL-8 by skeletal muscle in type 2 diabetes impairs tube growth: potential role of PI3K and the Tie2 receptor. *Am J Physiol Endocrinol Metab,* 309**,** E22-34.

ARIENS, R. A., LAI, T. S., WEISEL, J. W., GREENBERG, C. S. & GRANT, P. J. 2002. Role of factor XIII in fibrin clot formation and effects of genetic polymorphisms. *Blood,* 100**,** 743-54.

AURON, P. E. & WEBB, A. C. 1994. Interleukin-1: a gene expression system regulated at multiple levels. *Eur Cytokine Netw,* 5**,** 573-92.

BADOLATO, R. 1994. Serum amyloid A is a chemoattractant: induction of migration, adhesion, and tissue infiltration of monocytes and polymorphonuclear leukocytes. *Journal of Experimental Medicine,* 180**,** 203-209.

BANNER, D. W., D'ARCY, A., JANES, W., GENTZ, R., SCHOENFELD, H. J., BROGER, C., LOETSCHER, H. & LESSLAUER, W. 1993. Crystal structure of the soluble human 55 kd TNF receptor-human TNF beta complex: implications for TNF receptor activation. *Cell,* 73**,** 431-45.

BEALL, C. J., MAHAJAN, S., KUHN, D. E. & KOLATTUKUDY, P. E. 1996. Site-directed mutagenesis of monocyte chemoattractant protein-1 identifies two regions of the polypeptide essential for biological activity. *Biochem J,* 313 (Pt 2)**,** 633-40.

BEIJERS, H. J., FERREIRA, I., SPRONK, H. M., BRAVENBOER, B., DEKKER, J. M., NIJPELS, G., TEN CATE, H. & STEHOUWER, C. D. 2012. Impaired glucose metabolism and type 2 diabetes are associated with hypercoagulability: potential role of central adiposity and low-grade inflammation--the Hoorn Study. *Thromb Res,* 129**,** 557-62.

BENOIST, C. & MATHIS, D. 1997. Cell death mediators in autoimmune diabetes--no shortage of suspects. *Cell,* 89**,** 1-3.

BERNARD, G. R., ELY, E. W., WRIGHT, T. J., FRAIZ, J., STASEK, J. E., JR., RUSSELL, J. A., MAYERS, I., ROSENFELD, B. A., MORRIS, P. E., YAN, S. B. & HELTERBRAND, J. D. 2001. Safety and dose relationship of recombinant human activated protein C for coagulopathy in severe sepsis. *Crit Care Med,* 29**,** 2051-9.

BERTRAM, M. Y., JASWAL, A. V. S., VAN WYK, V. P., LEVITT, N. S. & HOFMAN, K. J. 2013. The non-fatal disease burden caused by type 2 diabetes in South Africa, 2009. *Global Health Action,* 6**,** 19244.

BESTER, J., MATSHAILWE, C. & PRETORIUS, E. 2018. Simultaneous presence of hypercoagulation and increased clot lysis time due to IL-1beta, IL-6 and IL-8. *Cytokine,* 110**,** 237-242.

BESTER, J. & PRETORIUS, E. 2016. Effects of IL-1β, IL-6 and IL-8 on erythrocytes, platelets and clot viscoelasticity. *Scientific Reports,* 6**,** 32188.

BEUTLER, B. 2004. Innate immunity: an overview. *Mol Immunol,* 40**,** 845-59.

BIANCALANA, M. & KOIDE, S. 2010. Molecular mechanism of Thioflavin-T binding to amyloid fibrils. *Biochim Biophys Acta,* 1804**,** 1405-12.

BIANCALANA, M., MAKABE, K., KOIDE, A. & KOIDE, S. 2009. Molecular mechanism of thioflavin-T binding to the surface of beta-rich peptide self-assemblies. *J Mol Biol,* 385**,** 1052-63.

BLACK, R. A., RAUCH, C. T., KOZLOSKY, C. J., PESCHON, J. J., SLACK, J. L., WOLFSON, M. F., CASTNER, B. J., STOCKING, K. L., REDDY, P., SRINIVASAN, S., NELSON, N., BOIANI, N., SCHOOLEY, K. A., GERHART, M., DAVIS, R., FITZNER, J. N., JOHNSON, R. S., PAXTON, R. J., MARCH, C. J. & CERRETTI, D. P. 1997. A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature,* 385**,** 729-33.

BLAHA, V., ANDRYS, C., SMAHELOVA, A., KNIZEK, J., HYSPLER, R., SOLICHOVA, D., BLAHA, M. & ZADAK, Z. 2006. Effect of atorvastatin on soluble CD14, CD40 Ligand, sE- and sP-selectins

and MCP-1 in patients with type 2 diabetes mellitus: relationship to cholesterol turnover. *Pharmacol Res,* 54**,** 421-8.

BOMBELI, T. & SPAHN, D. R. 2004. Updates in perioperative coagulation: physiology and management of thromboembolism and haemorrhage. *Br J Anaesth,* 93**,** 275-87.

BORISSOFF, J. I., SPRONK, H. M. & TEN CATE, H. 2011. The hemostatic system as a modulator of atherosclerosis. *N Engl J Med,* 364**,** 1746-60.

BOSE, E. & HRAVNAK, M. 2015. Thromboelastography: A Practice Summary for Nurse Practitioners Treating Hemorrhage. *J Nurse Pract,* 11**,** 702-709.

BOZINOVSKI, S., HUTCHINSON, A., THOMPSON, M., MACGREGOR, L., BLACK, J., GIANNAKIS, E., KARLSSON, A. S., SILVESTRINI, R., SMALLWOOD, D., VLAHOS, R., IRVING, L. B. & ANDERSON, G. P. 2008. Serum amyloid a is a biomarker of acute exacerbations of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 177**,** 269-78.

BRAATVEDT, G. D., GAMBLE, G., OCKELFORD, P., BAGG, W., FERRI, C. & DESIDERI, G. 2001. The Influences of Obesity and Glycemic Control on Endothelial Activation in Patients with Type 2 Diabetes. *The Journal of Clinical Endocrinology & Metabolism,* 86**,** 5491-5497.

BRADSHAW, D., NORMAN, R., PIETERSE, D. & LEVITT, N. S. 2007. Estimating the burden of disease attributable to diabetes in South Africa in 2000. *S Afr Med J,* 97**,** 700-6.

BRAY, C., BELL, L. N., LIANG, H., HAYKAL, R., KAIKSOW, F., MAZZA, J. J. & YALE, S. H. 2016. Erythrocyte Sedimentation Rate and C-reactive Protein Measurements and Their Relevance in Clinical Medicine. *Wmj,* 115**,** 317-21.

BRUNO, G., RUNZO, C., CAVALLO-PERIN, P., MERLETTI, F., RIVETTI, M., PINACH, S., NOVELLI, G., TROVATI, M., CERUTTI, F. & PAGANO, G. 2005. Incidence of type 1 and type 2 diabetes in adults aged 30-49 years: the population-based registry in the province of Turin, Italy. *Diabetes Care,* 28**,** 2613-9.

BURKE, H. B. 2016. Predicting Clinical Outcomes Using Molecular Biomarkers. *Biomark Cancer,* 8**,** 89-99.

CABANA, V. G., REARDON, C. A., WEI, B., LUKENS, J. R. & GETZ, G. S. 1999. SAA-only HDL formed during the acute phase response in apoA-I+/+ and apoA-I-/- mice. *J Lipid Res,* 40**,** 1090-103.

CAI, K., QI, D., HOU, X., WANG, O., CHEN, J., DENG, B., QIAN, L., LIU, X. & LE, Y. 2011. MCP-1 upregulates amylin expression in murine pancreatic β cells through ERK/JNK-AP1 and NF-κB related signaling pathways independent of CCR2. *PLoS One,* 6**,** e19559.

CARLSEN, E., FLATMARK, A. & PRYDZ, H. 1988. Cytokine-induced procoagulant activity in monocytes and endothelial cells. Further enhancement by cyclosporine. *Transplantation,* 46**,** 575-80.

CARR, M. E. 2001. Diabetes mellitus: a hypercoagulable state. *J Diabetes Complications,* 15**,** 44-54.

CATALAN, V., GOMEZ-AMBROSI, J., RAMIREZ, B., ROTELLAR, F., PASTOR, C., SILVA, C., RODRIGUEZ, A., GIL, M. J., CIENFUEGOS, J. A. & FRUHBECK, G. 2007. Proinflammatory cytokines in obesity: impact of type 2 diabetes mellitus and gastric bypass. *Obes Surg,* 17**,** 1464-74.

CEFALU, W. T. 2009. Inflammation, insulin resistance, and type 2 diabetes: back to the future? *Diabetes,* 58**,** 307-8.

CELI, A., PELLEGRINI, G., LORENZET, R., DE BLASI, A., READY, N., FURIE, B. C. & FURIE, B. 1994. P-selectin induces the expression of tissue factor on monocytes. *Proc Natl Acad Sci U S A,* 91**,** 8767-71.

CERF, M. E. 2013. Beta cell dysfunction and insulin resistance. *Front Endocrinol (Lausanne),* 4**,** 37.

CHACON, M. R., FERNANDEZ-REAL, J. M., RICHART, C., MEGIA, A., GOMEZ, J. M., MIRANDA, M., CAUBET, E., PASTOR, R., MASDEVALL, C., VILARRASA, N., RICARD, W. & VENDRELL, J. 2007. Monocyte chemoattractant protein-1 in obesity and type 2 diabetes. Insulin sensitivity study. *Obesity (Silver Spring),* 15**,** 664-72.

CHEN, L., MAGLIANO, D. J. & ZIMMET, P. Z. 2011. The worldwide epidemiology of type 2 diabetes mellitus--present and future perspectives. *Nat Rev Endocrinol,* 8**,** 228-36.

CHITI, F. & DOBSON, C. M. 2006. Protein misfolding, functional amyloid, and human disease. *Annu Rev Biochem,* 75**,** 333-66.

CHIU, J. J., WUNG, B. S., SHYY, J. Y., HSIEH, H. J. & WANG, D. L. 1997. Reactive oxygen species are involved in shear stress-induced intercellular adhesion molecule-1 expression in endothelial cells. *Arterioscler Thromb Vasc Biol,* 17**,** 3570-7.

CHOI, G., SCHULTZ, M. J., LEVI, M. & VAN DER POLL, T. 2006. The relationship between inflammation and the coagulation system. *Swiss Med Wkly,* 136**,** 139-44.

CICARELLI, D. D., VIEIRA, J. E. & BENSEÑOR, F. E. 2008. Comparison of C-reactive protein and serum amyloid a protein in septic shock patients. *Mediators Inflamm,* 2008**,** 631414.

CIMINI, F. A., BARCHETTA, I., PORZIA, A., MAINIERO, F., COSTANTINO, C., BERTOCCINI, L., CECCARELLI, V., MORINI, S., BARONI, M. G., LENZI, A. & CAVALLO, M. G. 2017.

Circulating IL-8 levels are increased in patients with type 2 diabetes and associated with worse inflammatory and cardiometabolic profile. *Acta Diabetol,* 54**,** 961-967.

CNOP, M., LANDCHILD, M. J., VIDAL, J., HAVEL, P. J., KNOWLES, N. G., CARR, D. R., WANG, F., HULL, R. L., BOYKO, E. J., RETZLAFF, B. M., WALDEN, C. E., KNOPP, R. H. & KAHN, S. E. 2002. The concurrent accumulation of intra-abdominal and subcutaneous fat explains the association between insulin resistance and plasma leptin concentrations : distinct metabolic effects of two fat compartments. *Diabetes,* 51**,** 1005-15.

CNOP, M., WELSH, N., JONAS, J. C., JORNS, A., LENZEN, S. & EIZIRIK, D. L. 2005. Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. *Diabetes,* 54 Suppl 2**,** S97-107.

COLLER, B. S. & SHATTIL, S. J. 2008. The GPIIb/IIIa (integrin alphaIIbbeta3) odyssey: a technology-driven saga of a receptor with twists, turns, and even a bend. *Blood,* 112**,** 3011-25.

CONWAY, E. M. 2012. Thrombomodulin and its role in inflammation. *Semin Immunopathol,* 34**,** 107-25.

COOK-MILLS, J. M., MARCHESE, M. E. & ABDALA-VALENCIA, H. 2011. Vascular cell adhesion molecule-1 expression and signaling during disease: regulation by reactive oxygen species and antioxidants. *Antioxid Redox Signal,* 15**,** 1607-38.

COX, C. L., STANHOPE, K. L., SCHWARZ, J. M., GRAHAM, J. L., HATCHER, B., GRIFFEN, S. C., BREMER, A. A., BERGLUND, L., MCGAHAN, J. P., KEIM, N. L. & HAVEL, P. J. 2011. Circulating concentrations of monocyte chemoattractant protein-1, plasminogen activator inhibitor-1, and soluble leukocyte adhesion molecule-1 in overweight/obese men and women consuming fructose- or glucose-sweetened beverages for 10 weeks. *J Clin Endocrinol Metab,* 96**,** E2034-8.

CRAY, C., ZAIAS, J. & ALTMAN, N. H. 2009. Acute phase response in animals: a review. *Comp Med,* 59**,** 517-26.

CREELY, S. J., MCTERNAN, P. G., KUSMINSKI, C. M., FISHER F, M., DA SILVA, N. F., KHANOLKAR, M., EVANS, M., HARTE, A. L. & KUMAR, S. 2007. Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes. *Am J Physiol Endocrinol Metab,* 292**,** E740-7.

DE BEER, M. C., WEBB, N. R., WROBLEWSKI, J. M., NOFFSINGER, V. P., RATERI, D. L., JI, A., VAN DER WESTHUYZEN, D. R. & DE BEER, F. C. 2010. Impact of serum amyloid A on high density lipoprotein composition and levels. *J Lipid Res,* 51**,** 3117-25.

DE BEER, M. C., YUAN, T., KINDY, M. S., ASZTALOS, B. F., ROHEIM, P. S. & DE BEER, F. C. 1995. Characterization of constitutive human serum amyloid A protein (SAA4) as an apolipoprotein. *J Lipid Res,* 36**,** 526-34.

DE VRIESE, A. S., VERBEUREN, T. J., VAN DE VOORDE, J., LAMEIRE, N. H. & VANHOUTTE, P. M. 2000. Endothelial dysfunction in diabetes. *Br J Pharmacol,* 130**,** 963-74.

DELANGHE, J. R., LANGLOIS, M. R., DE BACQUER, D., MAK, R., CAPEL, P., VAN RENTERGHEM, L. & DE BACKER, G. 2002. Discriminative value of serum amyloid A and other acute-phase proteins for coronary heart disease. *Atherosclerosis,* 160**,** 471-6.

DESHMANE, S. L., KREMLEV, S., AMINI, S. & SAWAYA, B. E. 2009. Monocyte chemoattractant protein-1 (MCP-1): an overview. *J Interferon Cytokine Res,* 29**,** 313-26.

DEVARAJ, S. & JIALAL, I. 2000. Low-density lipoprotein postsecretory modification, monocyte function, and circulating adhesion molecules in type 2 diabetic patients with and without macrovascular complications: the effect of alpha-tocopherol supplementation. *Circulation,* 102**,** 191-6.

DINARELLO, C. A. 1996. Biologic basis for interleukin-1 in disease. *Blood,* 87**,** 2095-147.

DONATH, M. Y., STORLING, J., MAEDLER, K. & MANDRUP-POULSEN, T. 2003. Inflammatory mediators and islet beta-cell failure: a link between type 1 and type 2 diabetes. *J Mol Med (Berl),* 81**,** 455-70.

DUAN, J., CHUNG, H., TROY, E. & KASPER, D. L. 2010. Microbial colonization drives expansion of IL-1 receptor 1-expressing and IL-17-producing gamma/delta T cells. *Cell Host Microbe,* 7**,** 140-50.

EBEID, S. A., SADEK, N. A., EL-BENHAWY, S. A. & MAHMOUD, G. N. 2014. Plasma substance P and soluble P-selectin as biomarkers of β-thalassemia induced hypercoagulability. *Alexandria Journal of Medicine,* 50**,** 107-111.

ECKEL, R. H., KAHN, S. E., FERRANNINI, E., GOLDFINE, A. B., NATHAN, D. M., SCHWARTZ, M. W., SMITH, R. J. & SMITH, S. R. 2011. Obesity and Type 2 Diabetes: What Can Be Unified and What Needs to Be Individualized? *Diabetes Care,* 34**,** 1424-1430.

EHSES, J. A., PERREN, A., EPPLER, E., RIBAUX, P., POSPISILIK, J. A., MAOR-CAHN, R., GUERIPEL, X., ELLINGSGAARD, H., SCHNEIDER, M. K., BIOLLAZ, G., FONTANA, A., REINECKE, M., HOMO-DELARCHE, F. & DONATH, M. Y. 2007. Increased number of islet-associated macrophages in type 2 diabetes. *Diabetes,* 56**,** 2356-70.

EIZIRIK, D. L. & CNOP, M. 2010. ER stress in pancreatic beta cells: the thin red line between adaptation and failure. *Sci Signal,* 3**,** pe7.

EKLUND, K. K., NIEMI, K. & KOVANEN, P. T. 2012. Immune functions of serum amyloid A. *Crit Rev Immunol,* 32**,** 335-48.

ENGELMAN, J. A., BERG, A. H., LEWIS, R. Y., LISANTI, M. P. & SCHERER, P. E. 2000. Tumor necrosis factor alpha-mediated insulin resistance, but not dedifferentiation, is abrogated by MEK1/2 inhibitors in 3T3-L1 adipocytes. *Mol Endocrinol,* 14**,** 1557-69.

ERNOFSSON, M. & SIEGBAHN, A. 1996. Platelet-derived growth factor-BB and monocyte chemotactic protein-1 induce human peripheral blood monocytes to express tissue factor. *Thromb Res,* 83**,** 307-20.

ESMON, C. T. 2005. The interactions between inflammation and coagulation. *Br J Haematol,* 131**,** 417-30.

ESSAYAN, D. M., FOX, C. C., LEVI-SCHAFFER, F., ALAM, R. & ROSENWASSER, L. J. 1998. Biologic activities of IL-1 and its role in human disease. *Journal of Allergy and Clinical Immunology,* 102**,** 344-350.

ETIENNE-MANNEVILLE, S., CHAVEROT, N., STROSBERG, A. D. & COURAUD, P. O. 1999. ICAM-1-coupled signaling pathways in astrocytes converge to cyclic AMP response element-binding protein phosphorylation and TNF-alpha secretion. *J Immunol,* 163**,** 668-74.

FALATI, S., GROSS, P., MERRILL-SKOLOFF, G., FURIE, B. C. & FURIE, B. 2002. Real-time in vivo imaging of platelets, tissue factor and fibrin during arterial thrombus formation in the mouse. *Nat Med,* 8**,** 1175-81.

FATY, A., FERRE, P. & COMMANS, S. 2012. The acute phase protein Serum Amyloid A induces lipolysis and inflammation in human adipocytes through distinct pathways. *PLoS One,* 7**,** e34031.

FEINSTEIN, R., KANETY, H., PAPA, M. Z., LUNENFELD, B. & KARASIK, A. 1993. Tumor necrosis factor-alpha suppresses insulin-induced tyrosine phosphorylation of insulin receptor and its substrates. *J Biol Chem,* 268**,** 26055-8.

FELDMANN, M., BRENNAN, F. M., ELLIOTT, M., KATSIKIS, P. & MAINI, R. N. 1994. TNF alpha as a therapeutic target in rheumatoid arthritis. *Circ Shock,* 43**,** 179-84.

FESTA, A., D'AGOSTINO, R., TRACY, R. P. & HAFFNER, S. M. 2002. Elevated Levels of Acute-Phase Proteins and Plasminogen Activator Inhibitor-1 Predict the Development of Type 2 Diabetes. *The Insulin Resistance Atherosclerosis Study,* 51**,** 1131-1137.

FINGAR, V. H., TABER, S. W., BUSCHEMEYER, W. C., TEN TIJE, A., CERRITO, P. B., TSENG, M., GUO, H., JOHNSTON, M. N. & WIEMAN, T. J. 1997. Constitutive and stimulated expression of ICAM-1 protein on pulmonary endothelial cells in vivo. *Microvasc Res,* 54**,** 135-44.

FLYNN, J. L., GOLDSTEIN, M. M., CHAN, J., TRIEBOLD, K. J., PFEFFER, K., LOWENSTEIN, C. J., SCHREIBER, R., MAK, T. W. & BLOOM, B. R. 1995. Tumor necrosis factor-alpha is required in the protective immune response against Mycobacterium tuberculosis in mice. *Immunity,* 2**,** 561-72.

FOLEY, J. H. & CONWAY, E. M. 2016. Cross Talk Pathways Between Coagulation and Inflammation. *Circ Res,* 118**,** 1392-408.

FOWLER, M. J. 2008. Microvascular and Macrovascular Complications of Diabetes. *Clinical Diabetes,* 26**,** 77-82.

FRAME, N. M. & GURSKY, O. 2016. Structure of serum amyloid A suggests a mechanism for selective lipoprotein binding and functions: SAA as a hub in macromolecular interaction networks. *FEBS Lett,* 590**,** 866-79.

FRANCIS, J. L., FRANCIS, D. A. & GUNATHILAGAN, G. J. 1994. Assessment of hypercoagulability in patients with cancer using the Sonoclot Analyzer and thromboelastography. *Thromb Res,* 74**,** 335-46.

FROST, R. A., NYSTROM, G. J. & LANG, C. H. 2002. Lipopolysaccharide regulates proinflammatory cytokine expression in mouse myoblasts and skeletal muscle. *Am J Physiol Regul Integr Comp Physiol,* 283**,** R698-709.

FUKUSHIMA, K., OGAWA, H., KITAYAMA, T., YAMADA, T., NAITO, H., FUNAYAMA, Y., SHIBATA, C., MATSUNO, S. & SASAKI, I. 2000. Essential role of epithelial serum amyloid a (SAA) in lipopolysaccharide (LPS)-associated mucosal inflammation. *Gastroenterology,* 118**,** A1342.

GALE, A. J. 2011. Current understanding of hemostasis. *Toxicol Pathol,* 39**,** 273-80.

GAULDIE, J., RICHARDS, C., HARNISH, D., LANSDORP, P. & BAUMANN, H. 1987. Interferon beta 2/B-cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. *Proc Natl Acad Sci U S A,* 84**,** 7251-5.

GAUR, U. & AGGARWAL, B. B. 2003. Regulation of proliferation, survival and apoptosis by members of the TNF superfamily. *Biochem Pharmacol,* 66**,** 1403-8.

GERSHOV, D., KIM, S., BROT, N. & ELKON, K. B. 2000. C-Reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains an antiinflammatory innate immune response: implications for systemic autoimmunity. *J Exp Med,* 192**,** 1353-64.

GETZ, G. S., KRISHACK, P. A. & REARDON, C. A. 2016. Serum amyloid A and atherosclerosis. *Curr Opin Lipidol,* 27**,** 531-5.

GILBERT, E. R. & LIU, D. 2012. Epigenetics: the missing link to understanding beta-cell dysfunction in the pathogenesis of type 2 diabetes. *Epigenetics,* 7**,** 841-52.

GIMBRONE, M. A., JR., OBIN, M. S., BROCK, A. F., LUIS, E. A., HASS, P. E., HEBERT, C. A., YIP, Y. K., LEUNG, D. W., LOWE, D. G., KOHR, W. J. & ET AL. 1989. Endothelial interleukin-8: a novel inhibitor of leukocyte-endothelial interactions. *Science,* 246**,** 1601-3.

GOKULAKRISHNAN, K., DEEPA, R., MOHAN, V. & GROSS, M. D. 2006. Soluble P-selectin and CD40L levels in subjects with prediabetes, diabetes mellitus, and metabolic syndrome--the Chennai Urban Rural Epidemiology Study. *Metabolism,* 55**,** 237-42.

GRIFFIN, M. E., MARCUCCI, M. J., CLINE, G. W., BELL, K., BARUCCI, N., LEE, D., GOODYEAR, L. J., KRAEGEN, E. W., WHITE, M. F. & SHULMAN, G. I. 1999. Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade. *Diabetes,* 48**,** 1270-4.

GRIFFITHS, K., PAZDERSKA, A., AHMED, M., MCGOWAN, A., MAXWELL, A. P., MCENENY, J., GIBNEY, J. & MCKAY, G. J. 2017. Type 2 Diabetes in Young Females Results in Increased Serum Amyloid A and Changes to Features of High Density Lipoproteins in Both HDL2 and HDL3. *J Diabetes Res,* 2017**,** 1314864.

GRUYS, E., TOUSSAINT, M. J., NIEWOLD, T. A. & KOOPMANS, S. J. 2005. Acute phase reaction and acute phase proteins. *J Zhejiang Univ Sci B,* 6**,** 1045-56.

GUEST, C. B., PARK, M. J., JOHNSON, D. R. & FREUND, G. G. 2008. The implication of proinflammatory cytokines in type 2 diabetes. *Front Biosci,* 13**,** 5187-94.

GUO, S., AL-SADI, R., SAID, H. M. & MA, T. Y. 2013. Lipopolysaccharide causes an increase in intestinal tight junction permeability in vitro and in vivo by inducing enterocyte membrane expression and localization of TLR-4 and CD14. *Am J Pathol,* 182**,** 375-87.

GUPTA, A. & PANSARI, K. 2003. The association between blood coagulation markers, atherothrombosis and dementia. *Int J Clin Pract,* 57**,** 107-11.

GUPTA, A., WATKINS, A., THOMAS, P., MAJER, R., HABUBI, N., MORRIS, G. & PANSARI, K. 2005. Coagulation and inflammatory markers in Alzheimer's and vascular dementia. *Int J Clin Pract,* 59**,** 52-7.

GURGUL-CONVEY, E., MEHMETI, I., LORTZ, S. & LENZEN, S. 2011. Cytokine toxicity in insulin-producing cells is mediated by nitro-oxidative stress-induced hydroxyl radical formation in mitochondria. *J Mol Med (Berl),* 89**,** 785-98.

HAN, C. Y., TANG, C., GUEVARA, M. E., WEI, H., WIETECHA, T., SHAO, B., SUBRAMANIAN, S., OMER, M., WANG, S., O'BRIEN, K. D., MARCOVINA, S. M., WIGHT, T. N., VAISAR, T., DE BEER, M. C., DE BEER, F. C., OSBORNE, W. R., ELKON, K. B. & CHAIT, A. 2016. Serum amyloid A impairs the antiinflammatory properties of HDL. *J Clin Invest,* 126**,** 266-81.

HARMAN-BOEHM, I., BLUHER, M., REDEL, H., SION-VARDY, N., OVADIA, S., AVINOACH, E., SHAI, I., KLOTING, N., STUMVOLL, M., BASHAN, N. & RUDICH, A. 2007. Macrophage infiltration into omental versus subcutaneous fat across different populations: effect of regional adiposity and the comorbidities of obesity. *J Clin Endocrinol Metab,* 92**,** 2240-7.

HAWRYLOWICZ, C. M., HOWELLS, G. L. & FELDMANN, M. 1991. Platelet-derived interleukin 1 induces human endothelial adhesion molecule expression and cytokine production. *J Exp Med,* 174**,** 785-90.

HEEMSKERK, J. W., BEVERS, E. M. & LINDHOUT, T. 2002. Platelet activation and blood coagulation. *Thromb Haemost,* 88**,** 186-93.

HEINRICH, J., SCHULTE, H., SCHONFELD, R., KOHLER, E. & ASSMANN, G. 1995. Association of variables of coagulation, fibrinolysis and acute-phase with atherosclerosis in coronary and peripheral arteries and those arteries supplying the brain. *Thromb Haemost,* 73**,** 374-9.

HEINRICH, P. C., CASTELL, J. V. & ANDUS, T. 1990. Interleukin-6 and the acute phase response. *Biochem J,* 265**,** 621-36.

HERBERT, J. M., SAVI, P., LAPLACE, M. C. & LALE, A. 1992. IL-4 inhibits LPS-, IL-1 beta- and TNF alpha-induced expression of tissue factor in endothelial cells and monocytes. *FEBS Lett,* 310**,** 31-3.

HERCZENIK, E. & GEBBINK, M. F. 2008. Molecular and cellular aspects of protein misfolding and disease. *Faseb j,* 22**,** 2115-33.

HESSELVIK, J. F., BLOMBACK, M., BRODIN, B. & MALLER, R. 1989. Coagulation, fibrinolysis, and kallikrein systems in sepsis: relation to outcome. *Crit Care Med,* 17**,** 724-33.

HILDEBRAND, F., HUBBARD, W. J., CHOUDHRY, M. A., FRINK, M., PAPE, H. C., KUNKEL, S. L. & CHAUDRY, I. H. 2006. Kupffer cells and their mediators: the culprits in producing distant organ damage after trauma-hemorrhage. *Am J Pathol,* 169**,** 784-94.

HIRANO, T., YASUKAWA, K., HARADA, H., TAGA, T., WATANABE, Y., MATSUDA, T., KASHIWAMURA, S., NAKAJIMA, K., KOYAMA, K., IWAMATSU, A. & ET AL. 1986. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature,* 324**,** 73-6.

HOLMAN, N., YOUNG, B. & GADSBY, R. 2015. Current prevalence of Type 1 and Type 2 diabetes in adults and children in the UK. *Diabet Med,* 32**,** 1119-20.

HORADAGODA, N. U., KNOX, K. M., GIBBS, H. A., REID, S. W., HORADAGODA, A., EDWARDS, S. E. & ECKERSALL, P. D. 1999. Acute phase proteins in cattle: discrimination between acute and chronic inflammation. *Vet Rec,* 144**,** 437-41.

HOSOGAI, N., FUKUHARA, A., OSHIMA, K., MIYATA, Y., TANAKA, S., SEGAWA, K., FURUKAWA, S., TOCHINO, Y., KOMURO, R., MATSUDA, M. & SHIMOMURA, I. 2007. Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. *Diabetes,* 56**,** 901-11.

HOTAMISLIGIL, G. S., SHARGILL, N. S. & SPIEGELMAN, B. M. 1993. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science,* 259**,** 87-91.

HOWLEY, I. W., HAUT, E. R., JACOBS, L., MORRISON, J. J. & SCALEA, T. M. 2018. Is thromboelastography (TEG)-based resuscitation better than empirical 1:1 transfusion? *Trauma Surgery & Acute Care Open,* 3**,** e000140.

HSIEH, C. H., FRINK, M., HSIEH, Y. C., KAN, W. H., HSU, J. T., SCHWACHA, M. G., CHOUDHRY, M. A. & CHAUDRY, I. H. 2008. The role of MIP-1 alpha in the development of systemic inflammatory response and organ injury following trauma hemorrhage. *J Immunol,* 181**,** 2806-12.

HSU, H., XIONG, J. & GOEDDEL, D. V. 1995. The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. *Cell,* 81**,** 495-504.

HSU, H. Y. & WEN, M. H. 2002. Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. *J Biol Chem,* 277**,** 22131-9.

HU, F. B., MEIGS, J. B., LI, T. Y., RIFAI, N. & MANSON, J. E. 2004. Inflammatory markers and risk of developing type 2 diabetes in women. *Diabetes,* 53**,** 693-700.

HUA, S., SONG, C., GECZY, C. L., FREEDMAN, S. B. & WITTING, P. K. 2009. A role for acute-phase serum amyloid A and high-density lipoprotein in oxidative stress, endothelial dysfunction and atherosclerosis. *Redox Report,* 14**,** 187-196.

HUBBARD, A. K. & ROTHLEIN, R. 2000. Intercellular adhesion molecule-1 (ICAM-1) expression and cell signaling cascades. *Free Radic Biol Med,* 28**,** 1379-86.

HULKA, B. S. & WILCOSKY, T. 1988. Biological markers in epidemiologic research. *Arch Environ Health,* 43**,** 83-9.

HURLEY, J. C. 1995. Endotoxemia: methods of detection and clinical correlates. *Clin Microbiol Rev,* 8**,** 268-92.

HWANG, H., HWANG, B.-Y. & BUENO, J. 2018. Biomarkers in Infectious Diseases. *Disease Markers,* 2018**,** 2.

IBRAHIM, H. & KLEIMAN, N. S. 2017. Platelet pathophysiology, pharmacology, and function in coronary artery disease. *Coron Artery Dis,* 28**,** 614-623.

IDF. 2018. *What is Diabetes*
 [Online]. Available: https://www.idf.org/aboutdiabetes/what-is-diabetes/facts-figures.html [Accessed 08/10/2018 2018].

JAIN, S., GAUTAM, V. & NASEEM, S. 2011. Acute-phase proteins: As diagnostic tool. *J Pharm Bioallied Sci,* 3**,** 118-27.

JAVAID, K., RAHMAN, A., ANWAR, K. N., FREY, R. S., MINSHALL, R. D. & MALIK, A. B. 2003. Tumor necrosis factor-alpha induces early-onset endothelial adhesivity by protein kinase Czeta-dependent activation of intercellular adhesion molecule-1. *Circ Res,* 92**,** 1089-97.

JAYARAMAN, S., HAUPT, C. & GURSKY, O. 2015. Thermal transitions in serum amyloid A in solution and on the lipid: implications for structure and stability of acute-phase HDL. *J Lipid Res,* 56**,** 1531-42.

JOHNSON, B. D., KIP, K. E., MARROQUIN, O. C., RIDKER, P. M., KELSEY, S. F., SHAW, L. J., PEPINE, C. J., SHARAF, B., BAIREY MERZ, C. N., SOPKO, G., OLSON, M. B. & REIS, S. E. 2004. Serum amyloid A as a predictor of coronary artery disease and cardiovascular outcome in women: the National Heart, Lung, and Blood Institute-Sponsored Women's Ischemia Syndrome Evaluation (WISE). *Circulation,* 109**,** 726-32.

JOUBERT, J., NORMAN, R., BRADSHAW, D., GOEDECKE, J. H., STEYN, N. P. & PUOANE, T. 2007. Estimating the burden of disease attributable to excess body weight in South Africa in 2000. *S Afr Med J,* 97**,** 683-90.

KAMATH, S., BLANN, A. D., CAINE, G. J., GURNEY, D., CHIN, B. S. & LIP, G. Y. 2002. Platelet P-selectin levels in relation to plasma soluble P-selectin and beta-thromboglobulin levels in atrial fibrillation. *Stroke,* 33**,** 1237-42.

KAMIMURA, D., ISHIHARA, K. & HIRANO, T. 2003. IL-6 signal transduction and its physiological roles: the signal orchestration model. *Rev Physiol Biochem Pharmacol,* 149**,** 1-38.

KANDA, H., TATEYA, S., TAMORI, Y., KOTANI, K., HIASA, K., KITAZAWA, R., KITAZAWA, S., MIYACHI, H., MAEDA, S., EGASHIRA, K. & KASUGA, M. 2006. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J Clin Invest,* 116**,** 1494-505.

KANETO, H., KATAKAMI, N., MATSUHISA, M. & MATSUOKA, T. A. 2010. Role of reactive oxygen species in the progression of type 2 diabetes and atherosclerosis. *Mediators Inflamm,* 2010**,** 453892.

KARIMI, Z., KAHE, F., JAMIL, A., MARSZALEK, J., GHANBARI, A., AFARIDEH, M., KHAJEH, E., NOSHAD, S., ESTEGHAMATI, A. & CHI, G. 2018. Intercellular adhesion molecule-1 in diabetic patients with and without microalbuminuria. *Diabetes Metab Syndr,* 12**,** 365-368.

KATZ, F. E., PARKAR, M., STANLEY, K., MURRAY, L. J., CLARK, E. A. & GREAVES, M. F. 1985. Chromosome mapping of cell membrane antigens expressed on activated B cells. *European Journal of Immunology,* 15**,** 103-106.

KELL, D. B. & PRETORIUS, E. 2015. The simultaneous occurrence of both hypercoagulability and hypofibrinolysis in blood and serum during systemic inflammation, and the roles of iron and fibrin(ogen). *Integr Biol (Camb),* 7**,** 24-52.

KELL, D. B. & PRETORIUS, E. 2016. Substoichiometric molecular control and amplification of the initiation and nature of amyloid fibril formation: lessons from and for blood clotting. BioRXiv preprint.

KELL, D. B. & PRETORIUS, E. 2018. No effects without causes: the Iron Dysregulation and Dormant Microbes hypothesis for chronic, inflammatory diseases. *Biol Rev Camb Philos Soc,* 93**,** 1518-1557.

KHONDKARYAN, L., MARGARYAN, S., POGHOSYAN, D. & MANUKYAN, G. 2018. Impaired Inflammatory Response to LPS in Type 2 Diabetes Mellitus. *Int J Inflam,* 2018**,** 2157434.

KISILEVSKY, R. & MANLEY, P. N. 2012. Acute-phase serum amyloid A: perspectives on its physiological and pathological roles. *Amyloid,* 19**,** 5-14.

KRISTIANSEN, O. P. & MANDRUP-POULSEN, T. 2005. Interleukin-6 and Diabetes. *The Good, the Bad, or the Indifferent?,* 54**,** S114-S124.

KUMON, Y., SUEHIRO, T., ITAHARA, T., IKEDA, Y. & HASHIMOTO, K. 1994. Serum amyloid A protein in patients with non-insulin-dependent diabetes mellitus. *Clinical Biochemistry,* 27.

KURITA, N., YAMASHIRO, K., KUROKI, T., TANAKA, R., UENO, Y., URABE, T., NOMOTO, K., TAKAHASHI, T., TSUJI, H., ASAHARA, T., YAMASHIRO, Y. & HATTORI, N. 2017. Gut dysbiosis induces lipopolysaccharide-mediated inflammation after cerebral ischemia in type 2 diabetic mice. *Journal of the Neurological Sciences,* 381**,** 875.

LASNE, D., JUDE, B. & SUSEN, S. 2006. From normal to pathological hemostasis. *Can J Anaesth,* 53**,** S2-11.

LEVI, M. & VAN DER POLL, T. 2010. Inflammation and coagulation. *Crit Care Med,* 38**,** S26-34.

LI, Z., DELANEY, M. K., O'BRIEN, K. A. & DU, X. 2010. Signaling during platelet adhesion and activation. *Arterioscler Thromb Vasc Biol,* 30**,** 2341-9.

LIGTENBERG, P. C., HOEPELMAN, I. M., OUDE SOGTOEN, G. A., DEKKER, A. W., VAN DER TWEEL, I., ROZENBERG-ARSKA, M. & VERHOEF, J. 1991. C-reactive protein in the diagnosis and management of infections in granulocytopenic and non-granulocytopenic patients. *Eur J Clin Microbiol Infect Dis,* 10**,** 25-31.

LIU, J. J., YEOH, L. Y., SUM, C. F., TAVINTHARAN, S., NG, X. W., LIU, S., LEE, S. B., TANG, W. E. & LIM, S. C. 2015. Vascular cell adhesion molecule-1, but not intercellular adhesion molecule-1, is associated with diabetic kidney disease in Asians with type 2 diabetes. *J Diabetes Complications,* 29**,** 707-12.

LOPEZ-CASTEJON, G. & BROUGH, D. 2011. Understanding the mechanism of IL-1β secretion. *Cytokine Growth Factor Rev,* 22**,** 189-95.

LOW WANG, C. C., HESS, C. N., HIATT, W. R. & GOLDFINE, A. B. 2016. Clinical Update: Cardiovascular Disease in Diabetes Mellitus: Atherosclerotic Cardiovascular Disease and Heart Failure in Type 2 Diabetes Mellitus - Mechanisms, Management, and Clinical Considerations. *Circulation,* 133**,** 2459-502.

LUTOSŁAWSKA, G. 2012. Interleukin-6 As An Adipokine And Myokine: The Regulatory Role Of Cytokine In Adipose Tissue And Skeletal Muscle Metabolism. 13**,** 372.

MADEJ, M. P., TÖPFER, E., BORASCHI, D. & ITALIANI, P. 2017. Different Regulation of Interleukin-1 Production and Activity in Monocytes and Macrophages: Innate Memory as an Endogenous Mechanism of IL-1 Inhibition. *Front Pharmacol,* 8**,** 335.

MAEDLER, K., SERGEEV, P., RIS, F., OBERHOLZER, J., JOLLER-JEMELKA, H. I., SPINAS, G. A., KAISER, N., HALBAN, P. A. & DONATH, M. Y. 2002. Glucose-induced beta cell production of IL-1beta contributes to glucotoxicity in human pancreatic islets. *J Clin Invest,* 110**,** 851-60.

MAGNUSSON, K., SIMON, R., SJOLANDER, D., SIGURDSON, C. J., HAMMARSTROM, P. & NILSSON, K. P. 2014. Multimodal fluorescence microscopy of prion strain specific PrP deposits stained by thiophene-based amyloid ligands. *Prion,* 8**,** 319-29.

MAIER, R., WEGER, M., HALLER-SCHOBER, E. M., EL-SHABRAWI, Y., WEDRICH, A., THEISL, A., AIGNER, R., BARTH, A. & HAAS, A. 2008. Multiplex bead analysis of vitreous and serum concentrations of inflammatory and proangiogenic factors in diabetic patients. *Mol Vis,* 14**,** 637-43.

MALLE, E. & DE BEER, F. C. 1996. Human serum amyloid A (SAA) protein: a prominent acute-phase reactant for clinical practice. *Eur J Clin Invest,* 26**,** 427-35.

MARZI, C., HUTH, C., HERDER, C., BAUMERT, J., THORAND, B., RATHMANN, W., MEISINGER, C., WICHMANN, H. E., RODEN, M., PETERS, A., GRALLERT, H., KOENIG, W. & ILLIG, T. 2013. Acute-phase serum amyloid A protein and its implication in the development of type 2 diabetes in the KORA S4/F4 study. *Diabetes Care,* 36**,** 1321-6.

MATHERS, C. D. & LONCAR, D. 2006. Projections of global mortality and burden of disease from 2002 to 2030. *PLoS Med,* 3**,** e442.

MATTILA, P., MAJURI, M. L., MATTILA, P. S. & RENKONEN, R. 1992. TNF alpha-induced expression of endothelial adhesion molecules, ICAM-1 and VCAM-1, is linked to protein kinase C activation. *Scand J Immunol,* 36**,** 159-65.

MAURER, M. & VON STEBUT, E. 2004. Macrophage inflammatory protein-1. *Int J Biochem Cell Biol,* 36**,** 1882-6.

MAYEUX, R. 2004. Biomarkers: potential uses and limitations. *NeuroRx,* 1**,** 182-8.

MEDZHITOV, R. & JANEWAY, C., JR. 2000. Innate immunity. *N Engl J Med,* 343**,** 338-44.

MENKIN, V. 1941. ON THE MECHANISM OF ENHANCED DIABETES WITH INFLAMMATION. *American Journal of Physiology-Legacy Content,* 134**,** 517-541.

MICHEAU, O. & TSCHOPP, J. 2003. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell,* 114**,** 181-90.

MIGITA, K., ABIRU, S., NAKAMURA, M., KOMORI, A., YOSHIDA, Y., YOKOYAMA, T., DAIKOKU, M., UEKI, T., TAKII, Y., YANO, K., YASTUHASHI, H., EGUCHI, K. & ISHIBASHI, H. 2004. Lipopolysaccharide signaling induces serum amyloid A (SAA) synthesis in human hepatocytes in vitro. *FEBS Lett,* 569**,** 235-9.

MUÑOZ-CÁNOVES, P., SCHEELE, C., PEDERSEN, B. K. & SERRANO, A. L. 2013. Interleukin-6 myokine signaling in skeletal muscle: a double-edged sword? *Febs j,* 280**,** 4131-48.

MYERS, C. L., WERTHEIMER, S. J., SCHEMBRI-KING, J., PARKS, T. & WALLACE, R. W. 1992. Induction of ICAM-1 by TNF-alpha, IL-1 beta, and LPS in human endothelial cells after downregulation of PKC. *Am J Physiol,* 263**,** C767-72.

NAIKI, H., HIGUCHI, K., HOSOKAWA, M. & TAKEDA, T. 1989. Fluorometric determination of amyloid fibrils in vitro using the fluorescent dye, thioflavin T1. *Anal Biochem,* 177**,** 244-9.

NAYLOR, S. 2003. Biomarkers: current perspectives and future prospects AU - Naylor, Stephen. *Expert Review of Molecular Diagnostics,* 3**,** 525-529.

NG, M., FLEMING, T., ROBINSON, M., THOMSON, B., GRAETZ, N., MARGONO, C., MULLANY, E. C., BIRYUKOV, S., ABBAFATI, C., ABERA, S. F., ABRAHAM, J. P., ABU-RMEILEH, N. M., ACHOKI, T., ALBUHAIRAN, F. S., ALEMU, Z. A., ALFONSO, R., ALI, M. K., ALI, R., GUZMAN, N. A., AMMAR, W., ANWARI, P., BANERJEE, A., BARQUERA, S., BASU, S., BENNETT, D. A., BHUTTA, Z., BLORE, J., CABRAL, N., NONATO, I. C., CHANG, J. C., CHOWDHURY, R., COURVILLE, K. J., CRIQUI, M. H., CUNDIFF, D. K., DABHADKAR, K. C., DANDONA, L., DAVIS, A., DAYAMA, A., DHARMARATNE, S. D., DING, E. L., DURRANI, A. M., ESTEGHAMATI, A., FARZADFAR, F., FAY, D. F., FEIGIN, V. L., FLAXMAN, A., FOROUZANFAR, M. H., GOTO, A., GREEN, M. A., GUPTA, R., HAFEZI-NEJAD, N., HANKEY, G. J., HAREWOOD, H. C., HAVMOELLER, R., HAY, S., HERNANDEZ, L., HUSSEINI, A., IDRISOV, B. T., IKEDA, N., ISLAMI, F., JAHANGIR, E., JASSAL, S. K., JEE, S. H., JEFFREYS, M., JONAS, J. B., KABAGAMBE, E. K., KHALIFA, S. E., KENGNE, A. P., KHADER, Y. S., KHANG, Y. H., KIM, D., KIMOKOTI, R. W., KINGE, J. M., KOKUBO, Y., KOSEN, S., KWAN, G., LAI, T., LEINSALU, M., LI, Y., LIANG, X., LIU, S., LOGROSCINO, G., LOTUFO, P. A., LU, Y., MA, J., MAINOO, N. K., MENSAH, G. A., MERRIMAN, T. R., MOKDAD, A. H., MOSCHANDREAS, J., NAGHAVI, M., NAHEED, A., NAND, D., NARAYAN, K. M., NELSON, E. L., NEUHOUSER, M. L., NISAR, M. I., OHKUBO, T., OTI, S. O., PEDROZA, A., et al. 2014. Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet,* 384**,** 766-81.

NICAUD, V., EVANS, A., RUIDAVETS, J.-B., ARVEILER, D., LUC, G., MALLET, C., CAMBIEN, F., RICARD, S. & HERRMANN, S.-M. 1998. The P-Selectin Gene is Highly Polymorphic: Reduced Frequency of the Pro715 Allele Carriers in Patients with Myocardial Infarction. *Human Molecular Genetics,* 7**,** 1277-1284.

NICHOLSON-WELLER, A., MARCH, J. P., ROSEN, C. E., SPICER, D. B. & AUSTEN, K. F. 1985. Surface membrane expression by human blood leukocytes and platelets of decay-accelerating factor, a regulatory protein of the complement system. *Blood,* 65, 1237-44.

NIELSEN, V. G., AUDU, P., CANKOVIC, L., LYERLY, R. T., 3RD, STEENWYK, B. L., ARMSTEAD, V. & POWELL, G. 2007a. Qualitative thrombelastographic detection of tissue factor in human plasma. *Anesth Analg,* 104**,** 59-64.

NIELSEN, V. G., KIRKLIN, J. K., HOOGENDOORN, H., ELLIS, T. C. & HOLMAN, W. L. 2007b. Thrombelastographic method to quantify the contribution of factor XIII to coagulation kinetics. *Blood Coagul Fibrinolysis,* 18, 145-50.

NOTKINS, A. L. & LERNMARK, A. 2001. Autoimmune type 1 diabetes: resolved and unresolved issues. *J Clin Invest,* 108**,** 1247-52.

OLOKOBA, A. B., OBATERU, O. A. & OLOKOBA, L. B. 2012. Type 2 diabetes mellitus: a review of current trends. *Oman Med J,* 27**,** 269-73.

OSBORN, L., HESSION, C., TIZARD, R., VASSALLO, C., LUHOWSKYJ, S., CHI-ROSSO, G. & LOBB, R. 1989. Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. *Cell,* 59**,** 1203-11.

PADDOCK, S. W. 2000. Principles and practices of laser scanning confocal microscopy. *Mol Biotechnol,* 16**,** 127-49.

PAGE, M. J., BESTER, J. & PRETORIUS, E. 2018. The inflammatory effects of TNF-α and complement component 3 on coagulation. *Scientific Reports,* 8**,** 1812.

PAGE, M. J., THOMSON, G. J. A., NUNES, J. M., ENGELBRECHT, A.-M., NELL, T. A., DE VILLIERS, W. J. S., DE BEER, M. C., ENGELBRECHT, L., KELL, D. B. & PRETORIUS, E. 2019. Serum amyloid A binds to fibrin(ogen), promoting fibrin amyloid formation. *Scientific Reports,* 9**,** 3102.

PALABRICA, T., LOBB, R., FURIE, B. C., ARONOVITZ, M., BENJAMIN, C., HSU, Y. M., SAJER, S. A. & FURIE, B. 1992. Leukocyte accumulation promoting fibrin deposition is mediated in vivo by P-selectin on adherent platelets. *Nature,* 359**,** 848-51.

PALTA, S., SAROA, R. & PALTA, A. 2014. Overview of the coagulation system. *Indian J Anaesth,* 58**,** 515-23.

PANDEY, M., LOSKUTOFF, D. J. & SAMAD, F. 2005. Molecular mechanisms of tumor necrosis factor-alpha-mediated plasminogen activator inhibitor-1 expression in adipocytes. *Faseb j,* 19**,** 1317-9.

PANEE, J. 2012. Monocyte Chemoattractant Protein 1 (MCP-1) in obesity and diabetes. *Cytokine,* 60**,** 1-12.

PARAMESWARAN, N. & PATIAL, S. 2010. Tumor necrosis factor-α signaling in macrophages. *Crit Rev Eukaryot Gene Expr,* 20**,** 87-103.

PARK, J., MIN, J. S., KIM, B., CHAE, U. B., YUN, J. W., CHOI, M. S., KONG, I. K., CHANG, K. T. & LEE, D. S. 2015. Mitochondrial ROS govern the LPS-induced pro-inflammatory response in microglia cells by regulating MAPK and NF-kappaB pathways. *Neurosci Lett,* 584**,** 191-6.

PATALAKH, II & KUDINOV, S. O. 2008. Hemostasis system and inflammation acute-phase proteins in thrombogenic pathologies. *Ukr Biokhim Zh (1999),* 80**,** 5-12.

PATLAK, M. 2002. New weapons to combat an ancient disease: treating diabetes. *Faseb j,* 16**,** 1853.

PEPYS, M. B. & BALTZ, M. L. 1983. Acute phase proteins with special reference to C-reactive protein and related proteins (pentaxins) and serum amyloid A protein. *Adv Immunol,* 34**,** 141-212.

PEPYS, M. B. & HIRSCHFIELD, G. M. 2003. C-reactive protein: a critical update. *The Journal of Clinical Investigation,* 111**,** 1805-1812.

PERERA, F. P. & WEINSTEIN, I. B. 2000. Molecular epidemiology: recent advances and future directions. *Carcinogenesis,* 21**,** 517-24.

PETERS, V. A., JOESTING, J. J. & FREUND, G. G. 2013. IL-1 receptor 2 (IL-1R2) and its role in immune regulation. *Brain Behav Immun,* 32**,** 1-8.

PFEFFER, K., MATSUYAMA, T., KUNDIG, T. M., WAKEHAM, A., KISHIHARA, K., SHAHINIAN, A., WIEGMANN, K., OHASHI, P. S., KRONKE, M. & MAK, T. W. 1993. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. *Cell,* 73**,** 457-67.

PHEIFFER, C., PILLAY-VAN WYK, V., JOUBERT, J. D., LEVITT, N., NGLAZI, M. D. & BRADSHAW, D. 2018. The prevalence of type 2 diabetes in South Africa: a systematic review protocol. *BMJ Open,* 8**,** e021029.

PICKUP, J. 2004. Inflammation and Activated Innate Immunity in the Pathogenesis of Type 2 Diabetes. *DIABETES CARE,,* 27.

PICKUP, J. C., CHUSNEY, G. D., THOMAS, S. M. & BURT, D. 2000. Plasma interleukin-6, tumour necrosis factor alpha and blood cytokine production in type 2 diabetes. *Life Sci,* 67**,** 291-300.

PICKUP, J. C. & CROOK, M. A. 1998. Is type II diabetes mellitus a disease of the innate immune system? *Diabetologia,* 41**,** 1241-8.

PIETERS, M., COVIC, N., LOOTS DU, T., VAN DER WESTHUIZEN, F. H., VAN ZYL, D. G., RHEEDER, P., JERLING, J. C. & WEISEL, J. W. 2006. The effect of glycaemic control on fibrin network structure of type 2 diabetic subjects. *Thromb Haemost,* 96**,** 623-9.

PRADHAN, S., GHIMIRE, A., BHATTARAI, B., KHANAL, B., POKHAREL, K., LAMSAL, M. & KOIRALA, S. 2016. The role of C-reactive protein as a diagnostic predictor of sepsis in a multidisciplinary Intensive Care Unit of a tertiary care center in Nepal. *Indian J Crit Care Med,* 20**,** 417-20.

PRETORIUS, E. & BESTER, J. 2016. Viscoelasticity as a measurement of clot structure in poorly controlled type 2 diabetes patients: towards a precision and personalized medicine approach. *Oncotarget,* 7.

PRETORIUS, E., BESTER, J., VERMEULEN, N., ALUMMOOTTIL, J., SOMA, P., BUYS, A. V. & KELL, D. B. 2015. Poorly controlled type 2 diabetes is accompanied by significant morphological and ultrastructural changes in both erythrocytes and in thrombingenerated fibrin: implications for diagnostics. *Cardiovascular Diabetology,* 14.

PRETORIUS, E., MBOTWE, S., BESTER, J., ROBINSON, C. J. & KELL, D. B. 2016a. Acute induction of anomalous and amyloidogenic blood clotting by molecular amplification of highly substoichiometric levels of bacterial lipopolysaccharide. *J R Soc Interface,* 13.

PRETORIUS, E., MBOTWE, S. & KELL, D. B. 2017a. Lipopolysaccharide-binding protein (LBP) reverses the amyloid state of fibrin seen in plasma of type 2 diabetics with cardiovascular co-morbidities. *Sci Rep,* 7**,** 9680.

PRETORIUS, E., OLUMUYIWA-AKEREDOLU, O. O., MBOTWE, S. & BESTER, J. 2016b. Erythrocytes and their role as health indicator: Using structure in a patient-orientated precision medicine approach. *Blood Rev,* 30**,** 263-74.

PRETORIUS, E., PAGE, M. J., ENGELBRECHT, L., ELLIS, G. C. & KELL, D. B. 2017b. Substantial fibrin amyloidogenesis in type 2 diabetes assessed using amyloid-selective fluorescent stains. *Cardiovasc Diabetol,* 16**,** 141.

PRETORIUS, E., PAGE, M. J., HENDRICKS, L., NKOSI, N. B., BENSON, S. R. & KELL, D. B. 2018a. Both lipopolysaccharide and lipoteichoic acids potently induce anomalous fibrin amyloid formation: assessment with novel Amytracker stains. *J R Soc Interface,* 15.

PRETORIUS, E., PAGE, M. J., MBOTWE, S. & KELL, D. B. 2018b. Lipopolysaccharide-binding protein (LBP) can reverse the amyloid state of fibrin seen or induced in Parkinson's disease. *PLoS One,* 13, e0192121.

PRETORIUS, E., SWANEPOEL, A. C., BUYS, A. V., VERMEULEN, N., DUIM, W. & KELL, D. B. 2014. Eryptosis as a marker of Parkinson's disease. *Aging (Albany NY),* 6**,** 788-819.

PRETORIUS, E., SWANEPOEL, A. C., DEVILLIERS, S. & BESTER, J. 2017c. Blood clot parameters: Thromboelastography and scanning electron microscopy in research and clinical practice. *Thromb Res,* 154**,** 59-63.

PRETORIUS, L., THOMSON, G. J. A., ADAMS, R. C. M., NELL, T. A., LAUBSCHER, W. A. & PRETORIUS, E. 2018c. Platelet activity and hypercoagulation in type 2 diabetes. *Cardiovascular Diabetology,* 17**,** 141.

PRICE, D. T. & LOSCALZO, J. 1999. Cellular adhesion molecules and atherogenesis. *Am J Med,* 107**,** 85-97.

QURESHI, A. A., GUAN, X. Q., REIS, J. C., PAPASIAN, C. J., JABRE, S., MORRISON, D. C. & QURESHI, N. 2012. Inhibition of nitric oxide and inflammatory cytokines in LPS-stimulated murine macrophages by resveratrol, a potent proteasome inhibitor. *Lipids Health Dis,* 11**,** 76.

RAMBARAN, R. N. & SERPELL, L. C. 2008. Amyloid fibrils: abnormal protein assembly. *Prion,* 2**,** 112-7.

RANDERIA, S., THOMSON, G., NELL, T. A. & PRETORIUS, E. 2019. The inflammatory cytokine profile on Type 2 Diabetes Mellitus leads to hypercoagulation and abnormal clot formation

RANDLE, P. J., GARLAND, P. B., HALES, C. N. & NEWSHOLME, E. A. 1963. The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet,* 1**,** 785-9.

REGAZZETTI, C., PERALDI, P., GREMEAUX, T., NAJEM-LENDOM, R., BEN-SAHRA, I., CORMONT, M., BOST, F., LE MARCHAND-BRUSTEL, Y., TANTI, J. F. & GIORGETTI-PERALDI, S. 2009. Hypoxia decreases insulin signaling pathways in adipocytes. *Diabetes,* 58**,** 95-103.

REGAZZI, R., DALLE, S. & ABDERRAHMANI, A. 2014. Compensatory mechanisms of pancreatic beta cells: insights into the therapeutic perspectives for diabetes. *J Diabetes Res,* 2014**,** 217387.

REID, J., MACDOUGALL, A. I. & ANDREWS, M. M. 1957. Aspirin and diabetes mellitus. *Br Med J,* 2**,** 1071-4.

REMICK, D. G. 2005. Interleukin-8. *Crit Care Med,* 33**,** S466-7.

REN, Y., WANG, H., LU, D., XIE, X., CHEN, X., PENG, J., HU, Q., SHI, G. & LIU, S. 2014. Expression of serum amyloid A in uterine cervical cancer. *Diagn Pathol,* 9**,** 16.

RENNE, T., SCHMAIER, A. H., NICKEL, K. F., BLOMBACK, M. & MAAS, C. 2012. In vivo roles of factor XII. *Blood,* 120**,** 4296-303.

RICE, G. E. & BEVILACQUA, M. P. 1989. An inducible endothelial cell surface glycoprotein mediates melanoma adhesion. *Science,* 246**,** 1303-6.

RICKLES, F. R. & FALANGA, A. 2001. Molecular basis for the relationship between thrombosis and cancer. *Thromb Res,* 102**,** V215-24.

RICKLES, F. R. & LEVINE, M. N. 2001. Epidemiology of thrombosis in cancer. *Acta Haematol,* 106**,** 6-12.

ROGLIC, G. & UNWIN, N. 2010. Mortality attributable to diabetes: estimates for the year 2010. *Diabetes Res Clin Pract,* 87**,** 15-9.

ROSENFELD, M. A., BYCHKOVA, A. V., SHCHEGOLIKHIN, A. N., LEONOVA, V. B., KOSTANOVA, E. A., BIRYUKOVA, M. I., SULTIMOVA, N. B. & KONSTANTINOVA, M. L. 2016. Fibrin self-assembly is adapted to oxidation. *Free Radic Biol Med,* 95**,** 55-64.

ROTHLEIN, R., DUSTIN, M. L., MARLIN, S. D. & SPRINGER, T. A. 1986. A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. *J Immunol,* 137**,** 1270-4.

ROTTER, V., NAGAEV, I. & SMITH, U. 2003. Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is, like IL-8 and tumor necrosis factor-alpha, overexpressed in human fat cells from insulin-resistant subjects. *J Biol Chem,* 278**,** 45777-84.

ROUSSEAU, S., PAPOUTSOPOULOU, M., SYMONS, A., COOK, D., LUCOCQ, J. M., PRESCOTT, A. R., O'GARRA, A., LEY, S. C. & COHEN, P. 2008. TPL2-mediated activation of ERK1 and ERK2 regulates the processing of pre-TNF alpha in LPS-stimulated macrophages. *J Cell Sci,* 121**,** 149-54.

SAAD, M. J., FOLLI, F., KAHN, J. A. & KAHN, C. R. 1993. Modulation of insulin receptor, insulin receptor substrate-1, and phosphatidylinositol 3-kinase in liver and muscle of dexamethasone-treated rats. *J Clin Invest,* 92**,** 2065-72.

SALINI, V., SAGGINI, A., MACCAURO, G., A., C., SHAIK-DASTHAGIRISAHEB, Y. & CONTI, P. 2011. Inflammatory Markers- Serum Amyloid A, Fibrinogen and C-Reactive. *European Journal of Inflammation,* 9.

SALTZMAN, E. T., PALACIOS, T., THOMSEN, M. & VITETTA, L. 2018. Intestinal Microbiome Shifts, Dysbiosis, Inflammation, and Non-alcoholic Fatty Liver Disease. *Front Microbiol,* 9**,** 61.

SAMUEL, V. T., PETERSEN, K. F. & SHULMAN, G. I. 2010. Lipid-induced insulin resistance: unravelling the mechanism. *Lancet,* 375**,** 2267-77.

SAVOLAINEN, O., FAGERBERG, B., VENDELBO LIND, M., SANDBERG, A. S., ROSS, A. B. & BERGSTRÖM, G. 2017. Biomarkers for predicting type 2 diabetes development-Can metabolomics improve on existing biomarkers? *PLoS One,* 12**,** e0177738.

SCARPATI, E. M. & SADLER, J. E. 1989. Regulation of endothelial cell coagulant properties. Modulation of tissue factor, plasminogen activator inhibitors, and thrombomodulin by phorbol 12-myristate 13-acetate and tumor necrosis factor. *J Biol Chem,* 264**,** 20705-13.

SCARPATI, E. M., WEN, D., BROZE, G. J., JR., MILETICH, J. P., FLANDERMEYER, R. R., SIEGEL, N. R. & SADLER, J. E. 1987. Human tissue factor: cDNA sequence and chromosome localization of the gene. *Biochemistry,* 26**,** 5234-8.

SCHMITZ-PEIFFER, C., BROWNE, C. L., OAKES, N. D., WATKINSON, A., CHISHOLM, D. J., KRAEGEN, E. W. & BIDEN, T. J. 1997. Alterations in the expression and cellular localization of protein kinase C isozymes epsilon and theta are associated with insulin resistance in skeletal muscle of the high-fat-fed rat. *Diabetes,* 46**,** 169-78.

SCHRODER, K. & TSCHOPP, J. 2010. The inflammasomes. *Cell,* 140**,** 821-32.

SELLECK, M. J., SENTHIL, M. & WALL, N. R. 2017. Making Meaningful Clinical Use of Biomarkers. *Biomark Insights,* 12**,** 1177271917715236.

SHARMA, R., SHARMA, R., KHAKET, T. P., DUTTA, C., CHAKRABORTY, B. & MUKHERJEE, T. K. 2017. Breast cancer metastasis: Putative therapeutic role of vascular cell adhesion molecule-1. *Cell Oncol (Dordr),* 40**,** 199-208.

SHEN, Y. M. & FRENKEL, E. P. 2004. Thrombosis and a hypercoagulable state in HIV-infected patients. *Clin Appl Thromb Hemost,* 10**,** 277-80.

SHIRAKAWA, F., TANAKA, Y., ODA, S., ETO, S. & YAMASHITA, U. 1989. Autocrine stimulation of interleukin 1 alpha in the growth of adult human T-cell leukemia cells. *Cancer Res,* 49**,** 1143-7.

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SHOELSON, S. E., LEE, J. & GOLDFINE, A. B. 2006. Inflammation and insulin resistance. *J Clin Invest,* 116**,** 1793-801.

SIMEONI, E., HOFFMANN, M. M., WINKELMANN, B. R., RUIZ, J., FLEURY, S., BOEHM, B. O., MARZ, W. & VASSALLI, G. 2004. Association between the A-2518G polymorphism in the monocyte chemoattractant protein-1 gene and insulin resistance and Type 2 diabetes mellitus. *Diabetologia,* 47**,** 1574-80.

SIMPSON, R. J., HAMMACHER, A., SMITH, D. K., MATTHEWS, J. M. & WARD, L. D. 1997. Interleukin-6: structure-function relationships. *Protein Sci,* 6**,** 929-55.

SIMS, J. E., GAYLE, M. A., SLACK, J. L., ALDERSON, M. R., BIRD, T. A., GIRI, J. G., COLOTTA, F., RE, F., MANTOVANI, A., SHANEBECK, K. & ET AL. 1993. Interleukin 1 signaling occurs exclusively via the type I receptor. *Proc Natl Acad Sci U S A,* 90**,** 6155-9.

SIPE, J. D., BENSON, M. D., BUXBAUM, J. N., IKEDA, S., MERLINI, G., SARAIVA, M. J. & WESTERMARK, P. 2014. Nomenclature 2014: Amyloid fibril proteins and clinical classification of the amyloidosis. *Amyloid,* 21**,** 221-4.

SLYEPCHENKO, A., MAES, M., MACHADO-VIEIRA, R., ANDERSON, G., SOLMI, M., SANZ, Y., BERK, M., KÖHLER, C. & CARVALHO, A. 2016. *Intestinal Dysbiosis, Gut Hyperpermeability and Bacterial Translocation: Missing Links Between Depression, Obesity and Type 2 Diabetes?*

SMART, S. J. & CASALE, T. B. 1994. TNF-alpha-induced transendothelial neutrophil migration is IL-8 dependent. *Am J Physiol,* 266**,** L238-45.

SMITH, S. A., TRAVERS, R. J. & MORRISSEY, J. H. 2015. How it all starts: Initiation of the clotting cascade. *Crit Rev Biochem Mol Biol,* 50**,** 326-36.

SOINIO, M., MARNIEMI, J., LAAKSO, M., LEHTO, S. & RONNEMAA, T. 2006. High-sensitivity C-reactive protein and coronary heart disease mortality in patients with type 2 diabetes: a 7-year follow-up study. *Diabetes Care,* 29**,** 329-33.

SPENCER, C. G., FELMEDEN, D. C., BLANN, A. D. & LIP, G. Y. 2007. Haemorheological, platelet and endothelial indices in relation to global measures of cardiovascular risk in hypertensive patients: a substudy of the Anglo-Scandinavian Cardiac Outcomes Trial. *J Intern Med,* 261**,** 82-90.

SPRIGGS, D. R., DEUTSCH, S. & KUFE, D. W. 1992. Genomic structure, induction, and production of TNF-alpha. *Immunol Ser,* 56**,** 3-34.

STANDIFORD, T. J., KUNKEL, S. L., BASHA, M. A., CHENSUE, S. W., LYNCH, J. P., 3RD, TOEWS, G. B., WESTWICK, J. & STRIETER, R. M. 1990. Interleukin-8 gene expression by a pulmonary epithelial cell line. A model for cytokine networks in the lung. *J Clin Invest,* 86**,** 1945-53.

STEER, S. A., SCARIM, A. L., CHAMBERS, K. T. & CORBETT, J. A. 2006. Interleukin-1 stimulates beta-cell necrosis and release of the immunological adjuvant HMGB1. *PLoS Med,* 3**,** e17.

STEYN, K., KAZENELLENBOGEN, J. M., LOMBARD, C. J. & BOURNE, L. T. 1997. Urbanization and the risk for chronic diseases of lifestyle in the black population of the Cape Peninsula, South Africa. *J Cardiovasc Risk,* 4**,** 135-42.

SWANEPOEL, A. C., NIELSEN, V. G. & PRETORIUS, E. 2015. Viscoelasticity and Ultrastructure in Coagulation and Inflammation: Two Diverse Techniques, One Conclusion. *Inflammation,* 38**,** 1707-26.

SWAROOP, J. J., RAJARAJESWARI, D. & NAIDU, J. N. 2012. Association of TNF-α with insulin resistance in type 2 diabetes mellitus. *Indian J Med Res,* 135**,** 127-30.

SYLVIA, K. E. & DEMAS, G. E. 2018. Acute intraperitoneal lipopolysaccharide influences the immune system in the absence of gut dysbiosis. *Physiol Rep,* 6.

TAKEDA, K. & AKIRA, S. 2004. TLR signaling pathways. *Semin Immunol,* 16**,** 3-9.

TAKEUCHI, O. & AKIRA, S. 2010. Pattern recognition receptors and inflammation. *Cell,* 140**,** 805-20.

TAN, S. Z., OOI, D. S., SHEN, H. M. & HENG, C. K. 2014. The atherogenic effects of serum amyloid A are potentially mediated via inflammation and apoptosis. *J Atheroscler Thromb,* 21**,** 854-67.

TANAKA, T., NARAZAKI, M. & KISHIMOTO, T. 2014. IL-6 in inflammation, immunity, and disease. *Cold Spring Harb Perspect Biol,* 6**,** a016295.

TANTRY, U. S., BLIDEN, K. P., SUAREZ, T. A., KREUTZ, R. P., DICHIARA, J. & GURBEL, P. A. 2010. Hypercoagulability, platelet function, inflammation and coronary artery disease acuity: results of the Thrombotic RIsk Progression (TRIP) study. *Platelets,* 21**,** 360-7.

THOMPSON, D., PEPYS, M. B. & WOOD, S. P. 1999. The physiological structure of human C-reactive protein and its complex with phosphocholine. *Structure,* 7**,** 169-77.

THOMPSON, J. C., JAYNE, C., THOMPSON, J., WILSON, P. G., YODER, M. H., WEBB, N. & TANNOCK, L. R. 2015. A brief elevation of serum amyloid A is sufficient to increase atherosclerosis. *J Lipid Res,* 56**,** 286-93.

THOR, M., YU, A. & SWEDENBORG, J. 2002. Markers of inflammation and hypercoagulability in diabetic and nondiabetic patients with lower extremity ischemia. *Thromb Res,* 105**,** 379-83.

THORN, C. F., LU, Z. Y. & WHITEHEAD, A. S. 2004. Regulation of the human acute phase serum amyloid A genes by tumour necrosis factor-alpha, interleukin-6 and glucocorticoids in hepatic and epithelial cell lines. *Scand J Immunol,* 59**,** 152-8.

THORN, K. 2016. A quick guide to light microscopy in cell biology. *Molecular Biology of the Cell,* 27**,** 219-222.

THORNBERRY, N. A., BULL, H. G., CALAYCAY, J. R., CHAPMAN, K. T., HOWARD, A. D., KOSTURA, M. J., MILLER, D. K., MOLINEAUX, S. M., WEIDNER, J. R., AUNINS, J. & ET AL. 1992. A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. *Nature,* 356**,** 768-74.

TILLETT, W. S. & FRANCIS, T. 1930. SEROLOGICAL REACTIONS IN PNEUMONIA WITH A NON-PROTEIN SOMATIC FRACTION OF PNEUMOCOCCUS. *J Exp Med,* 52**,** 561-71.

UHLAR, C. M. & WHITEHEAD, A. S. 1999. Serum amyloid A, the major vertebrate acute-phase reactant. *Eur J Biochem,* 265**,** 501-23.

VADAKAYIL, A. R., DANDEKERI, S., KAMBIL, S. M. & ALI, N. M. 2015. Role of C-reactive protein as a marker of disease severity and cardiovascular risk in patients with psoriasis. *Indian Dermatol Online J,* 6**,** 322-5.

VALLABHAPURAPU, S. & KARIN, M. 2009. Regulation and function of NF-kappaB transcription factors in the immune system. *Annu Rev Immunol,* 27**,** 693-733.

VAN DAMME, J., VAN BEEUMEN, J., DECOCK, B., VAN SNICK, J., DE LEY, M. & BILLIAU, A. 1988. Separation and comparison of two monokines with lymphocyte-activating factor activity: IL-1 beta and hybridoma growth factor (HGF). Identification of leukocyte-derived HGF as IL-6. *J Immunol,* 140**,** 1534-41.

VANNIER, E. & DINARELLO, C. A. 1994. Histamine enhances interleukin (IL)-1-induced IL-6 gene expression and protein synthesis via H2 receptors in peripheral blood mononuclear cells. *J Biol Chem,* 269**,** 9952-6.

VIALE, P. H. 2005. Abnormal clotting in cancer: an overview of pathophysiology and etiology. *Semin Oncol Nurs,* 21**,** 12-20.

VOGIATZI, K., APOSTOLAKIS, S., AMANATIDOU, V. & SPANDIDOS, D. A. 2009. Interleukin 8 and cardiovascular disease. *Cardiovascular Research,* 84**,** 353-360.

VOLANAKIS, J. E. 2001. Human C-reactive protein: expression, structure, and function. *Mol Immunol,* 38**,** 189-97.

VORSTER, H. H., VENTER, C. S., WISSING, M. P. & MARGETTS, B. M. 2005. The nutrition and health transition in the North West Province of South Africa: a review of the THUSA (Transition and Health during Urbanisation of South Africans) study. *Public Health Nutr,* 8**,** 480-90.

WANG, B., WOOD, I. S. & TRAYHURN, P. 2007. Dysregulation of the expression and secretion of inflammation-related adipokines by hypoxia in human adipocytes. *Pflugers Arch,* 455**,** 479-92.

WANG, X., BAO, W., LIU, J., OUYANG, Y. Y., WANG, D., RONG, S., XIAO, X., SHAN, Z. L., ZHANG, Y., YAO, P. & LIU, L. G. 2013. Inflammatory markers and risk of type 2 diabetes: a systematic review and meta-analysis. *Diabetes Care,* 36**,** 166-75.

WASILUK, A. 2004. Markers of platelets activation, CD 62P and soluble P-selectin in healthy term neonates. *J Perinat Med,* 32**,** 514-5.

WAUGH, D. J. & WILSON, C. 2008. The interleukin-8 pathway in cancer. *Clin Cancer Res,* 14**,** 6735-41.

WEISEL, J. W. 2005. Fibrinogen and fibrin. *Adv Protein Chem,* 70**,** 247-99.

WEISSENBACH, J., CHERNAJOVSKY, Y., ZEEVI, M., SHULMAN, L., SOREQ, H., NIR, U., WALLACH, D., PERRICAUDET, M., TIOLLAIS, P. & REVEL, M. 1980. Two interferon mRNAs in human fibroblasts: in vitro translation and Escherichia coli cloning studies. *Proc Natl Acad Sci U S A,* 77**,** 7152-6.

WILD, S., ROGLIC, G., GREEN, A., SICREE, R. & KING, H. 2004. Global Prevalence of Diabetes. *Estimates for the year 2000 and projections for 2030,* 27**,** 1047-1053.

WITTCHEN, E. S. 2009. Endothelial signaling in paracellular and transcellular leukocyte transmigration. *Front Biosci (Landmark Ed),* 14**,** 2522-45.

WOLBERG, A. S. 2007. Thrombin generation and fibrin clot structure. *Blood Rev,* 21**,** 131-42.

WOLPE, S. D., DAVATELIS, G., SHERRY, B., BEUTLER, B., HESSE, D. G., NGUYEN, H. T., MOLDAWER, L. L., NATHAN, C. F., LOWRY, S. F. & CERAMI, A. 1988. Macrophages secrete a novel heparin-binding protein with inflammatory and neutrophil chemokinetic properties. *J Exp Med,* 167**,** 570-81.

WOLTMANN, G., MCNULTY, C. A., DEWSON, G., SYMON, F. A. & WARDLAW, A. J. 2000. Interleukin-13 induces PSGL-1/P-selectin-dependent adhesion of eosinophils, but not neutrophils, to human umbilical vein endothelial cells under flow. *Blood,* 95**,** 3146-52.

WOOLLARD, K. J., LUMSDEN, N. G., ANDREWS, K. L., APRICO, A., HARRIS, E., IRVINE, J. C., JEFFERIS, A. M., FANG, L., KANELLAKIS, P., BOBIK, A. & CHIN-DUSTING, J. P. 2014.

Raised soluble P-selectin moderately accelerates atherosclerotic plaque progression. *PLoS One,* 9**,** e97422.

WORLD HEALTH ORGANISATION. 2017. *Diabetes* [Online]. Available: http://www.who.int/news-room/fact-sheets/detail/diabetes [Accessed 25/07/2018 2018].

WORLD HEALTH ORGANISATION. 2018. *Cardiovascular Disease* [Online]. Available: http://www.who.int/cardiovascular_diseases/en/ [Accessed 25/07/2018].

XIA, D. & SAMOLS, D. 1997. Transgenic mice expressing rabbit C-reactive protein are resistant to endotoxemia. *Proc Natl Acad Sci U S A,* 94**,** 2575-80.

XU, H., BARNES, G. T., YANG, Q., TAN, G., YANG, D., CHOU, C. J., SOLE, J., NICHOLS, A., ROSS, J. S., TARTAGLIA, L. A. & CHEN, H. 2003. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *The Journal of Clinical Investigation,* 112**,** 1821-1830.

XU, L., BADOLATO, R., MURPHY, W. J., LONGO, D. L., ANVER, M., HALE, S., OPPENHEIM, J. J. & WANG, J. M. 1995. A novel biologic function of serum amyloid A. Induction of T lymphocyte migration and adhesion. *J Immunol,* 155**,** 1184-90.

YANG, H., KO, H. J., YANG, J. Y., KIM, J. J., SEO, S. U., PARK, S. G., CHOI, S. S., SEONG, J. K. & KWEON, M. N. 2013. Interleukin-1 promotes coagulation, which is necessary for protective immunity in the lung against Streptococcus pneumoniae infection. *J Infect Dis,* 207**,** 50-60.

YANG, L., FROIO, R. M., SCIUTO, T. E., DVORAK, A. M., ALON, R. & LUSCINSKAS, F. W. 2005. ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-alpha-activated vascular endothelium under flow. *Blood,* 106**,** 584-92.

YE, R. D. & SUN, L. 2015. Emerging functions of serum amyloid A in inflammation. *Journal of Leukocyte Biology,* 98**,** 923-929.

ZABCZYK, M., HONDO, L., KRZEK, M. & UNDAS, A. 2013. High-density cholesterol and apolipoprotein AI as modifiers of plasma fibrin clot properties in apparently healthy individuals. *Blood Coagul Fibrinolysis,* 24**,** 50-4.

ZEWINGER, S., DRECHSLER, C., KLEBER, M. E., DRESSEL, A., RIFFEL, J., TRIEM, S., LEHMANN, M., KOPECKY, C., SAEMANN, M. D., LEPPER, P. M., SILBERNAGEL, G., SCHARNAGL, H., RITSCH, A., THORAND, B., DE LAS HERAS GALA, T., WAGENPFEIL, S., KOENIG, W., PETERS, A., LAUFS, U., WANNER, C., FLISER, D., SPEER, T. & MARZ, W. 2015. Serum amyloid A: high-density lipoproteins interaction and cardiovascular risk. *Eur Heart J,* 36**,** 3007-16.

ZHANG, G., MEREDITH, T. C. & KAHNE, D. 2013. On the essentiality of lipopolysaccharide to Gram-negative bacteria. *Curr Opin Microbiol,* 16**,** 779-85.

ZHENG, Y., LEY, S. H. & HU, F. B. 2017. Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. *Nature Reviews Endocrinology,* 14**,** 88.

ZOU, C. & SHAO, J. 2008. Role of adipocytokines in obesity-associated insulin resistance. *J Nutr Biochem,* 19**,** 277-86.

Appendices

Appendix A: Ethical Approval

<p align="center">**Approved with Stipulations**</p>

<p align="center">**New Application**</p>

28/03/2018

Project ID: 6399

HREC Reference #: S18/02/036

Title: Investigating the amyloidogenic potential of serum amyloid a in type II diabetes mellitus

Dear Mr Greig Thomson

The **New Application** received on 14/02/2018 15:29 was reviewed by members of the **Health Research Ethics Committee** via Minimal Risk Review procedures on 28/03/2018 and was approved with stipulations.

Please note the following information about your approved research protocol:

Protocol Approval Period: **28-Mar-2018 – 27-Mar-2019**

The stipulations of your ethics approval are as follows:

The timeline for this study should be adjusted.

Please remember to use your **project ID 6399** on any documents or correspondence with the HREC/UREC concerning your research protocol.

Translation of the consent document(s) to the language(s) applicable to your study participants should now be submitted to the HREC.

Please note that this decision will be ratified at the next HREC full committee meeting. HREC reserves the right to suspend approval and to request changes or clarifications from applicants. The coordinator will notify the applicant (and if applicable, the supervisor) of the changes or suspension within 1 day of receiving the notice of suspension from HREC. HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review:

Please note you can submit your progress report through the online ethics application process, available at: https://apply.ethics.sun.ac.za and the application should be submitted to the Committee before the year has expired. Please see Forms and Instructions on our HREC website for guidance on how to submit a progress report.

The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility, permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Please consult the Western Cape Government website for access to the online Health Research Approval Process, see: https://www.westerncape.gov.za/general-publication/health-research-approval-process. Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research

Appendix B: English informed consent form

| Cardiovascular disease (mark with x) |
| Control (mark with x) |

| Type 2 diabetes (mark with x) |

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT:
Investigating the amyloidogenic potential of serum amyloid a in type II diabetes mellitus

REFERENCE NUMBER:

PRINCIPAL INVESTIGATOR: Prof E Pretorius

ADDRESS: Department of Physiological Sciences, Faculty of Science, Stellenbosch University, STELLENBOSCH

CONTACT NUMBER:
You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the **Health Research Ethics Committee at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

You are invited to participate in research study conducted by Prof Resia Pretorius. This information leaflet is to help you to decide if you would like to participate. Before you agree to take part, you should fully understand what is being involved in this research study. Your medical practitioner will explain the reason for the drawing of blood. The drawing of 4 blood tubes will be a part of research study. First tube will be sent to pathology laboratory to determine your inflammatory status by looking at inflammatory markers like CRP levels. (An increase in CRP levels may influence the shape of the red blood cells, platelets and fibrin) and 3 more tubes will be used for our

laboratory haematological (blood) analysis using specialized microscopic and biochemical analysis.

The research team members are investigating the physiology and functioning of cells and molecules in whole blood. This will be done by using specialized equipment suc as the flow cytometer and microscopes that can magnify up to 100 000x (called an electron microscope and a fluorescent microscope). We will also look at your blood using a nanobiosensors, developed by prof Willie Perold and his research students from the Engineering Department. We will store a part of your sample that is not used, for later analysis in similar studies, so that we do not waste samples.

Why have you been invited to participate?

We are recruiting either healthy or individuals with inflammation and cardiovascular disease, with specific focus on individuals with type 2 diabetes over 18 to participate in this study,

What will your responsibilities be?

You will donate 4 tubes of blood (20 ml). You have no responsibilities.

Will you benefit from taking part in this research?

There are no personal benefits but results generated from this study will allow researchers to determine the physiology of healthy clotting and red blood cell structure and will be used to test novel nanobiosensors for blood clotting analysis, constructed by the members of the Faculty of Engineering, Stellenbosch University.

Are there in risks involved in your taking part in this research?

The Venipuncture (i.e. drawing blood) is normally done as part of routine medical care and presents a slight risk and discomfort. Drawing blood may result in a bruise at the puncture site, or less commonly swelling of the vein, infection and bleeding from the site. For your protection, the procedures will be performed under sterile conditions by your medical practitioner or by a trained phlebotomist.

If you do not agree to take part, what alternatives do you have?

Who will have access to your medical records?

There will be access to any medical records only to determine previously obtained glucose levels and cholesterol levels. ***All information obtained during the course of this study is strictly confidential.*** *Data that may be reported in scientific journals will not include any information, which identifies you as a patient in this study. We will ONLY have your name on this informed consent form but will give your blood tubes a dedicated UNIQUE number so that you will not be identified in any way. Prof Pretorius and the team will only use the unique number in the data analysis. Samples that are not fully used, will be stored under the unique number for future (repeat or similar) tests.*

What will happen in the unlikely event of some form injury occurring as a direct result of your taking part in this research study?

Neither you nor your medical scheme will be expected to pay for the study. During a study-related injury, the Department of Physiological Sciences/ Prof Pretorius assume no obligation to pay for the medical treatment of other injuries. You may discuss this in detail with your medical practitioner or Pathology Laboratory who have insurance for injuries that might occur during routine blood drawing practices.

Will you be paid to take part in this study and are there any costs involved?

No you will not be paid to take part in the study. There will be no costs involved for you, if you do take part.

Is there any thing else that you should know or do?

➢ You can contact the Health Research Ethics Committee at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor.

➢ If you request it, you will receive a copy of this information and consent form for your own records.

HbA1c levels:

Cholesterol levels:

Other medication used:

Declaration by participant

By signing below, I ………………………………..………… agree to take part in a research study entitled *Investigating the amyloidogenic potential of serum amyloid a in type II diabetes mellitus*.

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.

- I have had a chance to ask questions and all my questions have been adequately answered.

- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.

- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.

- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) ....……......……………….. on (*date*) ………….…………..
2005.

....
Signature of participant   **Signature of witness**

Declaration by investigator

I *(name)* ………………………………………….……… declare that:

- I explained the information in this document to …………………………………..

- I encouraged him/her to ask questions and took adequate time to answer them.

- I am satisfied that he/she adequately understands all aspects of the research, as discussed above

- I did/did not use a interpreter. (*If a interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) ....……......……………….. on (*date*) ………….…………..
2005.

....
Signature of investigator   **Signature of witness**

Declaration by interpreter

I *(name)* ………………………………………….……… declare that:

- I assisted the investigator (*name*) …………………………………………… to

  explain the information in this document to (*name of participant*)

  ………………………………………….. using the language medium of

  Afrikaans/Xhosa.

- We encouraged him/her to ask questions and took adequate time to answer them.

- I conveyed a factually correct version of what was related to me.

- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) ....…….......……………. on (*date*) …………...……………...

....
Signature of interpreter

....
Signature of witness

Appendix C: Afrikaans informed consent form

UNIEKE ETIESE NOMMER: 6399
Oorblywende bloed sal gestoor word vir soortgelyke studies, met spoedgetalle:
6329; 1952

Kontrole (merk met 'n x)
Kardiovaskulêre siekte (merk met 'n x)

Tipe 2 diabetes (merk met 'n x)

DEELNEMERINLIGTINGSBLAD EN -TOESTEMMINGSVORM

TITEL VAN DIE NAVORSINGSPROJEK:
Ondersoek na die amyloïdogene potensiaal van serumamyloïde A in tipe II diabetes mellitus
"Investigating the amyloidogenic potential of serum amyloid A in type II diabetes mellitus"

VERWYSINGSNOMMER:

HOOFNAVORSER: Prof E Pretorius

ADRES: Departement Fisiologiese Wetenskappe, Fakulteit Natuurwetenskappe, Universiteit Stellenbosch, STELLENBOSCH

KONTAKNOMMER: 0829295041

U word uitgenooi om deel te neem aan 'n navorsingsprojek. Lees asseblief hierdie inligtingsblad op u tyd deur aangesien die detail van die navorsingsprojek daarin verduidelik word. Indien daar enige deel van die navorsingsprojek is wat u nie ten volle verstaan nie, is u welkom om die navorsingspersoneel of dokter daaroor uit te vra. Dit is baie belangrik dat u ten volle moet verstaan wat die navorsingsprojek behels en hoe u daarby betrokke kan wees. U deelname is ook **volkome vrywillig** en dit staan u vry om deelname te weier. U sal op geen wyse hoegenaamd negatief beïnvloed word indien u sou weier om deel te neem nie. U mag ook te eniger tyd aan die navorsingsprojek onttrek, selfs al het u ingestem om deel te neem.

Hierdie navorsingsprojek is deur die **Gesondheidsnavorsingsetiekkomitee (GNEK)** van die Universiteit Stellenbosch goedgekeur en sal uitgevoer word volgens die etiese riglyne en beginsels van die Internasionale Verklaring van Helsinki, Suid-Afrikaanse Riglyne vir Goeie Kliniese Praktyk en die Etiese Riglyne vir Mediese en Genetiese Navorsing van die Mediese Navorsingsraad (MNR) van Suid Afrika.

Wat behels hierdie navorsingsprojek?

U word uitgenooi om deel te neem aan navorsingsstudie uitgevoer deur Prof Resia Pretorius. Hierdie inligtingsblad is saamgestel om u te help om te besluit of u wil deelneem. Voordat u saamstem om deel te neem, moet u ten volle verstaan wat hierdie navorsingsstudie behels. Die mediese praktisyn sal aan u die rede vir die trekking van

bloed verduidelik. Die trekking van vier bloedbuise sal deel vorm van die navorsingstudie. Die eerste buis sal na 'n patologiese laboratorium gestuur word om u inflammatoriese status te bepaal deur na inflammatoriese merkers soos CRP te kyk ('n verhoging in CRP-vlakke kan die vorm van rooibloedselle, bloedplaatjies en fibrien beïnvloed). Die ander drie buise sal gebruik word vir hematologiese (bloed) analises in ons laboratorium deur gebruik te maak van gespesialiseerde mikroskopiese en biochemiese tegnieke.

Die navorsingspan van hierdie projek bestudeer die fisiologie en funksionering van selle in volbloed, wat bloedplaatjies genoem word. Dit word gedoen deur gebruik te maak van gespesialiseerdetoersuting soos die vloeisitometer en mikroskope wat 'n beeld tot 100 000 x kan vergroot (dit word 'n elektronmikroskoop en 'n fluoreserende mikroskoop genoem. Ons sal ook kyk na u bloed met nanobiosensors, ontwikkel deur Prof Willie Perold en sy navorsingsstudente van die Fakulteit Ingenieurswese, Universiteit Stellenbosch. Ons sal 'n deel van jou monster stoor wat nie gebruik word nie, vir latere analise in soortgelyke studies, sodat ons nie steekproewe mors nie

Waarom is u genooi om deel te neem?

Ons werf of gesonde of individue met inflammasie en kardiovaskulêre siekte, met spesifieke fokus op individue ouer as 18 jaar met tipe 2-diabetes om aan hierdie studie deel te neem,

Wat sal u verantwoordelikhede wees?

U sal 4 bloedbuisies (20 ml) bloed skenk. U het geen verantwoordelikhede nie.

Sal u voordeel trek deur deel te neem aan hierdie navorsingsprojek?

Daar is geen persoonlike voordele nie, maar resultate wat uit hierdie studie gegenereer word, sal navorsers in staat stel om die fisiologie van gesonde bloedstolling en rooibloedselstruktuur te bepaal en sal gebruik word om 'n nuwe nano-biosensor vir analise van bloedstolling te toets, wat deur die lede van die Fakulteit Ingenieurswese ontwikkel is.

Is daar enige risiko's betrokke by u deelname aan hierdie navorsing?

Die veneuse punktuur (d.w.s. bloedtrekking) word normaalweg gedoen as deel van roetine mediese sorg en veroorsaak geringe risiko en ongemak. Bloedtrekking kan lei tot 'n kneusplek by die punksieplek, of minder algemeen, swelling van die aar, of infeksie en bloeding van die area. Vir u beskerming sal die prosedures onder steriele toestande deur u mediese praktisyn of deur 'n opgeleide bloedlating tegnikus uitgevoer word.

As jy nie saamstem om deel te neem nie, watter alternatiewe het jy?

Geen.

Wie sal toegang hê tot u mediese rekords?

Toegang tot mediese rekords sal slegs gedoen word om voorheen verwerfde glukosevlakke en cholesterolvlakke te bekom. **Alle inligting wat tydens die studie verkry word, is streng vertroulik.** *Data wat in wetenskaplike vaktydskrifte gerapporteer word, sal nie enige inligting bevat wat u as pasiënt in hierdie studie identifiseer nie. Ons sal SLEGS u naam op hierdie ingeligte toestemmingsvorm hê, maar sal 'n UNIEKE nommer op u bloedbuis aanbring sodat u nie op enige manier*

geïdentifiseer sa kan word nie. Prof Pretorius en haar span sal slegs die unieke nommer tydens data-analise gebruik. Monsters wat nie ten volle gebruik word nie, sal onder die unieke nommer vir toekomstige (herhalende of soortgelyke) toetse gestoor word.

Wat sal gebeur in die onwaarskynlike geval dat enige vorm van besering plaasvind as 'n direkte gevolg van u deelname aan hierdie navorsingstudie?

Daar sal nie van u of u mediese skema verwag word om vir die studie te betaal nie. Tydens 'n studieverwante besering aanvaar die Departement Fisiologiese Wetenskappe/ Prof Pretorius geen verpligting om te betaal vir mediese behandeling van ander beserings nie. U kan dit in detail bespreek met u mediese praktisyn of patologiese laboratorium wat versekering het vir beserings wat tydens roetine bloedtrekkings mag voorkom.

Sal u finansieel word om aan hierdie studie deel te neem en is daar enige koste daaraan verbonde?

Nee, u sal nie betaal word om deel te neem aan die studie nie. Daar sal ook geen koste vir u wees as u deelneem nie.

Is daar enige iets anders wat u moet weet of doen?

➢ U kan die Gesondheidsnavorsingsetiekkomitee by 021 938 9207 kontak indien u enige besorgdheid of klagtes het wat nie behoorlik deur die studieleier aangespreek is nie.

➢ As u dit aanvra, sal u 'n afskrif van hierdie inligting- en toestemmingsvorm vir u eie rekords ontvang.

HbA1c vlakke:

Cholesterolvlakke:

Verklaring deur deelnemer

Met die ondertekening van hierdie dokument onderneem ek, ...……...............……………...……….. om deel te neem aan 'n navorsingsprojek getiteld **'n Nuwe nano-biosensor vir die opsporing van aktivering van bloedplaatjies in inflammasie en kardiovaskulêre siektes, met spesifieke fokus op tipe 2-diabetes deur die aktiveringskapasiteit van vyf bloedplaatjie-agoniste te ondersoek.**

Ek verklaar dat:

• Ek hierdie inligtings- en toestemmingsvorm gelees het of aan my laat voorlees het en dat dit in 'n taal geskryf is waarin ek vaardig en gemaklik mee is.

• Ek geleentheid gehad het om vrae te stel en dat al my vrae bevredigend beantwoord is.

• Ek verstaan dat deelname aan hierdie navorsingsprojek vrywillig is en dat daar geen druk op my geplaas is om deel te neem nie.

• Ek kan kies om die studie te eniger tyd te verlaat en sal op geen manier gepenaliseer of benadeel word nie.

- Ek mag gevra word om die studie te verlaat voordat dit klaar is, indien die studie dokter of navorser voel dat dit in my beste belang is, of as ek nie die studieplan volg soos ooreengekom nie.

Geteken te *(plek)* .... op *(datum)* ....

....
Handtekening van deelnemer   **Handtekening van getuie**
Verklaring deur navorser

Ek *(naam)* …………………………………………… verklaar dat:

- Ek die inligting in hierdie dokument verduidelik het aan ....…………..
- Ek hom/haar aangemoedig het om vrae te vra en voldoende tyd gebruik het om dit te beantwoord.
- Ek tevrede is dat hy/sy al die aspekte van die navorsingsprojek soos hierbo bespreek, voldoende verstaan.
- Ek 'n tolk gebruik het/nie 'n tolk gebruik het nie. (*Indien 'n tolk gebruik is, moet die tolk die onderstaande verklaring teken.)*

Geteken te *(plek)* .... op *(datum)* ....

....
Handtekening van navorser   **Handtekening van getuie**

Verklaring deur tolk

Ek *(naam)* …………………………………………… verklaar dat:

- Ek die navorser *(naam)* …………..………………… bygestaan het om die inligting in hierdie dokument in Engels/Xhosa aan *(naam van deelnemer)* …….……………………........................ te verduidelik.
- Ons hom/haar aangemoedig het om vrae te vra en voldoende tyd gebruik het om dit te beantwoord.
- Ek 'n feitelik korrekte weergawe oorgedra het van wat aan my vertel is.
- Ek tevrede is dat die deelnemer die inhoud van hierdie dokument ten volle verstaan en dat al sy/haar vrae bevredigend beantwoord is.

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Geteken te *(plek)* .... op *(datum)* ....

....
Handtekening van tolk   **Handtekening van getuie**

Appendix D: Turnitin report

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