The Role of Lipoteichoic Acid on Amyloidogenesis and Its Effect on Erythrocytes and Platelets

by

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DECLARATION OF ORIGINALITY

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December 2019

Abstract

Hypercoagulation and amyloidogenesis are commonly known as hallmarks for inflammation. Hypercoagulation and amyloidogenesis are linked to quite a few communicable and non-communicable ailments such as cancer, rheumatoid arthritis and several neuro-inflammatory conditions, like Parkinson's and Alzheimer's disease. Recently, it was discovered that adding low concentrations of bacterial components such as lipopolysaccharide (LPS) to platelet poor plasma (PPP) and whole blood (WB) brings about hypercoagulation and amyloidogenesis. The main protein that showed these structural changes, was soluble fibrinogen. Fibrinogen changes to insoluble fibrin, in the presence of thrombin during clot formation. Studies show that in the presence of specific plasma proteins and/or "mopping" agents, the presence hypercoagulation and amyloid might be removed or reduced. An example of such serum protein is the presence of LPS binding protein (LBP).

In this thesis, the aim is to investigate the effects of bacterial component lipoteichoic acid (LTA) when directly added to healthy WB and fibrinogen and compare the clotting profiles and ultrastructure of these samples to that of Type two diabetes mellites (T2DM) PPP. Furthermore, this thesis aims to investigate the potential role of Apolipoprotein A-1 (ApoA1) as a mopping agent, by adding ApoA1 to healthy WB, fibrinogen and T2DM PPP. Here we suggest that ApoA1 may reduce hypercoagulation and amyloidogenesis in healthy WB and fibrinogen with added amyloid forming molecule, LTA and T2DM PPP.

We hypothesis that by adding low concentrations of LTA (5ng/L) to healthy WB and fibrinogen, LTA will cause hyperactivation of platelets, eryptosis of red blood cells (RBC) and amyloidogenesis in fibrin(ogen) respectively. In addition to this, we hypothesis that by adding ApoA1 to WB and fibrinogen that has been spiked with LTA, ApoA1 will attenuate supposed hyperactivation, eryptosis and amyloidogenesis. Moreover, we hypothesise that by adding ApoA1 to T2DM PPP, it will decrease the coagulopathies and amyloid state typically associated with T2DM patients.

Thromboelastography was used to assess clotting parameters of WB and Scanning electron microscopy (SEM) was used to view any ultrastructural changes on RBC and platelets before and after the addition of LTA and LTA with ApoA1 to healthy WB. Confocal microscopy was used to investigate whether amyloid protein formation occurred in fibrin(ogen) in the presence of LTA and if this amyloid was reduced when ApoA1 was added. For our T2DM control's, SEM and Confocal were used to view the differences in the PPP clot formed before and after the addition of ApoA1. The results for WB from the healthy controls showed a trend towards hypercoagulation in the presence of LTA and a trend towards attenuation of hypercoagulation and amyloidogenesis in the presence of ApoA1. In the T2DM model, a significant change was observed between the naïve T2DM PPP and the ApoA1 treated T2DM PPP. These are significant findings because they provide an alternative

novel approach to the formation of a hypercoagulable environment and amyloidogenesis and these findings can be advantageous in finding a potential treatment of chronic inflammatory conditions.

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List of Abbreviations

AD	Alzheimer's Disease
ApoA1	Apolipoprotein A-1
Ca^{2+}	Calcium
CD	Cluster of Differentiation
CRP	C-Reactive Protein
CV	Coefficient of Variance
CVD	Cardiovascular Disease
DMD	Dense Matted Deposits
ECM	Endothelial Cell Matrix
HDL	High-Density Lipoprotein
IBS	Irritable Bowel Syndrome
IL	Interleukin
K	Kinetics of Fibrin Formation
LBP	LPS Binding Protein
LCAT	Lecithin Cholesterol Acyl Transferase
LDL	Low Density Lipoprotein
LPS	Lipopolysaccharide
LTA	Lipoteichoic Acid
MA-	Maximum Amplitude
MAPK	Mitogen-activated protein kinase
MD-2	Lymphocyte Antigen 96
MyD88	Myeloid differentiation primary response 88
NK_KB	Nuclear Factor kappa B
OCS	Open Canalicular System
PAI-1	Plasminogen Activator Inhibitor-1
PAMP	Pathogen- Associated Molecular Patterns
PD	Parkinson's Disease
PGD_2	Prostaglandin D2
PGE_2	Prostaglandin E2
PGI_2	Prostacyclin
PRR	Pattern Recognition Receptors

R	Reaction Time
RBC	Red Blood Cells
S. aureus	*Staphylococcus aureus*
T2DM	Type Two Diabetes Mellitus
TEG	Thromboelastogram/Thromboelstography
TF	Tissue Factor
TLR	Toll-Like Receptors
TNF-α	Tumour Necrosis Factor alpha
TRIF	TIR domain containing adapter-inducing interferon-β
VWF	von Willebrand Factor
WB	Whole Blood
α Angle	Alpha Angle

Chapter 1: Introduction

Lipoteichoic acid (LTA) is a major component of Gram-positive bacterial cell wall envelopes (Villéger *et al.*, 2014). It is the counterpart of lipopolysaccharide (LPS) of Gram-negative bacterial cell wall (Villéger *et al.*, 2014). Lipoteichoic acid is an amphiphilic polymer, anchored in the cytoplasmic membrane by means if its glycolipid component, typically consisting of a poly (glycerol-phosphate) chain with a D-alanine and/or glycosyl substitutes (Villéger *et al.*, 2014). It is formed by linking hydrophilic polyphosphate polymer to a glycolipid (Villéger *et al.*, 2014). The external surface of peptidoglycan of Gram-positive bacteria is covered with a layer of LTA (Villéger *et al.*, 2014). Lipoteichoic acid is anchored in the peptidoglycan substratum via diacylglycerol moiety and has a surface exposed polyanionic linked polyglycerophosphate appendage which varies in its subunit composition in LTA from various Gram-positive bacteria (Kang, Sim, *et al.*, 2016).

There are many variations of LTA structures and the variation type is dependent on the bacterial strain (Villéger *et al.*, 2014). The variation is mostly due to the length of the hydrophilic backbone with an average of 10-60 glycerol-phosphate units and the fatty acid constituents of the hydrophobic anchor (Villéger *et al.*, 2014). These variations may have the ability to modify interactions with cell receptors thus leading to differences in the induced immunological responses (Villéger *et al.*, 2014). In *Staphylococcus aureus* (*S. aureus*) the repeating subunit contains D-alanine and α-N-acetylglucosamine (Villéger *et al.*, 2014).

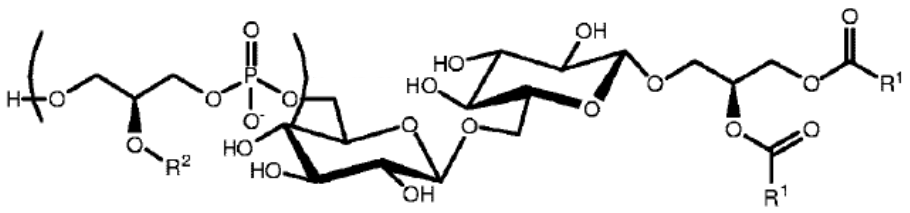

Figure 0.1 Chemical structure of LTA taken from (Villéger et al., 2014)

Lipoteichoic acid is important in human health because it is common knowledge that bacterial infections activate both the innate and adaptive branches of the immune system (Han *et al.*, 2003). Researchers have found that LTA is a potent inducer of inflammation due to its interactions with the hosts cells pattern recognition receptors (PRR) and/or hosts pathogen-associated molecular patterns (PAMP) molecules (Han *et al.*, 2003; Villéger *et al.*, 2014). The association of LTA with the hosts PRR and PAMP occurs via Toll-Like Receptor-2 (TLR-2) resulting in the activation of the Nuclear Factor kappa B (NF_kB) pathway, thus the secretion of proinflammatory mediators such as cytokines, nitric oxide and tumour necrosis factor alpha (TNF-α) ensues (Han *et al.*, 2003; Warshakoon, Burns and David, 2009; Villéger *et al.*, 2014).

Therefore, LTA and other bacterial inflammagens may have a prominent role in development of chronic systemic inflammation.

Although inflammation is a fundamental and critical part of the body's immune system, it is commonly referred to as a double-edged sword because of its ability to bring about both favourable and unfavourable outcomes (Lin and Kazmierczak, 2017). Favourable outcomes include the vital role that inflammation plays in host defence against pathogens and tissue repair as inflammation is necessary for these events to occur (Parolia *et al.*, 2014). Unfavourable outcomes of inflammation occur when the inflammatory system is dysregulated and it is unable to resolve itself, thus resulting in excessive inflammation (Serhan *et al.*, 2007). Excessive inflammation results in chronic low-grade systemic inflammation that may lead to unfavourable conditions such as excessive pain and the impairment of the tissue repair systems (Serhan *et al.*, 2007). Furthermore, researchers found that, chronic inflammation maybe the underlying cause of various conditions such as Alzheimer's disease (AD), asthma, stroke and T2DM just to mention a few (Pretorius, Mbotwe and Kell, 2017).

One of the worst-case-scenario of bacterial involvement in diseases is the development of sepsis (Bokhari and Patel, 2019). In the presence of systemic inflammation, sepsis easily leads to septic shock that results in multiple organ failure (Villéger *et al.*, 2014). Although sepsis is usually caused by Gram-negative bacteria, there is an increasing prevalence of sepsis as a result of Gram-positive bacteria due to the increasing occurrence of nosocomial infections because of invasive procedures, immunosuppression and cancer chemotherapy (Villéger *et al.*, 2014). The initiation and progression of systemic inflammatory responses are pathophysiological similar regardless of the causative organism, hence septic shock brought about as a result of Gram-negative bacteria is clinically indistinguishable from sepsis that originates from Gram-positive bacteria (Villéger *et al.*, 2014).

In inflammatory conditions, circulating pro-inflammatory cytokines are upregulated (Tian *et al.*, 2014). Cytokines are small signalling molecules that mediate the host's response to infections, trauma and inflammation and can either be pro-inflammatory or anti-inflammatory. Pro-inflammatory cytokines trigger or amplify systemic inflammation while anti-inflammatory cytokines mitigate inflammation and promote healing (Parolia *et al.*, 2014). Pro-inflammatory cytokines include molecules suchlike TNF-α, interleukin-1 (IL-1) and IL-12 and anti-inflammatory molecules include IL-10. Other cytokines have both pro and anti-inflammatory effects, such as IL-6. IL-6 can inhibit TNF-α and IL-1 while simultaneously activating IL-10. Both pro and anti-inflammatory cytokines work together to maintain homeostasis and prevent tissue damage due to constant inflammation (Parolia *et al.*, 2014). Therefore, in inflammatory conditions pro-inflammatory cytokines are upregulated and the pro/anti-inflammatory cytokine

balance in disrupted, resulting in constant inflammation. Returning to virulence factors, inflammagens such as LPS and LTA have the ability to activate the immune system by binding to cluster of differentiation-14 (CD14), which activates TLR that induces the secretion of pro-inflammatory cytokines IL-1, IL-8, IL-12 and TNF-α to name a few (Parolia *et al.*, 2014; Page, Bester and Pretorius, 2018). As pro-inflammatory molecules and inflammagens like LPS/LTA are upregulated during inflammation, the circulating molecules have a direct impact on the haematological system, particularly on platelets, red blood cells (RBC) and fibrin(ogen) (Page, Bester and Pretorius, 2018).

Another point worth noting regarding chronic inflammation is the negative impact it has on other systems of the body such as the coagulation system (Page, Bester and Pretorius, 2018). The coagulation system plays a vital role in the initiation of inflammatory system so the dysregulation of the inflammatory response affects the coagulation system too (Cheng *et al.*, 2011). In the presence of chronic inflammation, they are changes in the way normal blood clotting occurs resulting coagulopathies. The coagulation system becomes hyperactivated resulting in a hypercoagulable (thrombotic) and hypofibrinolytic environment (Pretorius, Mbotwe and Kell, 2017). A hypercoagulable environment means that the body is more susceptible to forming abnormal blood clots and a hypofibrinolytic environment means that the ability to lyse these colts is significantly diminished, thus a combination of the two is highly unfavourable (Kell and Pretorius, 2015b). It is thought that circulating bacterial inflammagens have a fundamental role in the development of hypercoagulation in inflammatory diseases (Ginsburg, 2002; Parolia *et al.*, 2014).

Various factors have an impact on creating a pro-inflammatory environment such as cytokines and environmental factors such as smoking and having high cholesterol levels (Cheng *et al.*, 2011). An important cytokine that links inflammation and to the coagulation system is IL-1. IL-1 induces fibrinogen synthesis in hepatocytes, thus excessive stimulation of IL-1 results in hyperactivation of the coagulation system resulting in hypercoagulation (Kell and Pretorius, 2015c). In Figure 1.0.2**Error! Reference source not found.** an overview diagram is shown of how the imbalance between circulating pro/anti -inflammatory cytokines and inflammagens may result in hypercoagulation as a marker of inflammation.

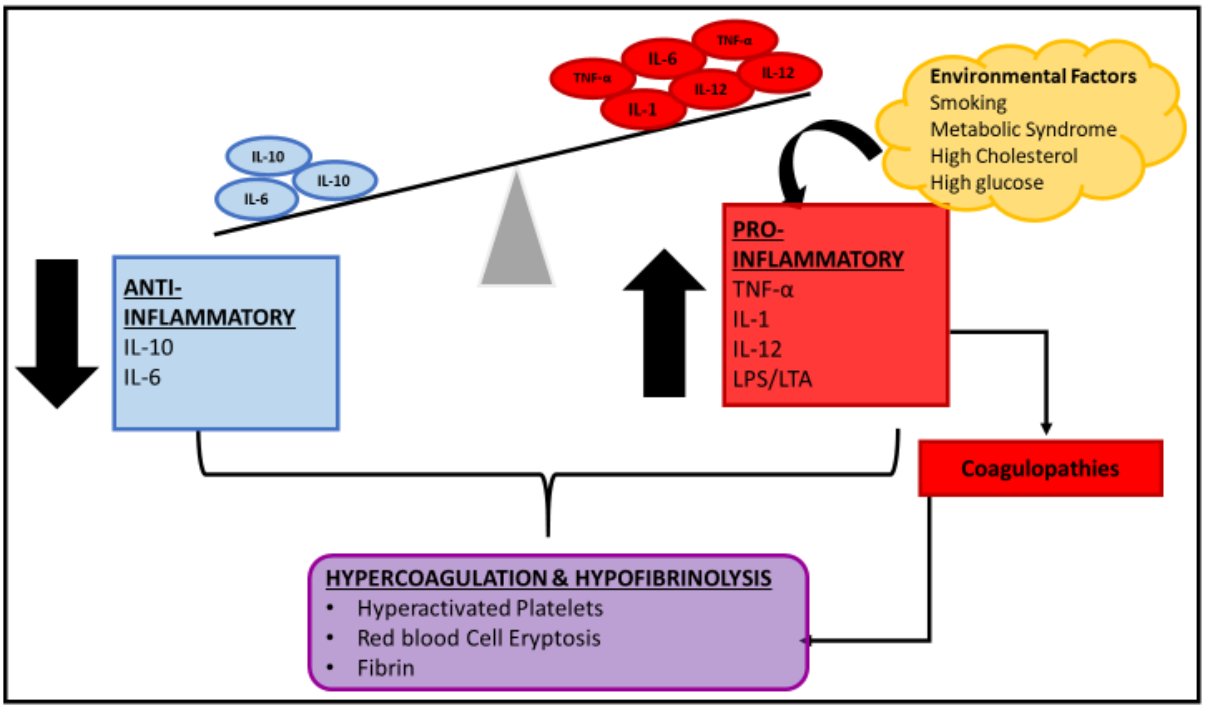

Figure 1.0.2 An overview of the cytokines involved in the inflammatory response. An imbalance in pro/anti-inflammatory cytokines results in overactivation of pro-inflammatory cytokines which directly affects the coagulation system.

How is hypercoagulation (also known as a hypercoagulable/prothrombotic state) defined? It is as a state where an individual has a higher prevalence to form clots/thrombus compared to the normal population (Grant, 2004; Kell and Pretorius, 2015c). It is characterised by hyperactivated platelets and eryptotic RBC. Research by Pretorius *et al.*, found that in the presence of chronic inflammatory conditions, diseases are accompanied by a hypercoagulable state as well as insoluble amyloid fibril formation (Pretorius, Mbotwe and Kell, 2017), which turned out to be amyloid in nature.

Amyloid is the general term used to describe abnormal β-sheet dominated insoluble, fibrous, extracellular *protein deposits* typically found in tissues and organs (Rambaran and Serpell, 2008; Eisenberg, David and Jucker, 2012). Amyloid fibrils are thought to cause disease by disrupting tissue structure and accumulating in organs thereby impairing organ function (Vaxman and Gertz, 2019). Thus, amyloid is associated with various inflammatory pathological conditions linked to high morbidity and mortality with the most common being AD and T2DM (Rambaran and Serpell, 2008). Recently our group discovered that the most prominent blood plasma protein ,fibrinogen, undergoes pathological protein structural changes and becomes amyloid-like when circulating inflammagens are present and inflammatory markers are dysregulated (Pretorius *et al.*, 2018). This is of great importance to development of many of the inflammatory diseases, that are linked to abnormal clotting and the broad

pathologies associated with cardiovascular disease in these illnesses (Pretorius, *et al.*, 2018). More on amyloid and its role in inflammatory conditions will be discussed in the next chapter.

From the information above concerning inflammation and its association with the coagulation system, and dysregulated circulating inflammatory markers, the question arose whether LTA is one of the fundamental causes of this hypercoagulation (considering that it might work closely together with dysregulated molecules like cytokines as seen in Figure 1.0.2). One of the methods that researchers from our group have employed to study this phenomenon, is to determine if bacterial mopping agents like LPS-binding protein (LBP), might have an effect on attenuating hypercoagulation in inflammatory conditions. LPS-binding protein is a serum glycoprotein belonging to the lipid-binding proteins family and is synthesised in the liver and intestinal epithelial cells (Gutsmann *et al.*, 2001). Under normal physiological conditions, serum LBP is present at 5-10µg/ml but rises to 200µg/ml during acute-phase response of an infection (Gutsmann *et al.*, 2001; Kopp, Kupsch and Schromm, 2016). LPS-binding protein has varying functions in immune regulation and lipid transfer (Kopp, Kupsch and Schromm, 2016). One of LBP's important functions is its ability to regulate and control bacterial infections. LPS-binding protein binds LPS endotoxin from Gram-negative bacteria and presents it to immune cells CD14, TLR4 or MD-2 signalling complex such as monocytes and macrophages. These immune cell are responsible for secreting inflammatory cytokines like TNF-α and IL-1 etc., to initiate an inflammatory response to fight off and eliminate the pathogen (Gutsmann *et al.*, 2001; Kopp, Kupsch and Schromm, 2016). Researchers have found that LBP also binds to other molecules such as LTA (Kopp, Kupsch and Schromm, 2016).

In various papers, our group found that either after adding LPS/LTA to healthy plasma, the fibrin(ogen) turned amyloid or hyper-clotted forming dense matted deposits (Pretorius *et al.*, 2016; Pretorius *et al.*, 2018). This was also found in clots from or inflammatory conditions like T2DM, AD and Parkinson's disease (PD). They found that when LPS/LTA is added to healthy individual platelet poor plasma (PPP), the clot that formed resembles a clot that is typically seen in individuals with chronic inflammatory diseases (Pretorius *et al.*, 2016). These types of clots are classified as dense matted deposits (DMD) and are not seen in healthy individuals. These clots have more of a netted structure compared to healthy clots which have more of a spaghetti like structure (Pretorius *et al.*, 2016; Pretorius, *et al.*, 2018).

Returning to LBP, we found that if it is added to healthy plasma (spiked with bacterial inflammagens) or plasma from inflammatory diseases such as T2DM, AD and PD, the hyperclottable and amyloid fibrin structure could be reduced (Pretorius *et al.*, 2018). After discovering the effects of LBP as a mopping molecule/reversing amyloid structure molecule,

the question arose if there might not be other similar agents. After much research, ApoA1 was identified as such a possible molecule.

Apolipoprotein A-1 is a human protein encoded by ApoA1 gene and is involved lipid metabolism. During the process of lipid metabolism, ApoA1 is converted to good cholesterol high density lipoprotein (HDL) in the human bloodstream and it is the predominant protein component of HDL (Sirniö *et al.*, 2017; 'APOA1 gene', 2018). High density lipoprotein (HDL) is referred to as good cholesterol because of its ability to transport cholesterol and phospholipids from body's peripheral tissues back to the liver where it is removed from the body or redistributed to other parts of the body ('APOA1 gene', 2018). Furthermore, ApoA1 has been found to have anti-oxidant functions, anti-apoptotic functions and protective anti-inflammatory properties. Additionally, ApoA1, has been found to attenuate LPS mediated neutrophilic airway inflammation (Yao *et al.*, 2016). Thus, from this information ApoA1 has been the chosen mopping agent for this thesis.

1. Problem statement

Based on my literature review and research that points to the involvement of LPS and other inflammagens that result in hypercoagulation and amyloid formation in whole blood and fibrin(ogen). It was noticed that the there is very limited information on the specific potential role of LTA in hypercoagulation and amyloid formation when added to healthy blood. In a disease model, like T2DM, the potential advantages of ApoA1 as a treatment for T2DM also had very limited information available. Therefore, this thesis will investigate the following hypotheses:

1.1. Hypothesis

We hypothesise that adding LTA WB of healthy individuals will cause hypercoagulation of fibrin(ogen) and that the damage will be amyloidogenic. We also hypothesise that the addition of LTA to healthy WB will cause platelets to become hyperactivated and that RBC will become eryptotic. Our second hypothesis is that by adding mopping agent ApoA1 to healthy WB already spiked with LTA, ApoA1 will decrease the hypercoagulation and amyloid signal observed in the above-mentioned entities. The third hypothesis is that adding mopping agent ApoA1 to T2DM PPP, ApoA1 will decrease coagulopathies and amyloid signal seen T2DM plasma.

Thus, the aim of this research study is to answer the following questions:

1.2. RESEARCH MODEL BASED ON HEATLHY BLOOD

1. Will adding LTA to healthy whole blood cause a hypercoagulable environment?

2. Will this hypercoagulable state cause amyloid formation in fibrin(ogen) when LTA is added to fibrinogen?
3. Will the addition of ApoA1 to fibrinogen reverse the effect of LTA i.e. hypercoagulable state and amyloid formation?
4. Will the addition of LTA to healthy whole blood cause RBC to become eryptotic and platelets to be hyperactivated?
5. Will the addition ApoA1 to the whole blood of healthy individuals after addition of LTA attenuate the effect of the LTA to platelets and RBC?

1.3. RESEARCH MODEL BASED ON TYPE TWO DIABETES BLOOD

6. Here we want to determine if the addition of ApoA1 to T2DM PPP, will reverse the effect of hypercoagulation and amyloidogenesis.

Chapter 2: Literature Review

This chapter will discuss and critically review literature pertaining to the inflammatory response in disease, the reasons for the response and the effects of the response on plasma proteins, platelets and RBC. The first paragraphs will give a basic background on inflammation, and Figure 2.1 will show a basic layout of this chapter.

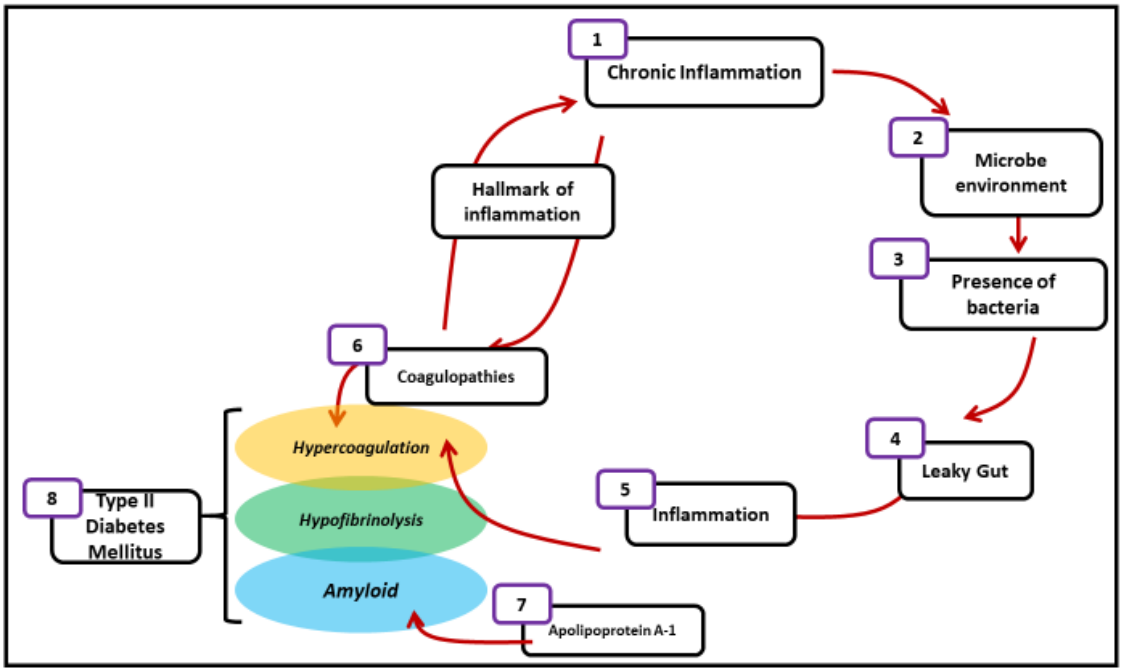

Figure 2.1 A flow diagram showing the main points that will be discussed this the literature review and order of the literature review.

Inflammation is a fundamental part of the immune system. The host initiates an immune response as a reaction to a potentially hazardous stimuli such as tissue injury, irritants and pathogens in order to set the foundation for the healing process. (Sherwood, 2010; Beck, 2013; Chandler and Ernst, 2017). The inflammatory response is classified as either acute inflammation or chronic (long-term) inflammation. Acute inflammation occurs rapidly and within minutes of tissue damage and is part of the first-line of defence against microbial pathogens such as parasites, bacteria, viruses and fungi (Chandler and Ernst, 2017). Acute immune response includes blood vessels dilation, immune cell activation and molecular mediators like chemokines and cytokines that together work to remove damaged cells and pathogen and initiate the healing and repair of injured tissue (Sherwood, 2010). Acute inflammation is characterised by swelling, pain, redness and heat, which is necessary to initiate healing after tissue injury and eliminating harmful micro-organisms (Beck, 2013). An example of acute inflammation is seen in the case of skin infections from *S. aureus* (Punchard *et al.,*2004).

On the other hand, chronic inflammation is slow and occurs for days, months and sometimes years due to continued interactions with toxic micro-organisms (Beck, 2013). Chronic inflammation is closely associated with countless infectious and non-infectious ailments that include but are not limited to cancers, T2DM and conditions associated with the central nervous system like AD and PD (Hunter, 2012). As mentioned in the introduction, these ailments have a tendency to share common pro-inflammatory cytokines like interleukins and they coincide with hypofibrinolysis, a hypercoagulable state, changes to RBC and possible eryptosis and platelet hyperactivation (Bester and Pretorius, 2016; Mu *et al.*, 2017).

From this information it is nited that although the primary function of the inflammatory response system is protective, its constant stimulation and activation is associated with pathologies in humans (Beck, 2013). It is for this reason that the inflammatory response system is regarded as a double-edged sword. In summary, short-lived inflammation has protective roles and is necessary to set the foundation for recovery however, prolonged inflammation is harmful, because of the continuous activation of pro-inflammatory cytokines (Festoff *et al.*, 2017).

This thesis willl focus on chronic (systemic) inflammation. The following paragraphs will now discuss novel ideas with regards to the development of inflammation, Particularly the role of bacterial inflammagens like LPS and LTA. The question that arises is how do LPS and LTA, in the first place, end up in circulation in order to have an effect on the haematological system and the developent of inflammation. One of the possibilities is the presence of a permeable gut, often referred to as a “leaky gut” which is a hallmark of most inflammatory conditions (Mu, Kirby, Christopher M Reilly, *et al.*, 2017). The next paragraphs will discuss leaky gut in the context of the development of inflammation.

2. Novel ideas on the development of inflammation

2.1 The Role of Bacteria in The Development of Leaky Gut

The physiological role of the gastrointestinal lining is to form a physical barrier that allows absorption of nutrients and water while selectively limiting permeation of gut by luminal toxins and microbes (Ahmad *et al.*, 2017). The gut luminal surface of human intestines has approximately 3.8×10^{13} commensal bacteria that perform functions to maintain gut homeostasis. Examples of these functions include metabolising food and drugs, synthesising vitamin B12 and K in addition to preventing pathobiotic invasions (Vindigni *et al.*, 2016; Tsai *et al.*, 2019). These functions together with the epithelial lining form a barrier that separates the host environment from the microbial environment (Mu, *et al.*, 2017).

In a healthy gut environment bacterial cells in the colon lumen are separated from the host’s body by a single layer of absorptive epithelium cells and a mucosal barrier (mucins) containing

lectins that prevent direct interactions between the host and the gut microbiota (Lau, Kalantar-Zadeh and Vaziri, 2015; Yu, 2018). Studies show that bacteria in the lumen interact less with the immune system compared to mucosal bacteria (Macfarlane, 2008). Mucins, are glycoproteins that are responsible for forming this protective barrier (Corfield, 2018). Studies have found that dysbiosis -alterations in gut microbiota- may result in serious pathological conditions as is the case with irritable bowel syndrome (IBS) (Torrazza, M.D. and Neu, 2012; Vindigni *et al.*, 2016).

Research shows that in pathophysiological inflammatory conditions, dysbiosis precedes the permeability of the epithelial lining becoming compromised, therefore allowing the passage of toxins, antigens, and bacteria from the gut lumen to enter the blood stream subsequently, creating a "dysbiosis-leaky gut complex." (Vindigni *et al.*, 2016; Mu, *et al.*, 2017). A leaky gut is defined as the increased permeability of the intestinal mucosa, that allows for the translocation (leak) of bacteria, their DNA and endotoxins into the bloodstream due to a breach in the mucosal barrier (epithelial tight junctions) of the colon due to inflammation (Lau, Kalantar-Zadeh and Vaziri, 2015; Ahmad *et al.*, 2017; Obrenovich, 2018). Other factors can also cause a shift in microbial composition. Factors such as, antibiotics, oral contraceptives, stress, the Western diet which is mostly processed foods high in fat, protein, and contain little to no fibre all contribute to the formation of the dysbiosis-leaky gut complex (Vindigni *et al.*, 2016). To substantiate this further, some studies found that in humans, a diet high in fat, increased LPS translocation into the bloodstream from the gut, which intensified inflammation therefore further amplifying leaky-gut (Molin, 2010).

As previously mentioned, leaky gut allows for interactions between the host and microbes which further exacerbates both local and systemic inflammatory responses, because of the changes in the gut microbiome i.e. a decrease in species diversity. This decrease in bacterial species diversity causes a loss in stability and is strongly associated with inflammation, causing a disruption in the mucosal barrier and translocation of the intestinal microbiota into the bloodstream, therefore stimulating the immune system (Ahmad *et al.*, 2017; Mu, *et al.*, 2017). Researchers have found evidence that links gut bacterial endotoxins, with increased levels of inflammation (Piya *et al.,* 2013). Gut bacterial DNA has been detected in blood and the presence of this bacteria correlated with increased C-reactive protein (CRP), IL, and D-lactate which is a common marker for gut permeability (Lau, Kalantar-Zadeh and Vaziri, 2015). Scientists are not certain whether changes in the gut barrier function precede inflammation or occurs because of inflammation, however what is known is that inflammation and dysbiosis are mutually reinforcing in patients (Vindigni *et al.*, 2016). This is the case especially in patients with a genetic predisposition to specific ailments, the presence of a leaky gut may allow environmental factors to enter the body and trigger the initiation and development of theses

genetically predisposed diseases like colorectal cancer, the metabolic syndrome, enteric infections, obesity, and IBS (Vindigni *et al.*, 2016; Mu, Kirby, Christopher M. Reilly, *et al.*, 2017). A compromised intestinal barrier is linked to the development of a number of chronic inflammatory disorders and systemic diseases such as T2DM, CVDs, cancers and neurodegenerative diseases (Yu, 2018). These diseases maybe caused or worsened by the presence of a leaky gut. Studies have found that the translocation of bacterial components further promotes systemic inflammation and has adverse CVD outcomes (Lau, Kalantar-Zadeh and Vaziri, 2015).

It has now been established that inflammation together with dysbiosis and leaky gut are closely connected (Torrazza, M.D. and Neu, 2012; Vindigni *et al.*, 2016). Importantly, the most potent inflammatory stimulus from bacteria entering via the dysbiosis-leaky gut complex are the membranes/wall components, LPSs and LTAs. These molecules are the potent inflammagens that can further trigger inflammation when they entre circulation (Lau, Kalantar-Zadeh and Vaziri, 2015). Lipopolysaccharide and LTA further drive inflammation because their debris trigger the immune system via TLR activation (Molin, 2010; Hunter, 2012).

In conclusion, healthy individuals may get a leaky gut, when microbes enter circulation, however, the immune system is able to eliminate these microbes and their membrane inflammagens (Sastalla, Monack and Kubatzky, 2016). However, diseases like T2DM etc., together with genetic predisposition, dysregulated insulin, increased glucose levels, high cholesterol, the immune response function of the individual is compromised or at best delayed (Kell and Pretorius, 2015a). Consequently, when dysbiosis and leaky gut is present and is driven by the gut permeability as discussed in the paragraphs above, together with a decreased immune function, bacterial inflammagens might become important role players as the drivers of the inflammatory process, in the presence of leaky gut, dysbiosis, and compromised immune function.

The next paragraphs will discuss the possible role of LTA as potent bacterial inflammagen, after Gram-positive bacteria enter the body via e.g. a leaky gut, followed by the shedding of LTAs, and how it could induce inflammation.

2.2. Lipoteichoic Acid and Its Potential Role in Inflammation

Microbes are made up of lipoproteins and lipids that are important for propagation, membrane integrity, signalling and responding to environmental stress. Gram-positive bacteria are typically comprised of structurally unique lipoproteins, poly-N-acetyl glucosamine, wall of teichoic acid, peptidoglycan and LTA (Chandler and Ernst, 2017). The main constituent of Gram-positive bacterial cell wall is LTA. Lipoteichoic acid is a surface-associated adhesion amphiphile (Ginsburg, 2002; Dai *et al.*, 2014; Kang, Ju-Ri Sim, *et al.*, 2016). Amongst other

things, LTA regulates autolytic wall enzymes muramidases and it is mainly released from bacterial cell walls after bacteriolysis (bacterial shedding) a method utilised by the immune system to eliminate bacteria from circulation (Ginsburg, 2002).

Lipoteichoic acid is thought to be a virulence factor because it plays a significant role in infections and in post infectious sequelae caused by Gram-positive bacteria (Ginsburg, 2002). Researchers found that in animal studies, LTA induces periodontal lesions, arthritis, and triggers cascades responsible for multi-organ failure and septic shock (Ginsburg, 2002). In human studies LTA is linked to various inflammatory conditions from minor skin ailments to meningitis and severe sepsis (Ginsburg, 2002; Kang, Ju-Ri Sim, *et al.*, 2016).

Pathogen-associated molecular patterns are found on microbes and not on mammals. Lipoteichoic acid is able to bring about infections and post infections sequelae because it is a type of PAMP molecule and induces bacterial pathogenesis by binding to host's factors or regulating intracellular signalling pathways (Dai *et al.*, 2014). Lipoteichoic acid stimulates the host's immune system resulting in the initiation of the hosts innate immune response system through TLR-2 (Dai *et al.*, 2014; Kang, Ju-Ri Sim, *et al.*, 2016). Therefore, LTA is an effective stimulator of the immune system and a malicious infectious agent, that if left untreated could have severe implications on a person's well-being (Dai *et al.*, 2014)Ì. The connection between inflammation and LTA will be discussed in more detail in the subsequent paragraphs.

2.3. How Does Circulating LTA Induce Inflammation?

Once in circulation and after bacterial shedding, LTA sets into motion an inflammatory response by binding non-specifically to either membrane phospholipids, target cells, or by binding specifically to TLR (Ginsburg, 2002). Toll like receptors are a class of proteins that form part of the innate immune system. Their main function is to recognise molecules from viruses and microbes such as LPS and LTA (Kang, Ju-Ri Sim, *et al.*, 2016). Toll like receptor-2 specifically recognises and binds to LTA leading to the activation of the innate immune and ultimately the triggering the activation of the adaptive immune system (Kang, Ju-Ri Sim, *et al.*, 2016). Lipoteichoic acid binding to target cells causes it to interact with circulating antibodies thus triggering the complement system (Kang, Ju-Ri Sim, *et al.*, 2016). Lipoteichoic acid is also identified as a stimulant for macrophages and neutrophils to secrete nitrogen species, cytotoxic cytokines and reactive oxygen species that further amplify cell damage (Ginsburg, 2002).

Toll like receptors are a type PRR that stimulates the immune system by identifying PAMP expressed by different microbes (Torrazza M.D. and Neu, 2012; Kawasaki and Kawai, 2014). Pathogen-associated molecular patterns are found on immune cells like macrophages and dendritic cells and non-immune cells such as epithelial cells and fibroblasts (Kawasaki and

Kawai, 2014). With regards to TLR, they are classified into two categories; intracellular TLR and cell surface TLR. Intracellular TLR are localised in the endosome and examples of them include TLR3, TLR7-9. TLR11-13. Intracellular TLR specifically recognise of virus and as virus are not the subject matter of interest for this thesis they will be no further discussion about them (Kawasaki and Kawai, 2014). Cell surface TLR specifically recognise microbe constituents such as lipoproteins, lipids and proteins, examples include TLR1, TLR2 and TLR4. TLR4 is specifically recognises Gram-negative bacterial component LPS and TLR2 specifically recognises Gram-positive bacterial component LTA (Kawasaki and Kawai, 2014).

The recognition of PAMP by TLR, triggers downstream signalling pathways and the recruitment of adaptor proteins Myeloid differentiation primary response 88 (MyD88) and TIR-domain containing adapter-inducing interferon-β (TRIF). TIR-domain containing adapter-inducing interferon-β responds to TLR activation after being exposed to a pathogen and triggers downstream signalling cascades that lead to specific cellular responses. Myeloid differentiation primary response 88 is a gene that is responsible for proving the instructions for proteins of the immune system that are responsible for responding early to pathogens. Myeloid differentiation primary response 88 and TRIF trigger signal transduction pathways responsible for the activate of NFκB and mitogen-activated protein kinase (MAPK) (Dai *et al.*, 2014; Kawasaki and Kawai, 2014). NFκB and MAPK control inflammation by regulating various genes responsible for inflammation such as chemokines and pro-inflammatory cytokine like TNF-α, IL-1β, IL-6 and IL-8 (Bester and Pretorius, 2016). These pro-inflammatory cytokines help to protect the host against microbial infections by stimulating inflammation that subsequently helps kill microbes (Kawasaki and Kawai, 2014). Some of the cytokines secreted in the presence of LTA are known to stimulate the coagulation system too (Bester and Pretorius, 2016a). Thus, over production of these cytokines will have an impact on the coagulation system. This will be discussed in more detail in the next paragraphs.

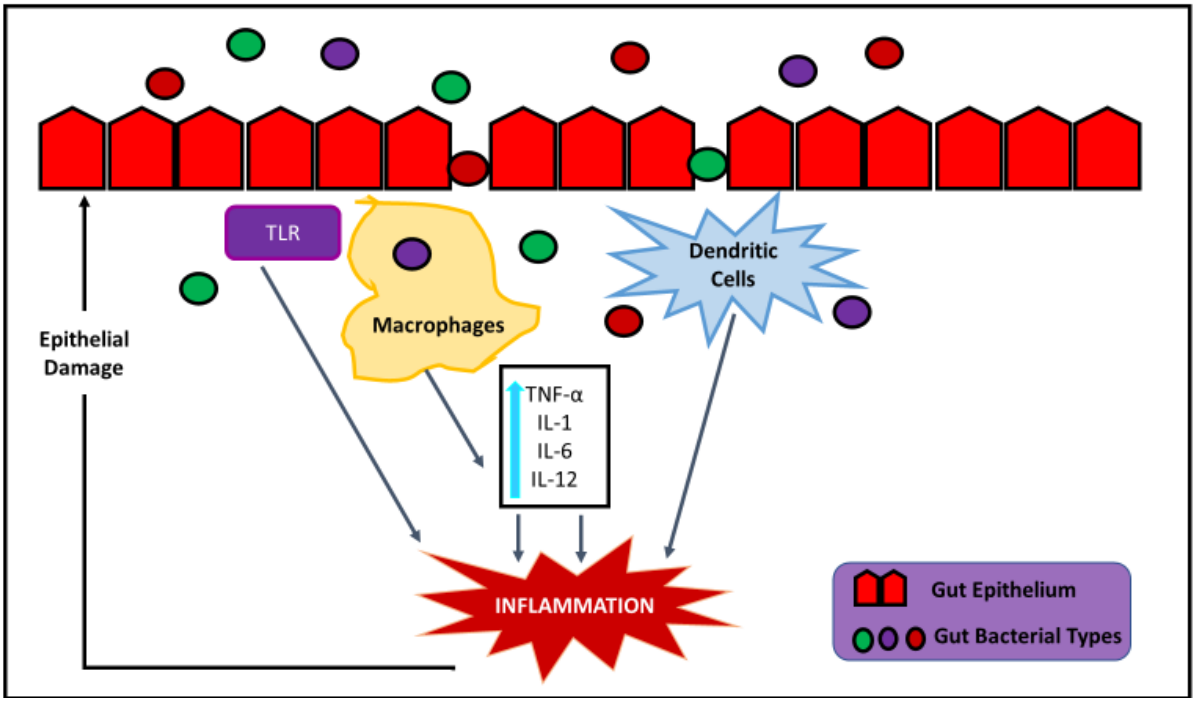

Figure 2.3.1 TLR signalling mechanisms that lead to inflammation adapted from (Ahmad et al., 2017)

2.4. Effects of Lipoteichoic Acid on Clotting Pathways

As noted in the above, dysregulated blood clotting is an important hallmark of inflammation. LTA, when in circulation, might be an important driver of the pathological clotting process. Before discussing pathological clotting, the following paragraphs will discuss coagulation in general.

2.4.1. Coagulation Cascade

In healthy people, blood clotting (also commonly referred to as thrombus formation/blood coagulation) ensues when blood transforms from a liquid-like state to a gel-like state, forming a blood clot (Sherwood, 2010). The coagulation cascade contributes to maintaining haemostasis by preserving the structure of the closed, high-pressure mammalian circulatory system after injury to the blood vessel (Furie and Furie, 1988). Clots that form after endothelial tissue damage comprise of circulating RBC, white blood cells and activated platelets that are integrated into the thrombus (Furie and Furie, 1988). The mechanically formed clot maintains haemostasis by hindering blood flow from the site of injury, therefore preventing mammals from bleeding to death (Furie and Furie, 1988).

Clot formation either occurs through the tissue factor (TF) cascade also known as the extrinsic pathway and is normally the outcome of tissue damage or it occurs by means of contact activation also known as the intrinsic pathway and is the outcome of endothelial damage (Sherwood, 2010). Both the intrinsic and the extrinsic pathways involve platelet activation,

intersect at factor X and terminate with the transformation of soluble fibrinogen into insoluble fibrin, finally forming a fibrin clot as seen in Figure 2.4.1.1 (Furie & Furie, 1988; Sherwood, 2010; Chapin & Hajjar, 2015; Pretorius, Mbotwe, *et al.*, 2016). Soluble fibrinogen protein circulates throughout the entire body at low plasma concentrations of between 2-4mg/mL and plays a key role in blood clot formation (Cortes-Canteli *et al.*, 2012; Chapin & Hajjar, 2015; Kell & Pretorius, 2017). In healthy individuals fibrinogen is approximately 340kDa elongated coiled plasma, 45nm in length and has a 55nm diameter, whereas insoluble fibrin's diameter is characteristically has a diameter of 80- 90nm (Kell and Pretorius, 2015).

Tissue factor initiates the conversion of prothrombin to thrombin and thrombin catalyses the polymerisation of soluble fibrinogen to an insoluble fibrin clot (Furie and Furie, 1988; Chapin and Hajjar, 2015). When fibrinogen is cleaved by thrombin, it is transformed from hydrophilic fibrinogen to hydrophobic fibrin which is polymerised into a fibrin clot (Cortes-Canteli *et al.*, 2012; van Oss, 1990). After the fibrin clot fulfils its purpose and the wound is healed, the clot is degraded into soluble fibrin degradation products by fibrinolysis (Rijken and Uitte De Willige, 2017). This occurs when inactive proenzyme plasminogen is converted into its active enzyme plasmin in the fibrinolytic system. The role of plasmin is to degrade insoluble fibrin into soluble fibrin degradation products which can be removed by the body (Rijken and Uitte De Willige, 2017). However, with chronic inflammation, the degradation process is limited because there is an increase in the production of coagulation proteins thus reducing the activity of the anticoagulant pathway, therefore preventing fibrinolysis. Additionally, with chronic inflammation fibrinogen is constantly being converted into fibrin (van Rooy *et al.*, 2015). More on this will be discussed in the upcoming paragraphs in greater detail.

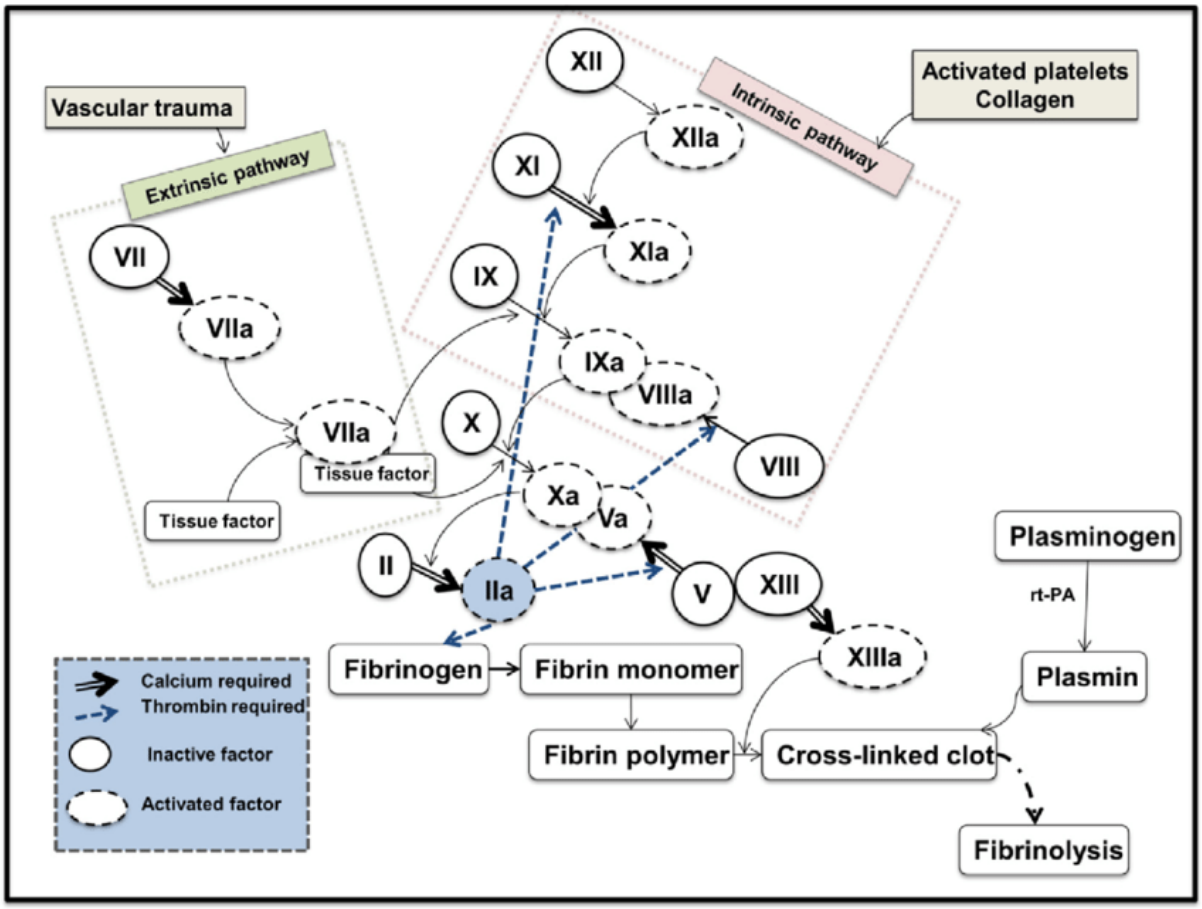

Figure 2.4.1.1 A schematic diagram of the coagulation cascade showing its key events taken form (Kell and Pretorius, 2017)

The next paragraphs will explain in detail how and why inflammation causes clotting abnormities in the coagulation cascade.

With inflammation, TF mediated thrombin generation is increased, anticoagulant mechanisms are downregulated, and fibrinolysis is inhibited (Levi *et al.*, 2003). The downregulation of fibrinolysis is mediated by anti-fibrinolytic (and pro-inflammatory) cytokines TNF-α, IL-1 and IL-8 (Poll, Jonge and an, 2000; Levi *et al.*, 2003). TNF-α and IL-1 are anti-fibrinolytic as it stimulates the production and release of plasminogen activator inhibitor-1 (PAI-1) (Poll, Jonge and an, 2000). Furthermore, in *in vitro* conditions, TNF-α and IL-1 induce vascular endothelial cells and endotoxins to produce significant amounts of IL-8 which is TF and thrombin dependant, thus more fibrinogen is being converted to fibrin clots (Levi *et al.*, 2003).

2.4.2 Hypercoagulation and Hypofibrinolysis

As previously mentioned, once the purpose of a fibrin clot is achieved, it needs to be degraded. However, this does not occur in the presence of inflammation. This phenomenon is known as hypofibrinolysis and mostly occurs simultaneously with hypercoagulation as discussed in the introduction. The following paragraphs will discuss this in more detail.

During inflammation, fibrinogen concentration increases and as a result fibrinolysis is reduced, causing a hypercoagulable environment (Muradashvili and Lominadze, 2013; Pretorius and Kell, 2014; Kell and Pretorius, 2015; Bester and Pretorius, 2016). As previously mentioned, hypercoagulation is the increased likelihood for the formation of blood clots and it is strongly linked to inflammation (Kell and Pretorius, 2015; Bester and Pretorius, 2016). A hypercoagulable environment is typically characterised with increased clot formation and decreased fibrinolysis (Bester & Pretorius, 2016; Pretorius, *et al.*, 2016). Hypercoagulation is one of the common pathologies underlying all thrombotic conditions such as disease venous thromboembolism, ischaemic heart attack and ischemic stroke (Bester & Pretorius, 2016; Pretorius, *et al.*, 2016).

As formerly mentioned, in healthy individuals, fibrin has a spaghetti-like appearance when fibrin fibrils form. However, in pathophysiological conditions like chronic-low grade systemic inflammation, fibrin forms dense matted deposits (DMD) rather than forming individual fibrin fibrils. Dense matted deposits have smaller diameters than healthy individual fibrin fibres and are not efficiently degraded, creating a thrombus-favouring environment such as is the case in hypercoagulability (Pretorius *et al.*, 2011, Pretorius *et al.*, 2013; Nielsen & Pretorius, 2014). Furthermore, studies found that higher fibrinogen concentrations results in shorter clot forming times and the clot that forms is often weaker than the clot formed under normal fibrinogen physiological concentrations (Cortes-Canteli *et al.*, 2012; Kell and Pretorius, 2015)**.** Therefore, increases in fibrinogen concentrations are linked to impaired fibrinolysis, a hypercoagulable state, upregulated inflammation and a changed clotting profile (Pretorius and Kell, 2014).

As platelets and RBC are closely associated with circulating fibrin, the next paragraphs will discuss healthy structure and function and how these cells change/are affected during inflammation. The role of bacterial inflammagens on their structure and function will also be discussed.

2.5. Inflammation and Its Effect on Platelets

Platelets are small, anucleate, “inactive” (in an inactive state) cells only present in mammals. They are derived from megakaryocytes and released into circulation in large numbers (Walsh, Metharom and Berndt, 2015; Koupenova and Ravid, 2018; Rossaint, Margraf and Zarbock, 2018). Platelets are key regulators of thrombus formation when acute vascular injury occurs, and their primary role is to maintain haemostasis by preventing interstitial bleeding (Ho-Tin-Noé, Demers and Wagner, 2011). Platelets adhere and aggregate at the site of injury therefore regulating and initiating thrombus formation in response to acute endothelium injury which eventually results in fibrin clot formation (Kang, 2013; Walsh, Metharom and Berndt, 2015). The secondary role of platelets is to help prevent the occurrence of wound infections entering

the blood circulation by creating a boundary that limits the entry of exogenous pathogens (Rossaint, Margraf and Zarbock, 2018).

As mentioned in the paragraph above, platelets are ”inactive” molecules, and they remain in an inactive state due to prostacyclin and they remain this an inactive state until they come into contact with adhesion triggering molecules such as collagen and von Willebrand factors (VWF) which are only accessible upon endothelial damage (Rossaint, Margraf and Zarbock, 2018). Platelet activation is the first step in clot formation in the coagulation cascade (van Rooy *et al.*, 2015). Platelet activation occurs either via binding to soluble platelet agonists such as ADP and thrombin or by platelet exposure to subendothelial extracellular matrix components such as collagen and pathogens (Rossaint, Margraf and Zarbock, 2018). When endothelial damage occurs, platelets become hyperactivated because the underlying endothelial cell matrix (ECM) releases an array of ligands that bind to different receptors on platelets, leading to signalling events within the platelets (Walsh, Metharom and Berndt, 2015; Rossaint, Margraf and Zarbock, 2018).

Upon platelet activation a positive feedback response is initiated; pro-coagulatory mediators such as ADP, thrombin and prostaglandins are secreted resulting in the activation and recruitment of more platelets at the site of damage leading to the formation of a leak-sealing thrombus (Rossaint, Margraf and Zarbock, 2018). The binding of platelets to ligands leads to the activation of platelet intracellular signalling pathways. These activated pathways lead to changes in platelet morphology, platelet cytoskeletal rearrangements, open canalicular system (OCS) cell surface adhesion molecules activation and the release of platelet granule content which are a source of many pro-coagulant and pro-inflammatory mediators pathogens (Rossaint, Margraf and Zarbock, 2018). For platelet adhesion to be initiated, glycoprotein receptor must bind to VWF and collagen (Walsh, Metharom and Berndt, 2015).

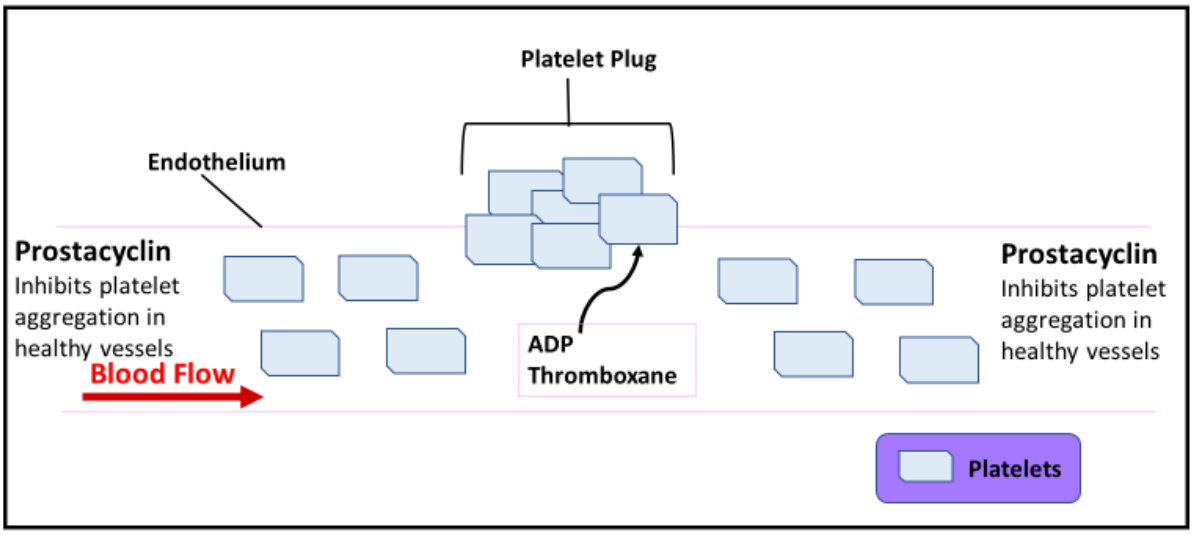

Figure 2.5.1 A diagram showing platelet activation and the various mediators and ligands involved adapted from (Ceraso and Spezzano, 2016).

Under normal physiological conditions, in the presence of acute inflammation, platelets release numerous inflammatory mediators that are necessary to initiate wound healing and for the recruitment of neutrophils and macrophages (Swanepoel and Pretorius, 2014; Thomas and Storey, 2015). However, continuous release of these inflammatory mediators become more detrimental than beneficial as long-term inflammation negatively affects platelet structure. Bester and Pretorius 2016a found that in the presence of pro-inflammatory cytokines platelets become hyperactivated, and clump together. In the presence of constant inflammation, platelets structure changes significantly. These platelets develop pseudopodia which is a sign of activation and look spiky (scrambled eggs appearance under a microscope) in appearance as seen in FFigure 2.5.2., furthermore, although not seen in Figure 2.5.2, literature has shown that platelets clump together in the presence of inflammation. Chronic inflammation has pathological impact on platelets thus causing pathological coagulation and hypercoagulation (Bester & Pretorius, 2016). Since, platelets make up approximately 80% of the thrombus formed and they are responsible for the elasticity and rigidity of the clot formed, any alterations in their membrane characteristics affects the quality of thrombus formed (van Rooy *et al.*, 2015).

Researchers have found that human platelets also have receptors for TLRs 1,2,4,6 and 8 on their surfaces (Bester and Pretorius, 2016a; Kerrigan, 2018). From the information mentioned earlier in this chapter, we know that LPS and LTA initiate immune responses by binding to TLRs 4 and 2 respectively, thus LPS and LTA could directly bind to platelets and induce an inflammatory like response (Kang *et al.*, 2016, ref for LPS). Furthermore, bacterial induced platelet aggregation is an all-or-nothing response, thus regardless of the bacterial concentration added the extent of platelet aggregation is always be great (Kerrigan, 2018).

When LPS/LTA bind to their respective TLR on platelets, platelets are stimulated to secret granular content such as P-selectin. P-selectin is necessary for the formation platelet leukocyte complex as it assists with the adhesion of platelets to monocytes, neutrophils and lymphocytes resulting in further platelet spreading and aggregation (Kerrigan, 2018).

In the presence of bacterial endotoxins, platelets interact with bacteria by binding bacteria for pathogen delivery and presentation thereby stimulating an immune response (Rossaint, Margraf and Zarbock, 2018). The mechanisms in which bacteria interact with platelets can be classified as direct or indirect interactions. Direct interactions occur when bacteria express proteins that directly interact with platelet surface receptors, thus activating platelets (Kerrigan, 2018). An example of a bacterial species that uses this is *Streptococcus sanguinis* which is implicated in endocarditis and periodontal disease (Nijjer and Dubrey, 2010). Indirect interactions include bridging protein which is when bacteria coat itself with plasma protein and uses this as a bridge to its reciprocal platelet receptor (Kerrigan, 2018). *Staphylococcus aureus* is known to use this specific method to bind to and activate platelets and inducing aggregation (Waller *et al.*, 2013; Kerrigan, 2018). Another indirect interaction includes secretion, which is when bacteria secret products that activate platelets and *E.coli* is known to use this method (Kerrigan, 2018). Therefore, platelet activation due to the presence of bacterial endotoxins, perpetuates the inflammatory response, which bring about adverse side effects.

In conclusion, platelets have a vital role in clot formation, protecting against foreign molecule invasions, initiating the coagulation cascade and initiating inflammation. However, under systemic inflammation due to endotoxins, platelets are negatively affected, and they are hyperactivated. In the next paragraphs, RBC and their involvement in the coagulation cascade and the effect of inflammation on them will be discussed.

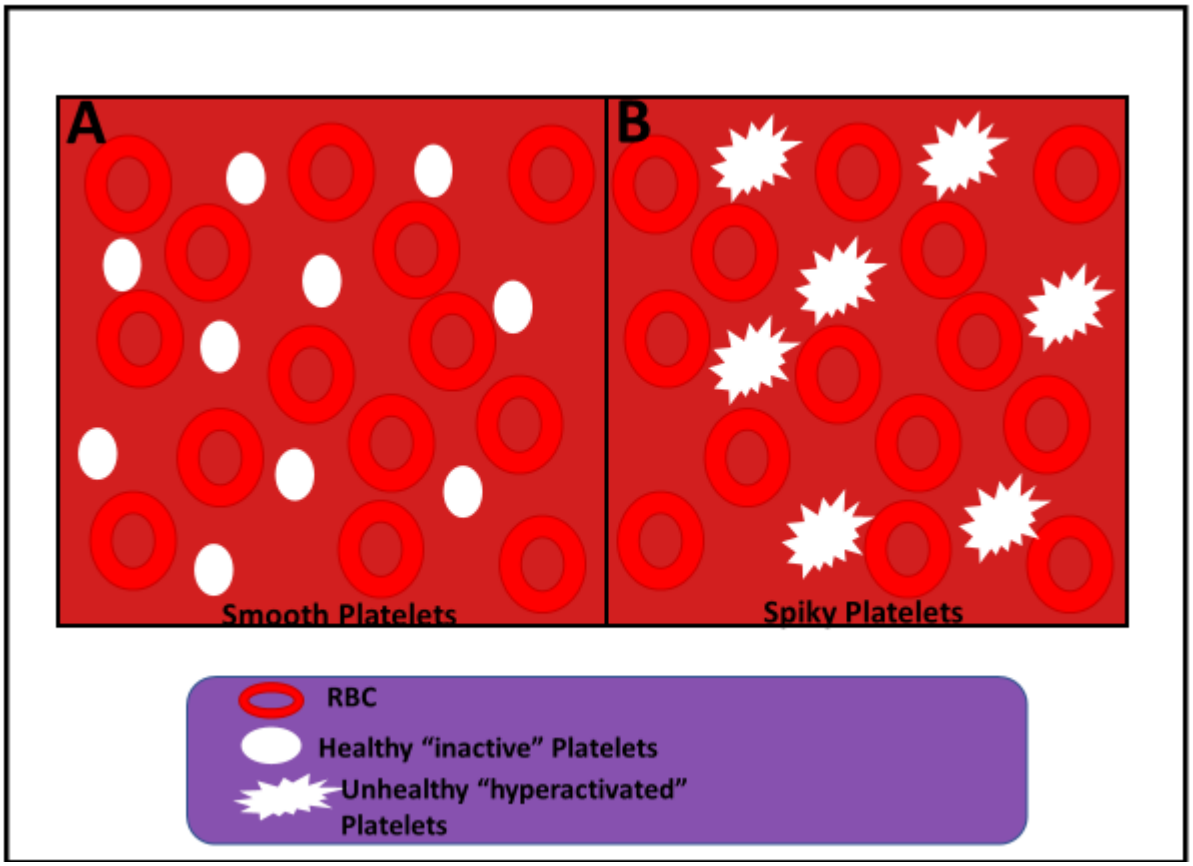

Figure 2.5.2 An image showing healthy and unhealthy platelets (white). Image A) is an example of a platelets in their inactivate state. B) is an example of activated platelets with pseudopodia and granules. Adapted from (*Poor blood circulation*, date accessed: 31 March 2019).

2.6. Inflammation and Its Effect on Erythrocytes (Red Blood Cells)

Erythrocytes or RBC are anucleate, biconcave discs that lack mitochondria (Helms, Gladwin and Kim-Shapiro, 2018). Their primary function is to transport oxygen to cells in the body and transport carbon dioxide away from the peripheral cells to the lungs where carbon dioxide is expelled from the body. Their secondary function is being involved in the coagulation cascade (Lang, Syed M Qadri and Lang, 2012; Pretorius, Du Plooy and Bester, 2016; Helms, Gladwin and Kim-Shapiro, 2018). Red blood cells play a role in thrombus formation and influence vascular tone (Lang, Syed M. Qadri and Lang, 2012; Helms, Gladwin and Kim-Shapiro, 2018). The lifespan of RBC is limited to 100-120 days by senescence. However, RBC death can occur before the aforementioned period in a process known as eryptosis, which will be discussed in detail in later paragraphs (Lang, Syed M. Qadri and Lang, 2012).

Red blood cells membrane is composed of three layers, mainly the exterior, the lipid bilayer and the inner surface. The exterior surface consists of a carbohydrate rich glycocalyx, the lipid bilayer consists of transmembrane proteins and interacts through integral membrane proteins and the inner surface consists of the membrane skeleton that is a structural network of proteins

(Pretorius, 2013; Pretorius, Du Plooy and Bester, 2016). The functionality of RBC is greatly dependent on membrane conformation (Pretorius, 2013). For example, the functional status of RBC is correlated with the roughness of the RBC membrane. In diseased individuals there is an increase in roughness of the RBC membrane (Pretorius, 2013). Furthermore, distressed RBC tend to agglutinate and form aggregates (Page, Bester and Pretorius, 2018).

Recently it's been discovered that RBC are affected by inflammation and are sensitive to high glucose levels (Pretorius, 2013). Studies have also found that they are morphological changes in the membrane of RBC after glucose exposure (Pretorius, 2013). Additionally, RBC in T2DM are smaller and have a reduced concave depth compared to healthy individuals (Pretorius, 2013).

Eryptosis is a method used by the body to remove damaged RBC and is similar to apoptosis (Pretorius *et al.,* 2016). However, eryptosis is the programmed suicidal cell death of RBC and is characterised by RBC shrinkage, the translocation of phosphatidylserine from the inner leaflet of the cell membrane to the outer surface of the RBC as well as cell membrane blebbing (Lang *et al.*, 2012; Lang & Lang, 2015). Excessive eryptosis contributes to various pathological conditions such as anaemia, heart failure, sepsis and iron deficiency (Pretorius *et al.,* 2016). There are various known triggers for eryptosis with oxidative stress and hyperosmotic shock being the most prominent (Pretorius *et al.,* 2016). The sequence of events that follow if eryptosis is triggered by oxidative stress as calcium (Ca^{2+}) enters into the RBC through permeable non-selective cation channels ultimately resulting cell shrinkage, membrane blebbing and cell membrane scrambling (Pretorius, Du Plooy and Bester, 2016). Alternatively, eryptosis can be triggered by hyperosmotic shock that also results in cell shrinkage, membrane blebbing and cell membrane scrambling as seen in Figure 2.6.1 (Pretorius, Du Plooy and Bester, 2016).

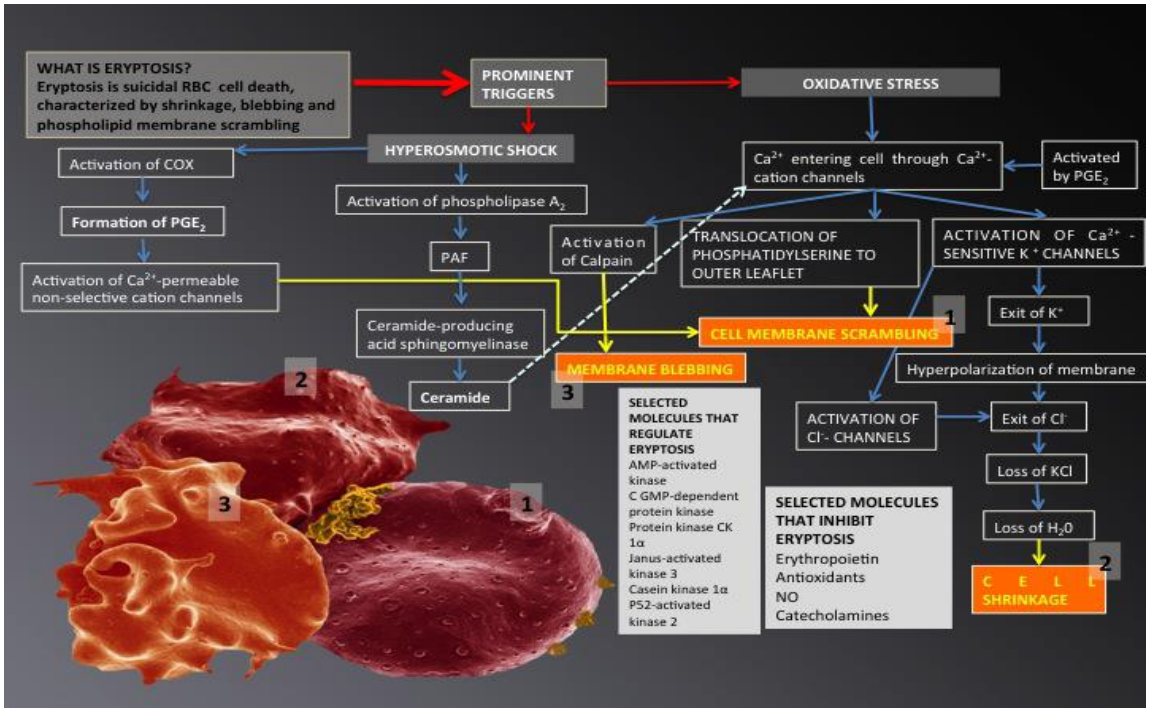

Figure 2.6.1 A detailed diagram showing eryptosis, its triggers and signalling pathways. Taken from (Pretorius et al., 2014)

In the presence of bacterial endotoxins, which possibly perpetuates inflammation, RBC are also affected which will be discussed in more detail.

2.7. Hypercoagulation and Amyloid Formation

Amyloid proteins are described as misfolded protein aggregates with cross β-sheets arranged perpendicular to the axis of the fibre conformation that aggregate under pathophysiological conditions (Romero, Kolter and Romero, 2014; Nizhnikov, Antonets and Inge-Vechtomov, 2015; Kisilevsky, Raimondi and Bellotti, 2016). The changes that occur in the conformational structure from healthy protein to amyloid protein, provides the amyloid protein with advantageous properties that enable them to survive under pathophysiological conditions. These amyloid proteins have been implicated in various incurable human sicknesses like PD, AD and recently T2DM (Kisilevsky, Raimondi and Bellotti, 2016). These advantageous properties consist of improved structural stability, amphiphilicity, adhesiveness, adsorption to hydrophobic-hydrophilic interfaces and insolubility (Romero, Kolter and Romero, 2014; Kisilevsky, Raimondi and Bellotti, 2016).

The definite cause of amyloid protein formation is still unknown, however it is thought that a pro-inflammatory environment together with the hyperactivated immune system might activate amyloid formation (Kisilevsky, Raimondi and Bellotti, 2016; Philippens *et al.*, 2016). Researches have found that increased levels of pro-inflammatory cytokine IL-1β amplifies the synthesis of Amyloid-β (Aβ) precursor protein, resulting in increased amyloidopathy

(Philippens *et al.*, 2016). However, the exact mechanism behind the relationship between inflammation and amyloid formation is not yet known (Philippens *et al.*, 2016).

As previously mention, fibrinolysis is impaired in the presence of hypercoagulation resulting in the formation of tighter fibrin networks (Cortes-Canteli *et al.*, 2012). In AD brains, it's been found that Aβ affects the structure of fibrin clots formed and interrupts clot lysis. Researchers have found that weaker fibrin fibres form in the presence of Aβ because Aβ interferes with the binding of plasminogen to fibrin (Cortes-Canteli *et al.*, 2012; Jean *et al.*,2017). Plasminogen is in control of fibrinolytic process, and a deficiency in it results in hypofibrinolysis consequently forming a hypercoagulable state. Researchers have shown that interactions between fibrin(ogen) and β-amyloid exist, however, the exact mechanism is not yet known (Ahn *et al.*, 2010; Cortes-Canteli *et al.*, 2012). It is already known that the inflammation brought about in the presence of bacterial components affects fibrin(ogen), in the next paragraphs we present a novel idea on amyloid formation in the presence of bacterial component LTA.

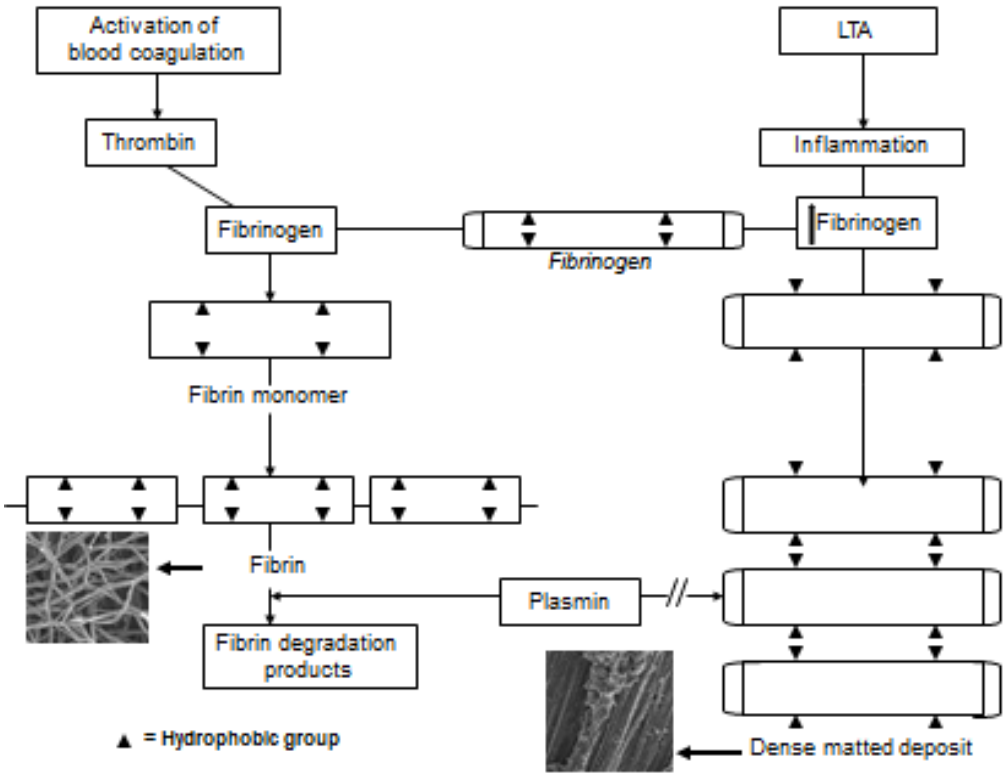

Figure 2.7.1 A diagram showing the coagulation cascade under normal physiological conditions and pathophysiological conditions. Adapted from (Pretorius and Kell, 2014).

The diagram in Figure 2.7.1, illustrates that under healthy physiological conditions, blood coagulation is not amyloidogenic in nature, however, when bacterial components like LTA are present, the clots formed favoured β-sheet conformation instead of an α-helical conformation (Pretorius and Kell, 2014; Pretorius *et al.*, 2018). On the left side of Figure 2.7.1, normal blood

coagulation occurs, and thrombin cleaves fibrinogen in to fibrin fibrils. On the right side of the diagram, blood clotting in the presence of LTA is demonstrated. Under pathophysiological conditions where LTA is present fibrin forms DMD instead of fibrin fibrils. Additionally, this pathway has been suggested in the causation of pathophysiological blood clot formation because LTA hinders normal fibrin(ogen) function and formation (Bester and Pretorius, 2016).

Understanding the implications of LTA on inflammation, hypercoagulation and amyloidogenesis better, scientists found possible a serum protein, namely LBP to reduce the amyloid signal that occurs in the presence of bacterial components. This will be discussed in more detail in the next paragraphs.

2.8. Novel Molecules That May Be Able to Prevent Abnormal Clotting During Inflammation and In the Presence of Bacterial Inflammagens

LPS binding protein is a human protein encoded by the LBP gene and synthesised by hepatocytes and intestinal epithelial cell response (Gutsmann *et al.*, 2001). Under normal conditions, LBP serum concentration is 5-10µg/ml conditions and rises to about 200µg/ml 24 hours after an acute immune response is initiated (Gutsmann *et al.*, 2001; Branescu *et al.*, 2013; Parolia *et al.*, 2014). The primary function of LBP is to regulate and control bacterial infections by binding LPS endotoxin from Gram-negative bacteria and presenting it to the immune system to be removed (Gutsmann *et al.*, 2001; Kopp, Kupsch and Schromm, 2016). LBP presents LPS endotoxins to CD14 and TLR-4, triggering macrophoges and neutrophiles to initiate an immune response by secreting IL-6, TNF-α and IL-1 and other inflammatory markers (Branescu *et al.*, 2013).

A recent study by Triantafilou *et al.* found that patients who died from sepsis had lower levels of LBP compared to survivors that had approximately 40% more LBP in their system than non-survivors (Triantafilou *et al.*, 2012). LBP is known to bind specifically to Gram-negative LPS, however, a recent study found that LBP interferes with LTA induced immune response by inhibiting its delivery to their target cells, thus it also binds to LTA (Triantafilou *et al.*, 2012; Kopp, Kupsch and Schromm, 2016). It is believed that in the absence of serum proteins like LBP, causes LPS/LTA directly bind to their cellular targets in high concentrations thus leading to high secretion of pro-inflammatory cytokines cells (Triantafilou *et al.*, 2012).

The same study also found that other serum protein can attenuate the effects of LTA and LPS in patients, and that the patients who survived sepsis had elevated levels of these protein serums. These serum proteins included transferrin, holotransferrin, low-density lipoprotein (LDL) and ApoA1 (Triantafilou *et al.*, 2012). They found that these serums significantly ($p<0.05$) neutralised LTA and LPS cytokine response cells (Triantafilou *et al.*, 2012). To further substantiate this, our group found that if LBP is added to healthy plasma (spiked with bacterial

inflammagens) or plasma from inflammatory diseases such as T2DM, AD and PD, the hyperclottable (amyloid) fibrin structure could be reversed or reduced (Pretorius *et al.*, 2018). After discovering the effects of LBP as a mopping molecule that has the potential to reverse amyloid structure molecule. The question arose if there might be other similar agents. Another novel molecule could produce similar results as LBP that we identified is ApoA1. This is the molecule of choice that we identified as a possible agent to prevent/remove/attenuate abnormal clotting found during inflammation/leaky gut/inflammatory conditions for this project. ApoA1 will be discussed in more details in the next paragraphs.

2.8.1. Apolipoprotein A-1

Apolipoprotein A-1 is human protein that is encoded by the *APOA1* gene, located on the eleventh chromosome and is involved in lipid metabolism. It is the main protein component of HDL in plasma comprising of 60-70% of HDL (Koldamova, Fitz and Lefterov, 2014). Apolipoprotein A-1 is relatively abundant in plasma proteins with a concentration of 1.0-1.5mg/ml or 125mg/dl and is synthesised in the liver and small intestines and is mainly degraded in the liver (Plump, Scorr and Breslow, 1994; Lu *et al.*, 2017; Sirniö *et al.*, 2017). The main function of ApoA1 is to transport surplus cholesterol from the peripheral tissues to the liver. Apolipoprotein A-1 has also been found to have anti-apoptotic, anti-oxidant, and anti-inflammatory functions (Koldamova, Fitz and Lefterov, 2014; Sirniö *et al.*, 2017). Apolipoprotein A-1 encourages ABCA1-mediated cholesterol and phospholipid efflux which initiates HDL synthesis. It is also a co-factor for lecithin cholesterol acyl transferase (LCAT) which is an enzyme that converts cholesterol to cholesterol esters (Sirniö *et al.*, 2017).

As mentioned above, ApoA1 has anti-inflammatory properties and various mechanisms in which ApoA1 potentially exerts these anti-inflammatory properties have been proposed. One such way is the ability of ApoA1 to reduce inflammation by preventing lymph node enlargement, cholesterol accumulation in lymphocytes as well preventing the activation and proliferation of lymphocytes thereby augmenting the effectiveness of lymph node T regulatory response (Mangaraj, Nanda and Panda, 2016). Another proposed mechanism that ApoA1 could use exert its anti-inflammatory effect is by inhibiting interplay between monocytes and T cells. Apolipoprotein A-1 interferes with T cell signalling of the monocytes therefore inhibiting the production of TNF-α and IL-1b which are known proinflammatory molecules (Mangaraj, Nanda and Panda, 2016). Furthermore, ApoA1 inhibits the monocyte inflammatory functions in peripheral blood mononuclear cells activated by specific anti-gens or lectins without affecting cell proliferation (Mangaraj, Nanda and Panda, 2016). Additionally, studies found that ApoA1 has the same structural homology as prostacyclin (PGI_2) stabilising factor and thus may have anti-clotting properties. In addition to this, the alpha helix structure of ApoA1 enables

it to bind to unstable PGI_2, thus causing its stabilisation. The stabilisation of PGI_2 results in the protective anti-aggregatory effects against platelet thrombin formation (Mangaraj, Nanda and Panda, 2016).

Apolipoprotein A-1 has beneficial properties such as having anti-inflammatory properties as previously mentioned. Recently ApoA1 has been recognized as having a glucose-controlling function. Studies have shown that HDL/ApoA1 induces glucose uptake in cultured muscles myotubes and stimulates insulin secretion from cultured β-cells (Namiri-Kalantari *et al.*, 2015). Upon further research, it was discovered that ApoA1 prevents the deposition of Aβ in cerebral blood vessels, and directly inhibits the aggregation of Aβ to lessen plaque formation and the absence of ApoA1 is associated with cerebral amyloid angiopathy (Mangaraj, Nanda and Panda, 2016).

The potential beneficial properties of ApoA1, led to the question on if it will work on an inflammatory condition, such as T2DM. The next paragraphs will discuss the relationship between coagulation and T2DM and the possible affect that ApoA1 might have.

2.9. Diabetes

Type two diabetes mellitus is a global public health epidemic affecting approximately 387 million people worldwide (Polonsky and Burant, 2016). The prevalence of T2DM is projected to double and affect more than half a billion people globally within the next twenty years. The most increase is predicted to be in developing countries as they adapt a more westernised lifestyle. The westernised lifestyle is one of an energy dense diet, accompanied a by lack of physical activity and sedentary behaviour which have been identified as risk factors for T2DM (Tabish, 2007).

Type two diabetes mellitus is a complex endocrine and metabolic disorder characterised by chronic hyperglycaemia due to its relative insulin deficiency and is a key source of morbidity, mortality and health costs globally (Caprio *et al.*, 2013; Zaccardi *et al.*, 2016; Pheiffer *et al.*, 2018). Chronic hyperglycaemia as observed in T2DM is due to the reduced ability of insulin to stimulate glucose uptake and supress glucose release (Smushkin and Vella, 2010). This is believed to be the result of prominent amyloid depositions and increased apoptosis of β-cells leading to a decrease in functional β-cells (Smushkin and Vella, 2010).

Type two diabetes mellitus is also characterised by a slow progressive degeneration of islet β-cells, which result in decreased insulin secretion and decreased insulin action on peripheral tissue (Miklossy *et al.*, 2010). The pathophysiological features of T2DM include but are not limited to inflammation, dysregulated angiogenesis, and atherosclerosis (Wong *et al.,* 2018). Our group has also found that another, previously unrecognised hallmark feature of T2DM is

amyloid and the formation of DMD (Pretorius, Mbotwe and Kell, 2017; Pretorius *et al.*, 2018). Islet amyloid deposits have been observed in more than 95% of all T2DM patients and the extent of the amyloid deposits is dependant severity of the disease (Zaccardi *et al.*, 2016). Furthermore, T2DMs has been associated with various vascular and nonvascular conditions such as cardiovascular diseases (CVD), cancer, liver diseases, mental and nervous system disorders (Zaccardi *et al.*, 2016).

A study by Stenkula showed that short term treatment of mice with ApoA1 lead to a significant increase in blood glucose clearance(Stenkula *et al.*, 2014). They found that the increase in glucose uptake in peripheral tissue coincided with an increase in glucose stimulated insulin secretion (Namiri-Kalantari *et al.*, 2015).

Figure 2.9.1 is a summary of this chapter. It is a diagram illustrating the possible role of bacteria in the inflammation-gut permeability complex, how LTA may contribute to hypercoagulation and amyloidogenesis and how it all connects to T2DM as discussed above.

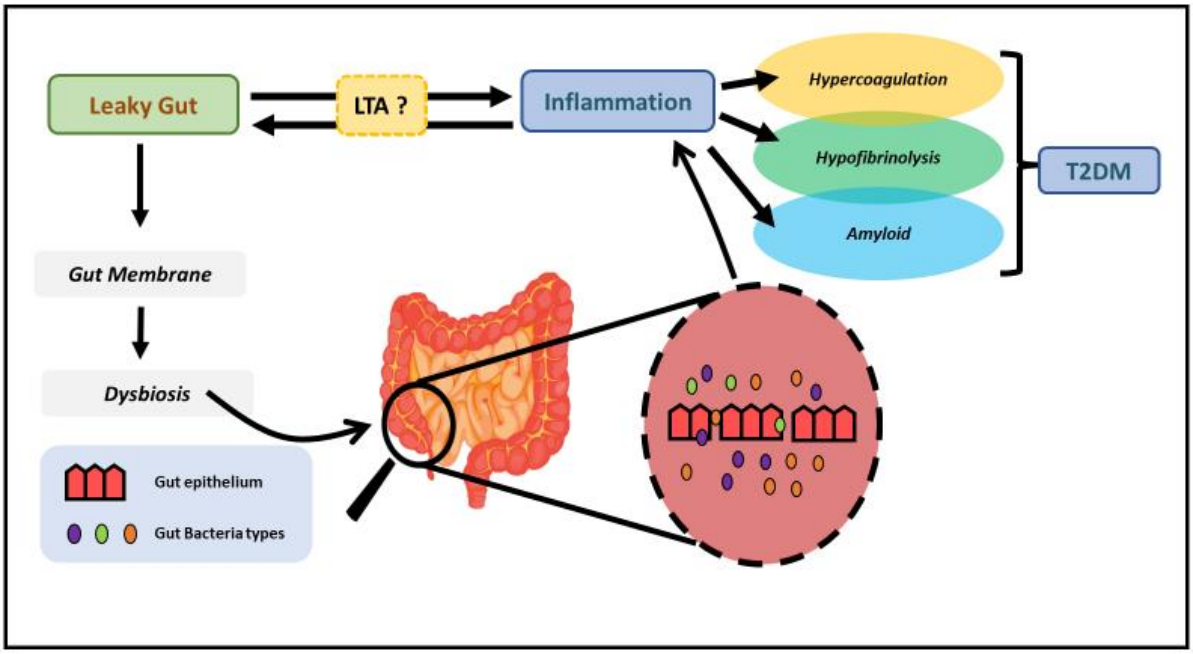

Figure 2.9.1 : A diagrammatic summary of the literature review.

Chapter 3: Sample Demographics and Collection for This Study

3.1. Introduction

This chapter will focus on the recruitment and selection of the study population. For this project, thirty healthy individuals between the ages of 18 and 35 were chosen. To determine the health profile of the individuals, blood was sent to Pathcare laboratories, where CRP, cholesterol, triglycerides and glucose levels were tested. For this study, every individual was used as their own control before and after the addition of LTA and LTA with ApoA1.

For our disease model, seven T2DM patients between the ages of 40 and 95 were chosen and only PPP from these individuals was used for this study. Experiments were done on PPP only for this part of the study. As with the healthy control, each individual was used as their own control before and after the addition of ApoA1.

3.2. Study Population Inclusion and Exclusion Criteria

For this study, a sample population of thirty healthy and seven T2DM individuals were included.

The exclusion criteria for healthy controls included:

- Any known chronic or acute inflammatory conditions such as Tuberculosis, asthma, Human immunodeficiency virus (HIV), endometriosis, the metabolic syndrome and its associated risk factors i.e. hypertension, obesity etc.
- Smokers
- Females on contraceptives or individuals on hormone replacement treatment
- Individuals on anti-inflammatory medication

The exclusion criteria for this project was based on the following reasons:

Inflammatory conditions- Inflammation is associated with a hypercoagulable/prothrombotic state (Silvio Danese *et al.*, 2007).

Smokers- Smoking is identified as a risk factor for almost all diseases associated with chronic or acute thrombosis (Nielsen, Hafner and Steinbrenner, 2013). Researchers found that smoking enhances clot strength and plasmatic coagulation by modulating the heme associated with fibrinogen (Nielsen, Hafner and Steinbrenner, 2013). Furthermore, smoking also induces a hypercoagulable state that involves increased circulating fibrinogen, increased circulation factor XIII and enhanced activation of platelets (Nielsen, Hafner and Steinbrenner, 2013).

Contraceptives/hormone treatment- Studies show that oestrogen is associated with various prothrombotic alterations in the proteins involved with coagulation. Examples of changes in procoagulant blood proteins include but are not limited to increased levels of factor II,VIII and X, increased fibrinogen concentrations and decreased levels of antithrombin (Trenor *et al.*, 2011).

The inclusion criteria included:

- All races and genders were welcome and accepted
- Controls were between the ages of 18-35 years old

3.3. Sample Size and Collection

Once informed consent was obtained a total of four blood tubes (two citrate tubes, one EDTA tube and one SST tube) were collected from each control. All drawing of blood was done by a qualified phlebotomist. Once blood was collected, citrate tubes was used for the study and any remaining blood from the citrate tubes was centrifuged into PPP and stored at -80-degree Celsius freezer.

Whole blood collected in EDTA and SST tubes was sent to Pathcare laboratories for analysis. The results were used to ensure that our controls were healthy and that they were no underlying inflammatory conditions. All blood drawing occurred in a clean environment and in an ethical manner.

3.4. Ethics

Ethical clearance for this research project was granted by the ethical committee of Stellenbosch University, South Africa. The ethical clearance number is as follows: 298/2016. We concur that we adhered strictly to the Declaration of Helsinki. (For a copy of the consent form please refer to the addendum.)

Table 3.4.1 Demographics of healthy controls used for this study.

Gender	Average Age
14 Female; 16 Male	23,5 Female; 23,5 Male

3.5. Pathcare Results

Blood obtained from each control was taken to PathCare laboratories where the following parameters were measured: Glucose (HbA1c), Cholesterol (S-HDL, S-LD L, S-non-HDL), cholesterol:ratio, CRP and triglycerides.

Table 3.5.1 Pathcare results of all participating individuals. Included in this table is HbA1c, Cholesterol (S-HDL, S-LD L, S-non-HDL), CRP and triglycerides levels of every control used for this project.

	HbA1c (%)	S-Cholesterol	S-HDL Cholesterol	S-LDL Cholesterol	S-Triglyceride	S-Non-HDL Cholesterol	CHOL:HDL ratio	CRP ultrasensitive
	5.6	5.20	1.80	3.00	0.64	3.4	2.90	0.94
	5.0	4.60	1.70	2.40	2.90	2.9	2.70	0.27
	5.2	3.10	1.10	1.50	0.36	2	2.80	0.22
	5.1	4.20	1.70	2.30	0.46	2.5	2.50	0.54
	5.4	4.20	1.10	2.70	0.78	3.1	3.80	0.97
	5.2	4.30	1.50	2.30	0.52	2.8	2.90	4.76
	*	3.80	1.30	1.90	0.51	2.5	2.90	1.78
	5.00	4.10	0.80	2.80	0.87	3.30	5.10	0.72
	5.40	5.10	1.40	3.20	0.68	3.70	3.60	1.33
	4.80	3.70	1.10	2.00	0.46	2.60	3.40	0.84
	4.80	4.60	2.30	2.00	0.63	2.30	2.00	0.98
	4.70	4.80	1.40	2.90	0.80	3.40	3.40	1.24
	3.40	3.80	1.50	2.00	0.62	2.30	2.50	0.49
	5.20	4.50	1.50	2.50	0.94	3.00	3.00	0.81
	5.4	3	1.8	1.1	0.63	3.8	1.7	0.34
	5.1	3.7	1.5	2	0.39	2.2	2.5	0.42
	4.9	5.9	1.4	3.9	1.53	4.5	4.2	0.49
	5.5	4.2	1.4	2.5	0.6	2.8	3	0.2
	5.5	4.8	1.2	3.2	4.8	3.8	4	0.81
	5.2	3.7	1.2	2.1	0.36	2.5	3.1	0.35

	5.3	**4.3**	**1.9**	**2.1**	**0.54**	**2.4**	**2.3**	**0.21**
	5.3	**6.2**	**2.1**	**3.5**	**0.55**	**4.1**	**3**	**1.27**
	5	**3.7**	**1.3**	**2**	**0.38**	**2.4**	**2.8**	**2.67**
	4.9	**4.1**	**1.1**	**2.4**	**0.64**	**3**	**3.7**	**0.43**
	4.9	**6.3**	**2.6**	**3.3**	**0.42**	**3.7**	**2.4**	**0.21**
	5.6	**4**	**1.4**	**2.1**	**1.16**	**2.6**	**2.9**	**1.96**
	4.9	**3.6**	**1.5**	**1.8**	**0.54**	**2.1**	**2.4**	**1.52**
	5.1	**5.4**	**1.4**	**3.6**	**0.5**	**4.0**	**3.9**	**0.35**
	5.7	**4.3**	**1.2**	**2.7**	**0.53**	**3.1**	**3.6**	**2.66**
	5.0	**5.00**	**1.3**	**3.2**	**1.39**	**3.7**	**3.8**	**1.77**
AVERAGE	**5.14**	**4.41**	**1.48**	**2.50**	**0.86**	**3.01**	**3.09**	**1.02**

***** Pathcare laboratories lost tube.

3.6. Molecules used in this study

In this chapter, only the molecules used for this study are mentioned. The particular protocols and methods will be discussed in more detail in the relevant chapters.

Lipoteichoic Acid

Lipoteichoic Acid from *Staphylococcus aureus* (Sigma, L3140) was used for this study.

A final exposure concentration of 5ng.L^{-1} was used in all experiments.

Apolipoprotein A1

For this study, two types of ApoA1 were used at various concentrations:

- Sigma Apolipoprotein A-1 from human plasma ->85% (SDS-PAGE), buffered aqueous solution. Product number A0722 at a concentration of 10ng/ml
- Purified American ApoA1 form sepsis individuals was used at a concentration of 30ng/ml and 164ul/ml

Thrombin

From the South African National Blood Service and was made in a concentration of 2U/ml and made up in a biological buffer containing 0.2% human serum albumin.

Purified Fibrinogen

Purified Fibrinogen 2mg/ml final concentration (Sigma, F3879)

0.2 $CaCl_2$

TEG ® Hemostasis System 0.2M $CaCl_2$

Amytracker™

Ebba Biotech Amytracker™ 480 ex vivo/100µL, Ebba Biotech Amytracker™630 ex vivo/100µL, Ebba Biotech Amytracker™680 ex vivo/100µL

Congo Red

Congo Red from (Sigma, C6277-25G)

3.7. Study History

This research study is continuation of my honours research. A paper about the results from my honours is attached in the addendum titled "Both lipopolysaccharide and lipoteichoic acids potently induce anomalous fibrin amyloid formation: assessment with novel Amytracker™ stains".

3.8. Conclusion

In this chapter, we reviewed whether the controls used for this study are healthy and had no underlying conditions that may have affected our results.

Chapter 4: Thromboelastography

4.1. Chapter Objectives

To answer research question:

- Will the addition of LTA to WB cause a hypercoagulable state?

4.2. Introduction

Thromboelastrogram (TEG) is a point of care device used to monitor coagulation by assessing viscoelastic properties of WB at the patient's body temperature (Ganter and Hofer, 2008; Pretorius *et al.*, 2017). Thromboelastrogram measures haemostatic components through all phases from clot initiation through to clot lysis (da Luz, Nascimento and Rizoli, 2013). These components include coagulation factors, platelets, RBC, rate of fibrin formation, clot strength and clot lysis (Pleym *et al.*, 1995; Ganter and Hofer, 2008). It assesses global haemostatic functions from a single blood sample under low shear (Ganter & Hofer., 2008). It achieves this by assessing the interactions between platelets and RBC giving information on platelet function (Ganter and Hofer, 2008; da Luz, Nascimento and Rizoli, 2013).

Thromboelastogram measures the physical properties of blood clots by using a stationary cylindrical cup that holds the blood sample and oscillates at an angle of 45° (Ganter and Hofer, 2008). A pin is suspended in the blood by a torsion wire as it monitors for motion. The torque of the rotation cup is transmitted to the immersed pin only after fibrin-platelet bonding has linked the pin and cup together (Pleym *et al.*, 1995). The magnitude of the pin's motion is determined by the strength of the fibrin-platelet bonds; therefore, the output is directly related to the strength of the clot formed (Ganter and Hofer, 2008; da Luz, Nascimento and Rizoli, 2013).

All the stages of the developing clot and clot lysis are measured and recorded numerically and graphically. These stages are Reaction time (R), kinetics of fibrin formation and clot development (K), clot formation time (α angle) and maximum amplitude (MA) which measures the maximum strength and stability of the clot, Maximum rate of thrombus generation (MRTG), Time to maximum rate of thrombis generation (TMRTG) and Total thrombus generation (TTG). A detailed explanation of each value is summarised in Table 4.2.1.

Table 4.2.1. Detailed explanation of each parameter measure by TEG

Parameters	**Explaination**
Reaction Time (R-value) measured in minutes	Initiation time i.e. time until initial clot/fibrin formation. Amplitude of (2mm).
Kinetics (K-value) measured in minutes	Kinectics of clot formation/time taken to achive a certain level of clot strength (20mm).
Alpha Angle (α) measured in degrees	Speed at which fibirn build up and cross linking takes place
Maximal Amplitude (MA) meausred in mm	Maximum strength/stiffness of clot. Reflects the ultimate strength of the fibrin clot formed. i.e. Ultimate strength & 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 thrombis generation (TMRTG) measured in minutes	The time interval observed before the maximum speed of the clot growth.
Total thrombus generation (TTG) measured in $Dyn.cm^{-2}$	Clot strength: the amount of total resistance (to the 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 adapted from (Ganter & Hofer., 2008; Pretoruis *et al., 2017*).

These TEG parameters can be used to determine hypercoagulability, which is not as easily detectable with conventional nonviscoelastic laboratory tests because they have a tendency to require markedly increased fibrinogen conertrations or platelet count (Pretorius *et al.*, 2017). TEG has the ability to determine whether a hypercoagulabe state is present in an individual.

In a hypercoagulable state, TEG parameters are altered from the normal ranges in the following manner (Pretorius *et al.*, 2017). A decrease in the R-value indicates that the clot forms faster. When the K-value decreases, the clot reaches the set stength of 20mm quicker. An increase in the alpha value indicates that there is an increase in thrombin burst resuting in more cross-linking of fibrin fibres. When the MA value increases, there is an increase in platelet or fibrin(ogen) interactions resulting in a denser clot that that is more rigid (Pretorius *et al.*, 2017). When the MRTG value increaes clot growth is increased. When TMRTG decreases, there is a decrease in time from clot initiation to maximum clot formation. TTG increases, thus the clot strength increases (Pretorius *et al., 2017*). A hypercoaguable state is diagnosed when R time is short and the MA increased or one of the TEG parameters is altered towarsds hypercoagulability (Ganter and Hofer, 2008; Pretorius *et al.*, 2017).

4.2. Materials and Methods

Blood was collected from individuals in sodium citrated collecting tubes. Once the WB was obtained from the individual, the blood was allowed to sit for at least 30 minutes, at room temperature before any treatment/analysis could be performed on it. This was done to allow complete Ca^{2+} binding to the blood. Analysis on the blood was done 24 hours of the blood draw.

For the Naïve sample the, following procedure was followed:

To study the clotting properties of the WB on the TEG for analysis, 340µL of untreated naive WB was placed in a TEG cup and 20µL of 0.2µL $CaCl_2$ was added to the sample. The $CaCl_2$ is added to initiate coagulation and thus reverse the effect of the sodium citrate in the collecting tubes. This Sample was then placed in a Thromboelastograph 5000 Hemostasis Analyzer.

For the LTA exposed blood the, follwing procedure was followed:

Whoel blood was treated with LTA at a final exposure of 5ng/L in an Eppendorf tube for 10 minutes. 340µL of the LTA treated WB was placed in the TEG cup and 20µL of 0.2 $CaCl_2$ was added to the sample. The $CaCl_2$ was added to initiate coagulation and thus reverse the effect of the sodium citrate found in the collecting tubes. The sample was then tested in a Thromboelastograph 5000 Hemostasis Analyzer.

For the LTA and ApoA1 blood, the following procedure was followed:

Whole blood was spiked with LTA at a final exposure of 5ng/L for 10 minutes, then treated with ApoA1 at a final exposure concetration of 10ng/ml (Sigma ApoA1) for 10 minutes and 30ng/ml (American ApoA1) for 30 minutes respectively in an Eppendorf tube. 340µL of the LTA and ApoA1 treated WB was placed in the TEG cup and 20µL of 0.2 $CaCl_2$ was added to

the sample. The $CaCl_2$ was added to initiate coagulation and thus reverse the effect of the sodium citrate found in the collecting tubes. The sample was then tested in a Thromboelastograph 5000 Hemostasis Analyzer.

Whole blood was treated with ApoA1 at a final exposure concetration of 164ul/ml (American ApoA1) for 30 minutes before LTA was added at a final exposure concentration of 5ng/L in an Eppendorf tube for 10 minutes. 340µL of the LTA and ApoA1 treated WB was placed in the TEG cup and 20µL of 0.2 $CaCl_2$ was added to the sample. The $CaCl_2$ was added to initiate coagulation and thus reverse the effect of the sodium citrate found in the collecting tubes. The sample was then tested in a Thromboelastograph 5000 Hemostasis Analyzer.

Blood that remained in the sodium citrate collecting tube was centrifuged to platelet poor plasma (PPP).

For the exact protocol followed please refer to the addendum.

4.3. Results

Table 4.3.1 The average value for each parameter that was tested with TEG, before and after the addition of LTA and LTA and ApoA1 to healthy control WB.

	Group	R	K	Angle	MA	MRTG	TMRTG	TTG
Control WB	10ng/ml	7,85	2,53	36,75	62,59	5,78	10,87	859,27
	30ng/ml	8,26	2,76	60,92	59,23	5,14	11,8	763,94
	164/ml	9,02	3,66	58,3	57,1	4,14	12,98	626,68
LTA Treated WB	10ng/ml	7,15	3,7	57,55	59,35	4,86	11,05	859,27
	30ng/ml	7,54	2,86	61,1	57,18	4,64	11,21	697,13
	164/ml	7,21	3,48	57,28	53,24	3,78	11,42	535,31
LTA & APOA1 Treated WB	10ng/ml	9,02	2,55	64,68	57,55	5,29	9,5	727,24
	30ng/ml	7,83	2,86	60,92	57,04	4,22	10,44	685,07
	164ug/ml	6,28	3,2	60,01	50,07	4,01	9,28	544,76

4.4. Statistical Analysis TEG

For statistical analysis, a One-way Anova was performed for TEG and Bonferroni was used as a correction test. Results are presented as mean/median ± standard deviation and p value.

Table 4.4.1 TEG results displayed as mean/median, standard deviation and p-value for all three treatment groups (including multiple comparisons p-values). p is significant when p>0.005 and significant p values are displayed in bold and in purple text. * trend towards significance.

TEG Parameters	Control	LTA	LTA+ ApoA1	p-value	p-value		
					Control vs LTA	Control vs. LTA+ ApoA1	LTA vs. LTA+ApoA1
Whole blood analysis (10ng/ml ApoA1)							
R-time	8.050± 2.232	7.600± 1.793	6.150± 0.725	0.1864	0.8593	0.0990*	0.8593
K-time	2.200± 0.9823	2.650± 1.952	2.600± 0.450	**0.0148**	**0.0156**	0.6563	0.3526
α angle	67.000± 7.363	54.700± 7.859	65.000± 5.145	0.1002	0.1650	>0.9999	0.1650
MA	64.800± 5.943	55.280± 6.728	55.500± 4.822	**0.0212**	0.2806	**0.0219**	0.9429
MRTG	6.055± 1.588	4.000± 1.765	4.870± 1.427	0.1352	0.1325	>0.9999	0.5391
TMRTG	10.690± 3.031	11.010± 1.754	9.458± 1.333	0.1338	>0.9999	0.5607	**0.0348**
TTG	821.200± 183.7	724.300± 201.200	694.900± 134.100	0.0937*	0.5747	**0.0073**	>0.9999
Whole blood analysis (30ng/ml ApoA1)							
R-time	8.26 ± 2.656	7.540 ± 1.184	7.210 ± 0.986	0.3202	>0.9999	0.8395	0.6735
K-time	2.76 ± 0.7043	2.860 ± 0.656	2.860 ± 0.747	0.8620	>0.9999	>0.9999	>0.9999
α angle	60.920 ± 6.47	61.100 ± 6.225	60.920 ± 6.547	0.9943	>0.9999	>0.9999	>0.9999
MA	59.23 ± 7.569	57.180 ± 6.411	57.040 ± 4.147	0.3171	0.6213	0.5372	>0.9999
MRTG	5.055 ± 2.232	4.520 ± 1.793	3.845 ± 0.725	0.1873	>0.9999	0.2209	0.3526
TMRTG	11.8 ± 3.222	11.210 ± 1.834	10.44 ± 1.530	0.2716	>0.9999	0.3364	>0.9999
TTG	763.9 ± 207.7	697.100 ± 161.8	685.100 ± 111.2	0.1720	0.4153	0.2515	>0.9999
Whole blood analysis (164µg/ml ApoA1)							
R-time	9.020 ± 2.762	7.830 ± 0.946	6.280 ± 1.527	**0.0202**	0.5782	**0.0179**	0.2848
K-time	3.660 ± 1.483	3.480 ± 0.699	3.310 ± 1.343	0.6992	>0.9999	>0.9999	>0.9999
α angle	55.900 ± 9.0110	55.250 ± 4.691	58.650 ± 7.171	0.159	0.7907	>0.9999	0.3526

MA	53.160 ± 11.220	53.240 ± 6.374	50.070 ± 11.65	0.6034	>0.9999	>0.9999	>0.9999
MRTG	3.435 ± 2.232	3.380 ± 1.793	3.590 ± 0.725	0.8302	>0.9999	>0.9999	>0.9999
TMRTG	12.980 ± 3.911	11.430 ±1.400	9.284 ± 2.283	**0. 0276**	0.6943	**0.0253**	0.3083
TTG	626.700± 279.200	535.300± 230.1	544.800± 234.8	0.3908	0.6508	0.7988	>0.9999

***Trend towards significance

10ng/ml

In the control vs LTA samples, the R-value, alpha angle, MA, MRTG, TMRTG and TTG decrease although no significance was found. There was a significant increase in the k- value of the control vs LTA samples with a significant p-value of 0.0148. In the multiple comparisons of the k-value, there was a significant increase found between the control vs LTA samples with a significant p-value of 0.0156 as seen in Table 4.4.1 and Figure 4.4.1.

From Table 4.4.1 and Figure 4.4.1, the LTA vs LTA+ApoA1 samples, there was a decrease found in the R-value, k-value, TMRTG and TTG, with a significant decrease in the TMRTG parameter with a p-value of 0.0348. An increase was observed in the alpha angle, MA and MRTG although no significance was found.

When looking at control vs LTA+ApoA1, there was a decrease in the R-value, k-value, alpha angle, MA, MRTG, TMRTG and TTG. With significant decreases observed in MA and TTG parameters with p-values of 0.0219 and 0.0073 respectively.

30 ng/ml

In Table 4.4.1 and Figure 4.4.2, no significance or trend towards significance was found in this group. The R-value, MA, MRTG, TMRTG and TTG decreases across all treatment groups compared to the control for both LTA and LTA+ApoA1 groups. The k-value increased from the control to LTA and remained the same for LTA vs LTA+ApoA1 group. The alpha angle increased between control vs LTA and decreased between LTA vs LTA+ApoA1.

164µg/ml

As seen in Table 4.4.1 and Figure 4.4.3, the R-value, K- value and TMRTG decreased across all sample groups compared to the control, with significance in R-value with a p-value of 0.0202 and TMRTG with a p-value of 0.0276. Significance was found between the LTA vs LTA+ApoA1 for both R-value and TMRTG with a p-value of 0.0179 with a p-value of 0.0253 respectively. The alpha angle decreased between control vs LTA and increased between LTA

vs LTA+ApoA1 and control vs LTA+ApoA1. MRTG and TTG decreased between the control and LTA exposed WB and increased from the LTA to LTA+ApoA1 group.

Table 4.4.2 TEG results displayed as arrows showing where parameters increased and decreased as well as significant values (including multiple comparison). p is significant when p>0.005 and significant p values are displayed in bold and in purple text. * trend towards significance

TEG Parameters	Control	LTA	LTA and ApoA1	P value	Significant difference		
					Control vs LTA	Control vs. LTA & ApoA1	LTA vs. LTA&ApoA1
Whole blood analysis (10ng/ml ApoA1)							
R-time	↔	↓	↓	0.1864	-	-	-
K-time	↔	↑	↑	**0.0148**	Yes		-
α angle	↔	↓	↓	0.1002	-	-	-
MA	↔	↓	↓	**0.0212**	-	Yes	-
MRTG	↔	↓	↓	0.1352	-	-	-
TMRTG	↔	↓	↓	0.1338	-	-	Yes
TTG	↔	↓	↓	0.0937*	-	Yes	-
Whole blood analysis (30ng/ml ApoA1)							
R-time	↔	↓	↓	0.3202	-	-	-
K-time	↔	↑	↑	0.8620	-	-	-
α angle	↔	↑	=	0.9943	-	-	-
MA	↔	↓	↓	0.3171	-	-	-
MRTG	↔	↓	↓	0.1873	-	-	-
TMRTG	↔	↓	↓	0.2716	-	-	-
TTG	↔	↓	↓	0.1720	-	-	-
Whole blood analysis (164µg/ml ApoA1)							
R-time	↔	↓	↓	**0.0202**	-	Yes	-
K-time	↔	↓	↓	0.6992	-	-	-
α angle	↔	↓	↑	0.1590	-	-	-
MA	↔	↑	↓	0.6034	-	-	-
MRTG	↔	↓	↑	0.8302	-	-	-
TMRTG	↔	↓	↓	**0.0276**	-	Yes	-
TTG	↔	↓	↓	0.3908	-	-	-

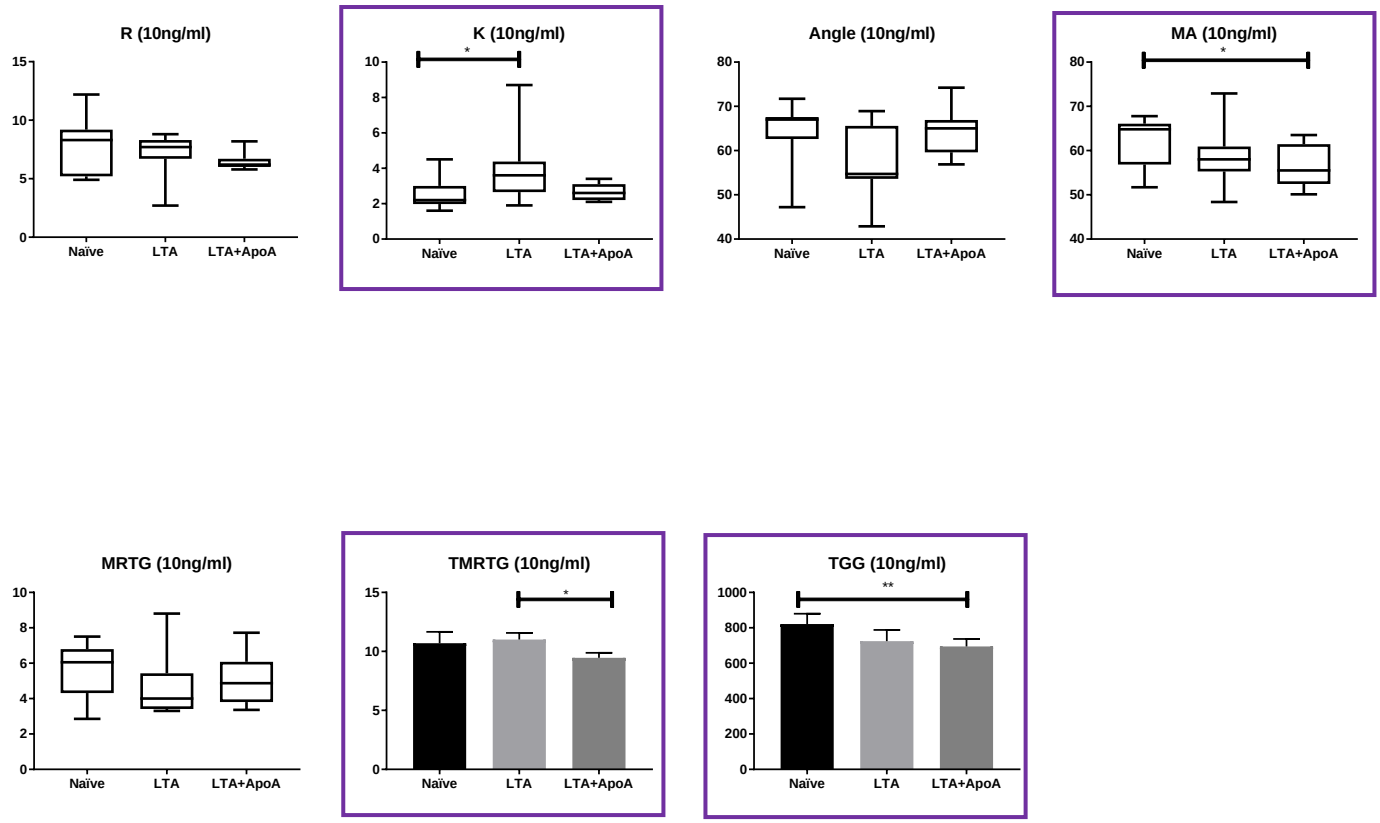

Figure 4.4.1 TEG graphs 10ng/ml. Box and whiskers graphs represent nonparametric data and bar graphs represent parametric data. Graphs in purple boxes are those with significant p-values p<0.005

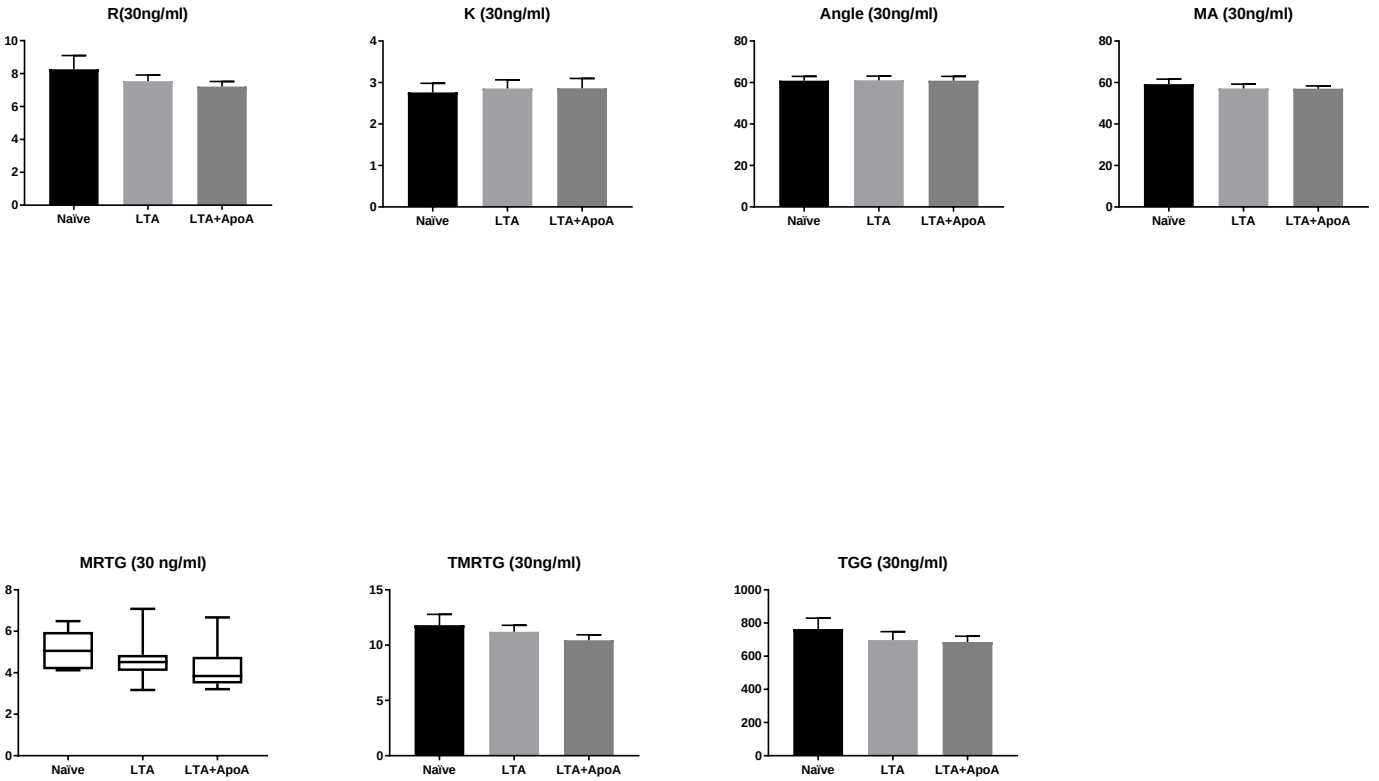

Figure 4.4.2 TEG Graphs 30ng/ml. Box and whiskers graphs represent nonparametric data and bar graphs represent parametric data.

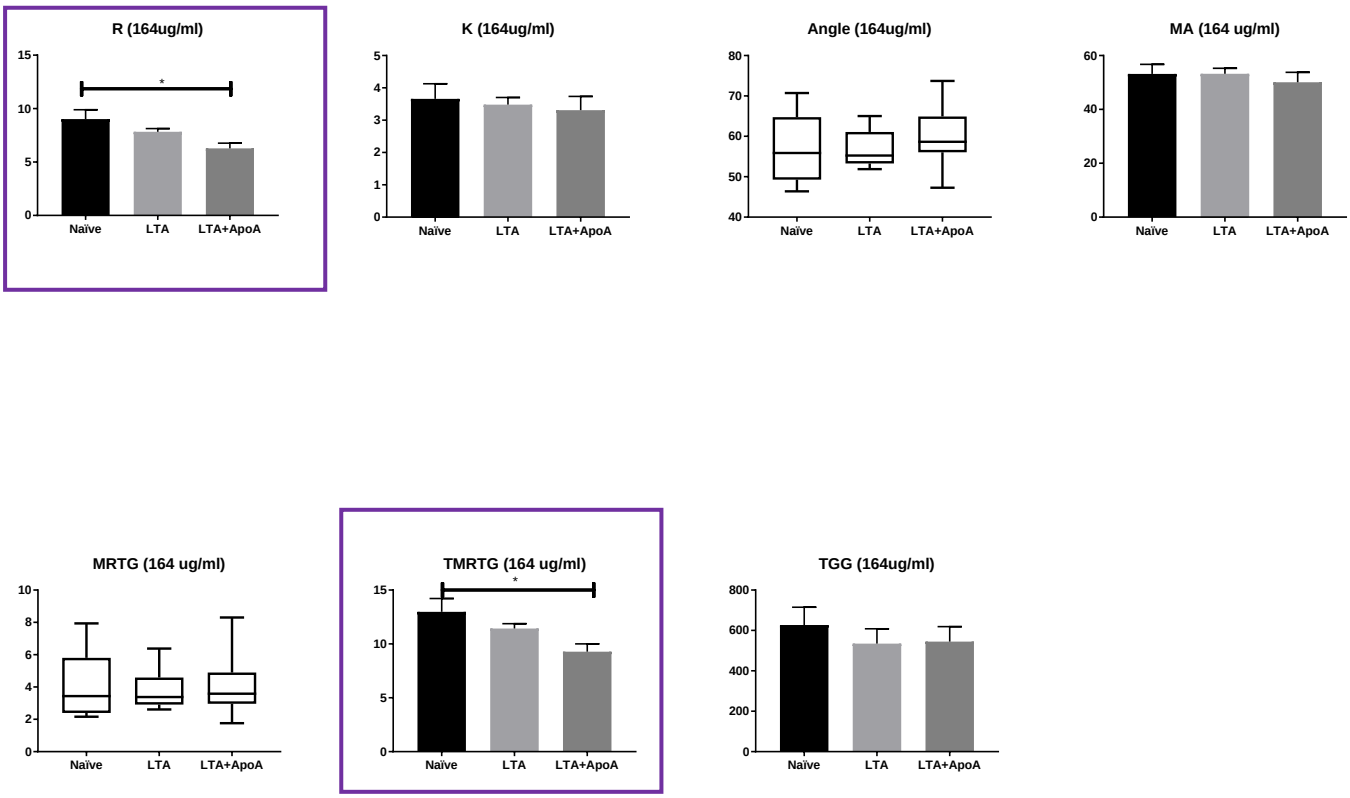

Figure 4.4.3 TEG graph 164µg/ml. Box and whiskers graphs represent nonparametric data and bar graphs represent parametric data. Graphs in purple boxes are those with significant p-values p<0.005

4.5. Discussion

In the introduction of this chapter, it was predicted that in the presence of bacterial components such as LPS and LTA, blood would become hypercoagulable and that by adding ApoA1 to WB with LTA will attenuate hypercoagulability. From the TEG results the following was conferred:

A significant change was found in the k-value of the 30ng/ml group particularly between the control vs LTA samples. There was a significant increase in the median of the control vs LTA exposed WB compared to the naïve control. This was an unexpected finding as it was expected that the k-value would decrease as that would be indicative of hypercoagulation according to literature (Pretorius *et al.*, 2017).

Significant changes were also found in MA (p-value=0.0212) particularly between the control vs control & LTA with ApoA1 samples (p-value=0.0219). There was a decrease in the median from the control to the LTA+ApoA1 exposed sample. This means that blood became less hypercoagulable. And the clot that formed in the presence of ApoA1 was weaker than the clot formed the healthy control as MA is indicative of clot strength (Pretorius *et al.*, 2017). This was unexpected finding because it was hypothesised that by adding ApoA1, it would attenuate the effects of LTA and return to a value closer to that of the naïve healthy control. However, according to these results, WB became hypercoagulable.

A significant decrease was found between LTA vs LTA+ApoA1 samples in TMRTG. This too was an unexpected finding as it was thought that TMRTG in the presence of ApoA1 would resemble that of the control TMRTG. A decrease in TMRTG is indicative of hypercoagulation thus in the presence of LTA +ApoA1 WB became hypercoagulable according to the results.

The last significant finding from the 10ng/ml group is between control vs LTA+ApoA1 of the TTG parameter with a p-value of 0.0073. Here, in the presence LTA+ApoA1, there is a decrease in TTG which means that clot strength is decreased. This finding was unexpected as it was expected that TEG value to resemble that of the healthy control.

In the 164µg/ml group, a significant decrease in the R-value (p-value=0.0202) and the TMRTG (p-value=0.0276) are observed particularly in the control vs LTA+ApoA1 samples suggesting that WB becomes more hypercoagulable in the presence of LTA+ApoA1. This is an unexpected finding as we expected that in by adding ApoA1 to the sample with LTA, ApoA1 would attenuate the hypercoagulable state caused by LTA. In the 164ug/ml group, WB was incubated with ApoA1 for 30 minutes before LTA was added, however we found that compared to the naïve WB, it still became more hypercoagulable.

A significant change towards hypercoagulation between the healthy control and LTA exposed WB was expected (Pretorius *et al.*, 2016, 2018). With regards to ApoA1, it was expected ApoA1 would attenuate the hypercoagulable state because literature states that ApoA1 has been found to have anti-inflammatory, anti-apoptotic and anti-oxidant functions as well as the ability to bind LTA (Density and Chung, 2012; Sirniö *et al.*, 2017). However, we suggest that the reason that ApoA1 failed to bring about the desired results was either because it was not exposed for a long enough period of time to allow for it to bind to LTA or the ApoA1 concentration used was too low to bring about effective attenuation. Thus, LTA was left to bind to the WB for longer period thus causing the hypercoagulation.

In the 30ng/ml group no significant p-values or trend towards significance were observed. However, by looking at the arrows in

Table 4.4.2, one notices that hypercoagulation was present as there was a decrease in R-value and TMRTG, an increase MA when LTA is added compared to the control samples. Changes like these are representative of hypercoagulation (Pretorius *et al.*, 2017). Hypercoagulation was also seen in the LTA+ApoA1 samples, these are unexpected findings for the same reasons explained above.

Although no significance was found between the naïve vs LTA samples, a closer look at the arrows in

Table , the parameters R and TMRTG across all treatment concentration decreased, and in the 30ng/ml treatment group, alpha angle increased, in the 164µg/ml treatment group k-value decreased and MA increased which are all changes towards hypercoagulation. Therefore, it is speculated that had the LTA been left to incubate for longer than 10 minutes maybe for 30 minutes to 1 hour, these results would have been significant.

4.6. Conclusion

To conclude on this chapter, it is found that in the presence of LTA, healthy control WB did not show significant hypercoagulation although there was a trend. Sample population number and or variability in inflammatory status of the individuals may have played an important role in giving these results. In the presence of LTA+ApoA1, healthy WB appears to become hypercoagulable. We speculate that these are the results because neither LTA nor ApoA1 was incubated in the WB for a long enough period of time for binding or the ApoA1 concentration used was too low to bring about the desired results.

Chapter 5: Scanning Electron Microscopy

5.1. Chapter Objectives

To answer research questions:

- Will the addition of LTA to healthy WB cause RBC to become eryptotic and platelets to be hyperactivated?
- Will the addition ApoA1 to control WB, after addition of LTA reverse the effect of the LTA?

5.2. Introduction

Scanning electron microscopy is used to study the morphological changes of cells (van Rooy *et al.*, 2015). This is because SEM has better magnification and zooming capabilities than light microscopy. Therefore, it allows one to see both the structural and morphological changes that occur in cells, such as interactions between cells and any cell membrane changes that may occur. Therefore, the research method of choice for this thesis was SEM to determine if LTA/ApoA1 had any structural effects on the cells of the haematological system as noted in chapter 4. The TEG results pointed to the fact that LTA/ApoA1 may cause hypercoagulability that might induce structural changes to blood cells. Research says that in hypercoagulability (as shown in the TEG), eryptosis which is membrane changes in RBC ensues and platelets become aggregated, form pseudopodia and OCS (Pretorius *et al.*, 2018).

Bacterial components such as LTA and LPS are known to be potent inducers of inflammation (Pretorius *et al.*, 2016). Researchers have found a that direct causative relationship between Gram-negative LPS and hypercoagulation exits (Pretorius *et al.*, 2016). However, whether a similar relationship exists between Gram-positive LTA and the cells of the haematological system is yet to be fully determined. Thus, for this study, the aim is to investigate this relationship, by investigating the effect that LTA will have on structural morphology of the cells, namely platelets and RBC.

Methods and Materials

To investigate whether the addition of LTA to WB will cause platelets to become hyperactivated and RBC to become eryptotic the following was done:

To see if concentration of ApoA1 would influence the morphology of platelets and RBC. We added:

1. 5ng/L^{-1} of LTA to fresh WB for at least *10 minutes before* being treated with a final exposure concentration of *10ng/ml* of Sigma ApoA1 for 10 minutes.

2. 5ng/L^{-1} of LTA to fresh WB for at least *10 minutes before* being treated with a final exposure concentration of *30ng/ml* of American ApoA1 for at least 10 minutes.

To determine if we added a higher concentration of ApoA1to WB before spiking WB with LTA, would LTA/ApoA1 have an influence on the morphology of platelets and RBC the following was done:

3. Whole blood was treated with a final exposure concentration of *164ul/ml* of American ApoA1 for at least *30 minutes* before 5ng/L^{-1} of LTA was added for at least *10 minutes* before analysis.

Once the concentrations step was done, followed the following procedure:

A drop of prepared WB was smeared onto a coverslip and left to dry for approximately three minutes before being covered with 0.075M sodium phosphate buffer (pH 7.4). The smear was fixed in 2.5% glutaraldehyde/formaldehyde (1:1) in PBS solution with a pH of 7.4 for 30 minutes, followed by rinsing 3 times in phosphate buffer for 5 minutes before being fixed for 30 minutes with 1% osmium tetroxide (OsO4). The samples were again rinsed 3 times with PBS for 5 minutes and dehydrated serially in 30%, 50%, 70%, 90% and three times with 100% ethanol. The material was critical point dried, mounted and coated with carbon. A Zeiss ULTRA plus FEG-SEM with InLens capabilities were used to study the surface morphology of platelets and micrographs were taken 1 kV.

5.3. Results

5.3.1. Platelets

Healthy platelets have no pseudopodia formation, do not aggregate and no membrane blebbling occurs as characteristics are commonly associated with hyperactivated platelets. As seen in Figure 5.3.1., the platelets in the naïve micrographs were in their inactive state as they had none of the above-mentioned features that are indicative of hyperactivity.

However, with the addition of LTA to healthy WB as seen in the SEM micrographs in Figure 5.3.1, platelets underwent structural changes and were hyperactivated. A distinct feature of hyperactivated platelets are pseudopodia (white arrows), OCS (blue arrows), platelet aggregation and clumping as indicated by the yellow circles, platelets attaching to RBC as indicated by blue circles and platelet spreading also know scrambled egg conformation as shown by black arrows.

With platelets that were treated with both LTA and ApoA1 it was observed that across all three treatment concentrations, platelets were more active than in their naïve state but less activate than the platelets exposed to LTA only. The platelets treated with both LTA and ApoA1 lost or

started to lose their “inactive” shape and were forming pseudopodia (white arrows). The 10ng/ml micrographs showed the platelets were still relatively active because of the visible scrambled egg conformation (black arrow). The 30ng/ml group showed the least platelet activation, as the platelets seem to have returned to their ‘inactive’ round conformation. The 164ug/ml, platelet had pseudopodia, however, the platelets were relatively inactive.

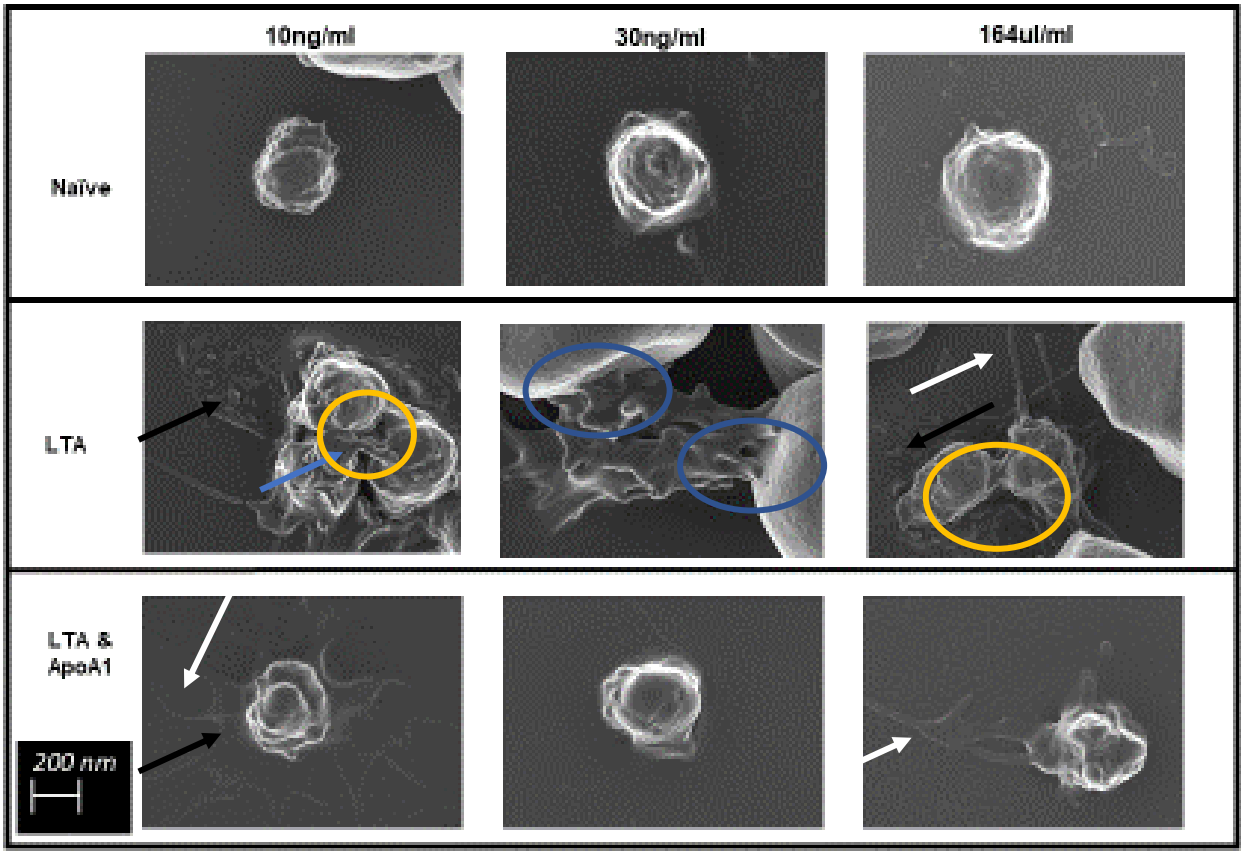

Figure 5.3.1 Scanning electron microscopy micrographs of platelets. Blue arrows are pointing to OCS, yellow circles indicate platelet clumping, blue circles show where platelets attach to RBC and white arrows are pointing towards platelet scrambling.

5.3.2. Red Blood Cells

Referring to Figure 5.3.2., in the healthy samples it was noticed that RBC were in their healthy biconcave disc shape and had smooth membranes. In the LTA samples, the structure of RBC membrane remained unchanged in the presence of LTA, however, it was observed that RBC agglutinated. In the LTA and ApoA1 treated groups RBC appeared to have returned to their healthy “naive” state. They had smooth membranes and were not clumped or fused together.

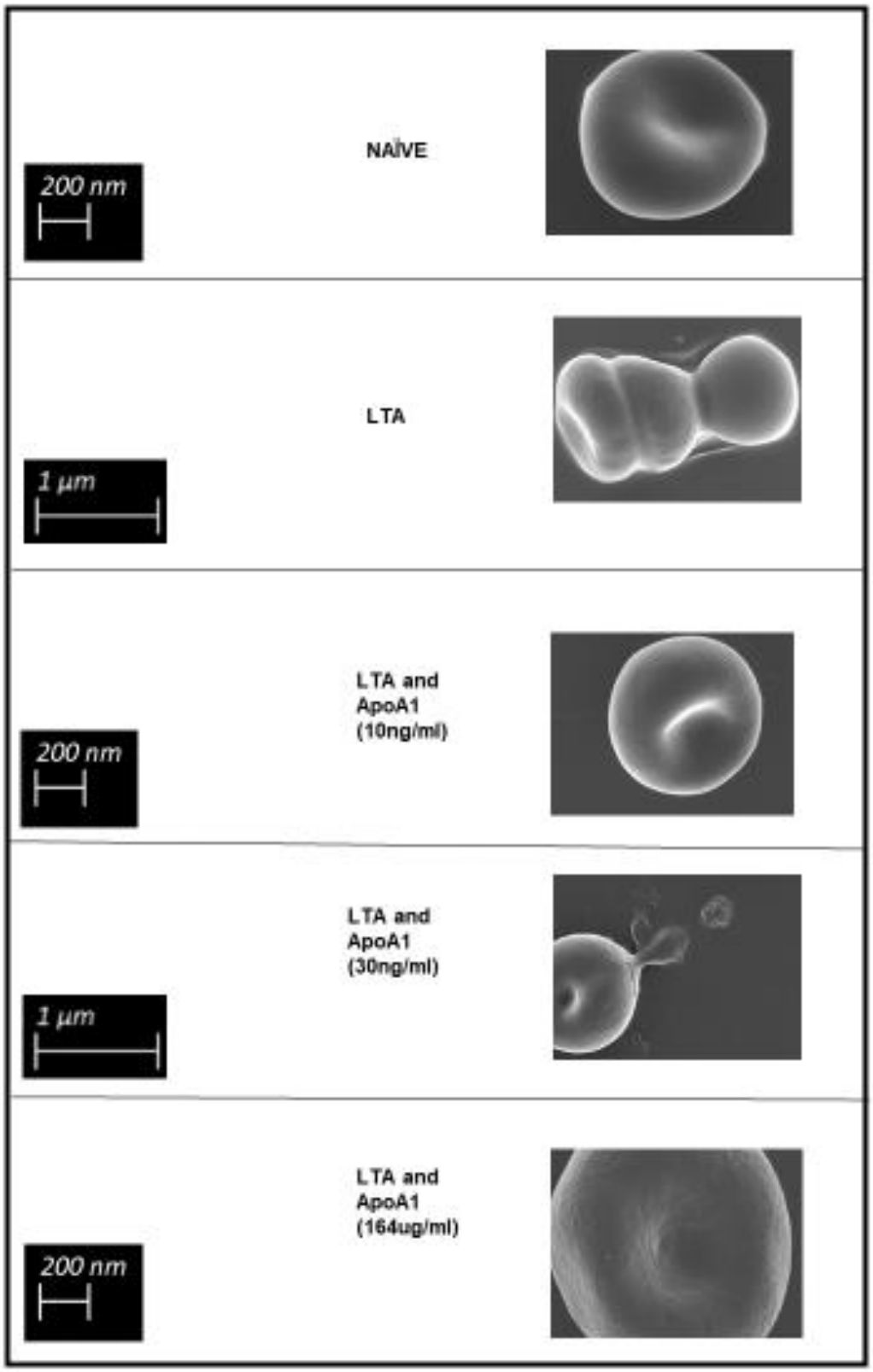

Figure 5.3.2 Scanning electron micrographs of RBC.

5.4. Discussion

5.4.1. Platelets

In the introduction, it was suggested that LTA may be a potent inducer of the coagulation cascade, and a trend towards this was seen with TEG results where parameters were upregulated suggesting hypercoagulation. In this chapter, it was visually confirmed that LTA brought about structurally changes to RBC and platelet morphology, therefore confirming the trend seen in the TEG results, showing a dysregulated coagulation cascade as seen in platelets and RBC in Figure 5.3.1 an Figure 5.3.2.

In Figure 5.3.2., that naïve platelets remained in their healthy “inactive” state which was a round shape form as expected. However, platelets that were exposed to LTA were found to be hyperactivated. They formed pseudopodia, attached to red blood cells and formed OCS, platelets clumped together, and the platelets interacted with RBC, these results were expected. These structural changes are often observed in the presence of dysregulated and circulating inflammagens like interleukins (Bester and Pretorius, 2016c), but in this case, no inflammagens were added suggesting that, LTA could be a potent independent stimulator for the coagulation system. We suggest that LTA brought about these changes by directly binding to the platelets, possibly on platelet TLR-2 that literature suggests is present on platelets (Pretorius. et al., 2014; Bester and Pretorius, 2016a; Kerrigan, 2018). Thus causing platelet hyperactivation as platelets are known to interact with bacterial components resulting in their direct activation (Rossaint, Margraf and Zarbock, 2018).

In the ApoA1 treatment groups SEM showed that by adding ApoA1 with LTA, the platelets became less activated across all treatment concentration groups. We found that the platelets had more pseudopodia and spreading and were more active than the healthy “naïve” platelets but had less pseudopodia and platelet spreading than the platelets exposed to LTA only thus suggesting hyperactivation in the presence of LTA thus confirming the trend seen in TEG in chapter 4. Platelets treated with ApoA1 were less clumped together and had fewer OCS than they did in the presence of LTA only.

In this chapter, platelets were exposed to three different concentrations of ApoA1. Two exposure concentrations (10ng/ml and 30ng/ml) where the ApoA1 was added after LTA had been added to the WB and one exposure concentration (164µl/ml) where the ApoA1 was added before LTA was added. In the first exposure of 10ng/ml, the platelets appeared to be relatively active as pseudopodia formation and membrane spreading was visible, thus this concentration maybe too low to completely attenuate the potent effects of LTA. The second exposure of 30ng/ml, the platelets appeared to have returned to their inactive state as no pseudopodia, aggregation or platelet spreading was visible. It was in its round conformation

characteristically associated with healthy inactive state platelets. With the last exposure of 164µg/ml, the platelet had visible pseudopodia, that could be due to contact activation, however, the platelets were not hyperactivated. This demonstrated that ApoA1 could have anti-inflammatory properties that could attenuate hypercoagulation and platelet hyperactivity. We would suggest that ApoA1 provides protection to the platelets against LTA, however the exact mechanism is not known and would require further investigations (Gordon *et al.*, 2016).

We hypothesised that in the presence of LTA, platelets would become hyperactivated and that ApoA1 would attenuate this hyperactivation. Both hypotheses were proven to be true in this chapter. From this we showed that, very low concentrations of LTA led to pathophysiological changes in the coagulation cascade that may contribute to the hypercoagulable state seen in inflammatory conditions.

5.4.2. Red Blood Cells

In Figure it is observed that although RBC maintained their membrane structure in the presence of LTA, they did clump as a result of agglutination therefore appearing as if they were fused together and this is characteristic of RBC that are distressed (Page, Bester and Pretorius, 2018). From Figure 5.3.2, it is also observed that RBC were less affected by LTA compared to platelets. The only distinguishable changes observed in RBC in the presence of LTA was agglutination. They maintained their membrane integrity and no shrinkage or other signs of eryptosis was observed. This could be because RBC are less sensitive to LTA or the concentration of LTA added was too low to induce eryptosis. With regards to RBC and ApoA1, RBC appeared to be less fused together in the presence of ApoA1 and LTA. This is a positive sign regarding the ability of ApoA1 to attenuate the effects of LTA on the RBC.

We hypothesised that in the presence of LTA, RBC would become eryptotic and ApoA1 would attenuate this eryptosis. However, the results showed that RBC did not become eryptotic, instead they agglutinated, thus proving our hypothesis to be not true. However, we did see that in the presence of ApoA1 RBC were less clumped.

5.5. Conclusion

For this chapter, it was hypothesised that in the presence of LTA, platelets and RBC would become hyperactivated and eryptotic respectively and that ApoA1 would attenuate the platelet hyperactivity and eryptoticness of RBC. The hypothesis was proven to be true regarding platelet hyperactivity and not true regarding RBC eryptosis. The second part of the hypothesis regarding ApoA1's potential attenuation effect was proven true in both platelets and RBC. Even though RBC did not become eryptotic, they did agglutinate in the presence of LTA and this was attenuated by the addition of ApoA1.

As previously mentioned, hypercoagulation is abnormal blood clotting. Agglutination causes blood to become sticky and attach to blood vessel walls, thus forming plaques and blood clots. Clinically these characteristics are associated with thrombosis and CVD like strokes, and heart attacks. Thus, the possible clinical implications for this chapter is that, in the presence of bacterial components LTA possibly via leaky gut, platelet hyperactivity and RBC agglutination may occur that may lead to heart attacks and strokes. And that, by adding ApoA1, the risk of thrombosis could be decreased.

Chapter 6: Confocal Microscopy

6.1. Chapter Objectives:

- To investigate whether a hypercoagulable state will cause amyloid formation in fibrin(ogen) when LTA is added.
- To investigate whether the addition of ApoA1 to fibrinogen will attenuate the effect of LTA.

6.2. Introduction

In chapter 4, using TEG it was determined that there is a trend towards a hypercoagulable state in the presence of LTA and this hypercoagulability was demonstrated by platelet hyperactivity and RBC agglutination in chapter 5. Considering that fibrinogen is part of the coagulation process, the question arose if a third technique is used to investigate amyloid formation in fibrinogen, could the TEG results be explained further.

Fibrinogen is a soluble plasma protein that is cleaved to insoluble fibrin by thrombin in the presence of vascular injury or inflammation (Kattula, Byrnes and Wolberg, 2017). Fibrinogen is present in high concentrations in plasma and its concentration significantly increases in the presence of inflammation (Hewett and Roth, 1995). As mentioned in chapter two, excessive fibrin(ogen) activity results in coagulopathies that become detrimental to health. One such coagulopathy is hypercoagulation, which is abnormal blood clotting (Caine *et al.*, 2002). Various factors contribute to this, but the one of interest for this project is the presence of bacterial endotoxin such as LPS and LTA.

Studies by Pretorius *et al.,* found that when adding low concentrations of LPS, to healthy individuals PPP/fibrin(ogen), abnormal amyloid-like clots formed (Pretorius *et al.*, 2016). The individual fibrin fibrils seen in healthy clots are not seen in the presence of LPS, instead DMD are formed and the resultant fibrin(ogen) is amyloidogenic in nature (Pretorius *et al.*, 2016). They also found that by adding LBP along with LPS, the potency of the LPS was reduced and healthier fibrin fibrils formed (Pretorius, Page, Mbotwe, *et al.*, 2018). The search for a product that would yield similar results in the presence of LTA lead to ApoA1. Apolipoprotein A-1 is a human protein that has been shown to potentially have both anti-inflammatory and anti-amyloid properties (Koldamova, Fitz and Lefterov, 2014; Sirniö *et al.*, 2017). Apolipoprotein A-1 may be involved in the defence against bacteria because ApoA1 concentrations significantly increase in the presence of bacterial infections (Gutsmann *et al.*, 2001; Branescu *et al.*, 2013; Parolia *et al.*, 2014). Based on this, ApoA1 was the chosen potential mopping agent for LTA endotoxin.

As previously mentioned, the fibrin(ogen) clot formed in the presence of bacterial endotoxins is amyloidogenic in nature. Amyloid proteins aggregate under non-physiological conditions and cause disease (Romero *et al.,* 2014; Nizhnikov *et al.,* 2015; Kisilevsky *et al.,* 2016). Amyloid proteins change from a mostly α- helical conformation to mostly β-sheet conformation that has negative impact on protein function (Kell and Pretorius, 2017). Historically, the presence of amyloid proteins in tissue was detected by various fluorescent dyes such as Congo Red, Tht, NIAD-4, H&E stains. For this study, Congo Red and Amytracker™ 630 are the dyes of choice. The next paragraph explain their specifc affinitities to amyloid proteins and why they maybe useful for this study.

Congo Red, is an old and common marker used for the identification of amyloid proteins and it is believed that it binds to hydrophobic areas and β-sheets (Khurana *et al.*, 2001). The other marker used in this study are the novel markers known as Amytracker™. Amytracker™ dyes are highly sensitive dyes that rapidly bind to amyloid proteins and fluoresce once bound to amyloid (Klingstedt *et al.*, 2013; Shirani *et al.*, 2015). They are markers that might give an indication of disease earlier than other markers. The exact binding sites of Amytracker™ is not clear yet, however, our group is in the process of determing the their biocemical interactions.

6.3. Methods and Materials

To determine whether the addition of LTA to fibrinogen would cause fibrin(ogen) to become amyloidogenic, the following procedures was followed:

On the day of analysis, stored frozen fibrinogen was thawed to room temperature, and aliquoted into Eppendorf tubes into two groups, the Amytracker™ group and the Congo Red group. The thawed fibrinogen was incubated with a final exposure concentration of 30µL/mg of American ApoA1[1] for at least 30 minutes. This was followed with the fibrinogen being spiked with a final exposure concentration of 5ng.L^{-1} LTA for 10 minutes. Lastly, a final exposure concentration of 0.1µL of Amytracker™ 630 was added to one group for 30 minutes to 100µL fibrinogen. The other group was incubated with a final exposure concentration of 10µL Congo Red for 30 minutes to 100µL fibrinogen. Naïve fibrinogen was only incubated with the markers Amytracker™ 630 and Congo Red respectively.

Just before viewing the fibrinogen on the confocal, thrombin was added in a 1:2 ratio of thrombin to PPP therefore 5µL thrombin and 10µL fibrinogen was added to create an extensive fibrin network, since thrombin is responsible for the formation of fibrin clots. Once the thrombin was added onto the fibrinogen, a coverslip is placed over the newly formed clot

[1] Only American ApoA1 was used for confocal microscopy as Sigma ApoA1 fluorescence intensity was too much. This could be due to the stabilisers used.

and the sample was viewed using Zeiss LSM 510 META confocal microscope with a Plan-Apochromat 63x/1.4 Oil DIC objective.

6.5. Statistical Analysis

To determine whether the difference in fluorescence intensity was significant, statistical analysis was performed using the histogram function on ImageJFiji, using the mean and standard deviation to calculate the coefficient of variance (CV). The CV was used to perform a one-way ANOVA on GraphPad Prism, with the Bonferroni test for correction for normally distributed data and the Tedd-Dunn test for correction for not normally distributed data.

6.6. Results

Confocal microscopy was used with the aim to determine whether clot formation in fibrinogen in the presence of LTA would be amyloidogenic in nature and if ApoA1 would attenuate the effects of the LTA. The following results were obtained.

Refer to Figure 6.6.1: In micrographs A and D there was low fluorescence intensity in naïve fibrinogen for both the Amytracker^{TM} 630 and Congo Red groups. In micrographs B and E, by adding LTA to healthy fibrinogen, there was an increase in fluorescence intensity compared to the naïve fibrinogen micrographs, suggesting that there was an increase in amyloid formation in the presence of LTA. In micrographs C and F, there was less fluorescence intensity in the fibrinogen micrographs that have been exposed to LTA with ApoA1 compared to the micrographs treated with LTA only.

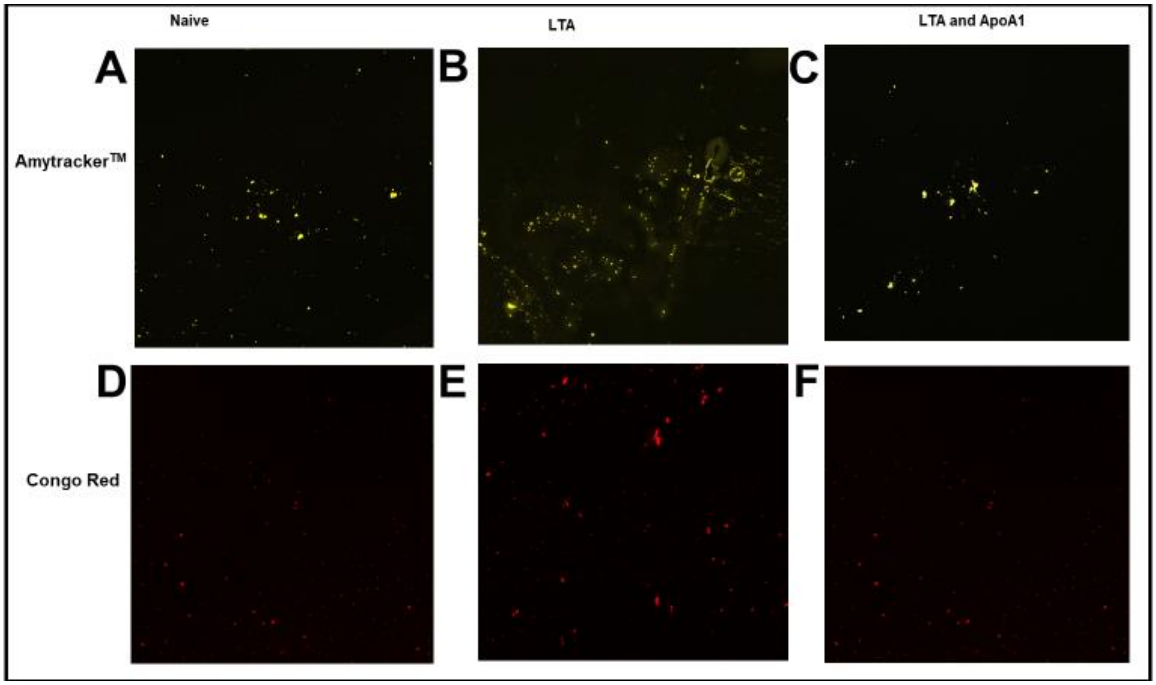

Figure 6.6.1 Fibrinogen micrographs: in micrographs A -C are stained using Amytracker™ 630 whereas micrographs D-F are stained using Congo Red ™. Micrographs A and D are naive fibrinogen; Micrographs B and E and exposed to LTA only; Micrographs C and F are treated with LTA and ApoA1.

To determine whether the difference in fluorescence intensity was significant, statistical analysis was performed. It was found that the difference in fluorescence was not statistically significant. However, from the graphs in Figure 6.6.2 there was a trend observed in both graph A and B with regards to fluorescence intensity. The trend observed concurred with what was seen in the micrographs. It was observed that the least fluorescence intensity was observed on the naïve group and the most fluorescence intensity was observed in the LTA treated group. While the LTA with ApoA1 fluorescence intensity was more than the naïve group, but less than the LTA only group.

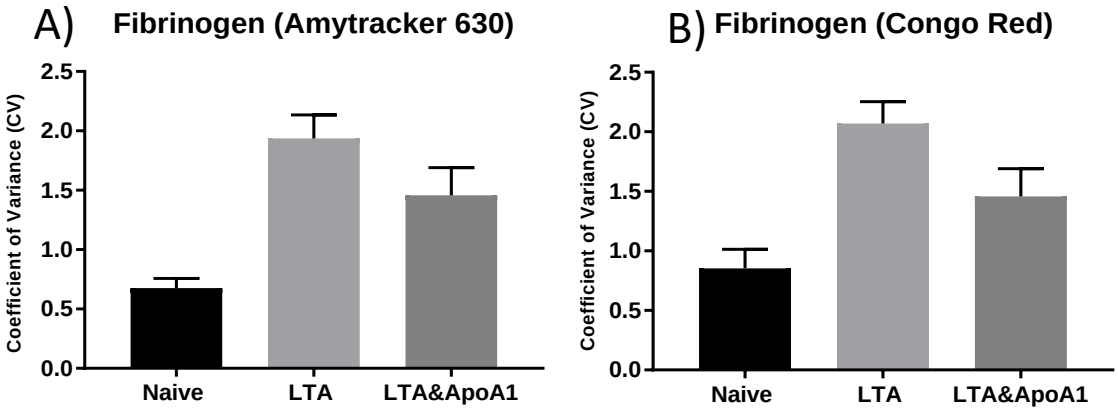

Figure 6.6.2 Statistical analysis graphs showing the intensity of the two dyes used A) Amytracker™ 630 and B) Congo Red™. From the above graph we see that a trend was observed in graph A and B with regards to fluorescence intensity. Higher fluorescence intensity was observed in LTA than the naïve fibrinogen and LTA and ApoA1 treated samples.

6.7. Discussion

Confocal microscopy was used with the aim to determine whether clot formation in fibrin(ogen) in the presence of LTA would become amyloidogenic. From the results obtained from confocal microscopy, the following can be deduced. Figure 6.6.1 gave a visual representation of the difference between fibrinogen that was exposed to LTA and LTA with ApoA1 compared to the naïve control that was not exposed to LTA or ApoA1. In the naïve panels, sporadic fluorescence was observed, indicating that little amyloid formation occurred in healthy individuals as was expected. In the LTA treated group, fluorescence was more enhanced compared to the naïve control panels, suggesting that there was an increase in amyloid formation in the presence of LTA. In the samples treated with both LTA and ApoA1, there was a reduced amount of fluorescence compared to the samples exposed to LTA, but more fluorescence than the naïve controls, as expected. This indicated that ApoA1 may attenuate amyloidogenesis in the presence of LTA. Confocal analysis indicated that LTA contributed to the formation of amyloid proteins. It is suspected that the direct binding of LTA to fibrin(ogen) would result in this type of clot forming.

To determine whether the difference in fluorescence intensity was significant, statistical analysis was performed and it was found that there was a difference, however, the difference in fluorescence was not statistically significant. From the graphs in Figure 6.6.2, a trend was observed in both graphs.

Previously published knowledge of our protein of interest fibrin(ogen) was translated, because there is very little published data on interactions of the specific fluorescent markers/dyes on fibrin(ogen). These results coincided with the novel findings of Pretorius and co-workers 2016 and 2017 study that in the presence of LPS and LTA PPP became amyloidogenic (Pretorius

et al., 2016; Pretorius, Mbotwe and Kell, 2017; Pretorius *et al.*, 2018). Even though, previous studies have shown that interaction between fibrin(ogen) and β-amyloid proteins took place, a causative relationship between fibrin(ogen), amyloid and bacterial components was never been known till recently. It is suspected that LTA directly binds to fibrin(ogen) thus causing these types of amyloid-like clots (Ahn *et al.*, 2010, 2014; Pretorius *et al.*, 2016; Pretorius, Mbotwe and Kell, 2017).

Furthermore, Congo Red is thought to bind to β-sheets in amyloid proteins, thus, this indicated that fibrinogen developed more β-sheets and lost its α-coils morphology in the presence of LTA, implying that it became amyloidogenic in nature. However, in the presence of ApoA1, it appeared the effect of LTA was attenuated as there was less fluorescence implying that Congo Red and Amytracker^{TM} 680 had less β-sheets to bind to. ApoA1 appears to have had a protective function here. It may have prevented the uncoiling of the fibrinogen α-coils, meaning that there was less areas for Congo Red and Amytracker^{TM} 680 to bind to, hence less fluorescence was seen. Therefore, fibrinogen retained more of its functional α-coil morphology in the presence of ApoA1 and LTA compared to LTA only.

These are significant findings because fibrin(ogen) was affected by inflammation. There was a positive feedback relationship between inflammation and fibrin(ogen) (Levi and van der Poll, 2010). Thus, when it was constantly stimulated by the presence of LTA, it may have resulted in changes in its structure that may affect the inflammatory profile of the individual. Thus, LTA may be the one of the links between chronic systemic inflammation and amyloid formation. The potential for ApoA1 (at such low concentrations 30ug/ml) to decrease the amyloid formation in the presence of LTA was also a significant finding because it may be used a potential treatment to slow down the formation rate of amyloid in amyloid-specific diseases such as Alzheimer's disease.

6.8. Conclusion

The aim of this chapter was to determine whether a hypercoagulable state will cause amyloid formation in fibrin(ogen) when LTA was added to fibrinogen. And to determine whether the addition of ApoA1 to fibrin(ogen) would attenuate the effect of LTA? We found that in the presence of LTA, there was a trend towards amyloid formation and that ApoA1 had the potential to attenuate this amyloid formation as seen in Figure 6.6.1 and Figure 6.6.2.

The clinical implication of this study is that if there was Gram-positive bacteria entering via dysbiosis, it means that they may shed via low levels of LTA and this might cause a hypercoagulable state, and this might be one of the reasons for a hypercoagulable state in the presence of inflammatory conditions.

Chapter 7: Type Two Diabetes Model

7.1. Chapter Objectives

- To investigate if the addition of ApoA1 to T2DM PPP, will reverse the effect of amyloidogenesis and coagulopathies observed in T2DM patients.

7.2. Introduction

As mentioned in chapter two, T2DM is chronic metabolic condition characterised by high glucose levels, both systemic and chronic inflammation, blood hypercoagulability and the presence of amyloid proteins (Pretorius *et al.*, 2018). Due to the dysregulation of the inflammatory system present in T2DM, coagulopathies present themselves as anomalous clot formations in the form of DMD that may occur in circulation (Pretorius *et al.*, 2018).

In the past our group used LBP to try reverse the amyloid seen in plasma fibrin of individuals with AD (Pretorius *et al.*, 2018). This led us to investigate the possibilities of other molecules that could reverse the coagulopathies seen in chronic inflammatory conditions such as T2DM. For this study, the molecule of choice is ApoA1 on the inflammatory condition T2DM.

Apolipoprotein A-1 is the major constituent of HDL (Besler, Lüscher and Landmesser, 2012). High density lipoprotein is a molecule that is primarily responsible for the efflux of cholesterol from peripheral tissue to the liver and is also shown to have anti-inflammatory properties (Gordon *et al.*, 2016). Apolipoprotein A-1 is believed have anti-inflammatory properties that may be beneficial to individuals with chronic low-grade inflammation such as T2DM patients (Gutsmann *et al.*, 2001; Branescu *et al.*, 2013; Parolia *et al.*, 2014).

In the previous chapters, we found that ApoA1 had favourable results when it came to attenuating the effects of LTA. In chapter 5 and chapter 6, it is shown that by adding ApoA1 to healthy WB and healthy fibrinogen spiked with LTA respectively, the detrimental effects of LTA were reduced. Therefore, in this chapter the aim was to investigate whether the addition of ApoA1 to a known inflammatory and hypercoagulable condition such as T2DM would yield similar results.

7.3. Methods and Materials

7.3.1. SEM

On the day of analysis, T2DM PPP from seven patients was thawed to room temperature, and aliquoted into Eppendorf tube. A drop (10µl) of prepared T2DM PPP was smeared onto a coverslip and left to dry for approximately three minutes them immersed in 0.075M sodium phosphate buffer (pH7.4) then. Smears were then fixed in 2.5% glutaraldehyde/formaldehyde in a 1:1 ration in PBS solution with a pH of 7.4 for 30 minutes. This was followed by rinsing 3

times in phosphate buffer for five minutes before being fixed for 30 minutes with 1% osmium tetroxide (OsO4). The samples will again be rinsed 3 times with PBS for five minutes and dehydrated serially in 30%, 50%, 70%, 90% and three times with 100% ethanol. The material was critical point dried, mounted and coated with carbon. A Zeiss ULTRA plus FEG-SEM with InLens capabilities were used to study the surface morphology of platelets and micrographs were taken 1 kV.

7.3.2. Confocal

On the day of analysis, stored T2DM PPP from seven patients was thawed to room temperature, and aliquoted into Eppendorf tube. The thawed T2DM PPP was incubated with a final exposure concentration of 30ng/ml of ApoA1 for at least 30 minutes before adding a final exposure concentration of 5µM of thioflavin t (Tht) and a final exposure of 0.1µL of Amytracker™ 480 and Amytracker™ 680 for 30 minutes.

Just before viewing the T2DM PPP on the confocal, thrombin was added to it in a 1:2 ratio of thrombin to PPP therefore 5µL thrombin and 10µL fibrinogen was added to create an extensive fibrin network, since thrombin is responsible for the formation of fibrin clots. Once the thrombin was added onto the PPP, a coverslip was placed over the newly formed clot and the sample was viewed using Zeiss LSM 510 META confocal microscope with a Plan-Apochromat 63x/1.4 Oil DIC objective.

7.4. Statistical Analysis

ImageJ Fiji was used to calculate histogram function of each image and the data obtain (mean and standard deviation) was used to calculate CV. The CV was used to perform a t-test on GraphPad Prism. This was done for both Confocal and SEM images.

7.5. Results

7.5.1. SEM

A healthy control clot resembles a bowl of cooked spaghetti, where there thick and thin fibres are visible as seen in Figure 7.5.1. Scanning electron microscopy was used to determine whether the addition of ApoA1 to T2DM PPP would reverse the hypercoagulable state observed in T2DM patients, the following results were obtained.

In Figure 7.5.1.2, T2DM PPP presented with, thick fibrin(ogen) fibres that are clumped together as indicated by blue circles as well as DMD as indicated by blue arrows. The fibre was also more mesh-like instead of being more like a bowl of cooked spaghetti. In Figure 7.5.1.3, T2DM PPP was treated with ApoA1 and a significant difference was observed compared to the untreated group. In Figure7.5.1.3, individual fibrin fibres were clearly visible,

there was significantly less mesh-like fibrin(ogen) and less DMD visible. The fibrin fibres resembled that of a bowl of cooked spaghetti.

Statistical analysis further verified that, the differences observed between the naïve T2DM PPP and ApoA1 treated PPP was significant. Statistical analysis was performed on the images and a significant p value of <0.0001 was obtained as seen in Figure 7.5.1.4.

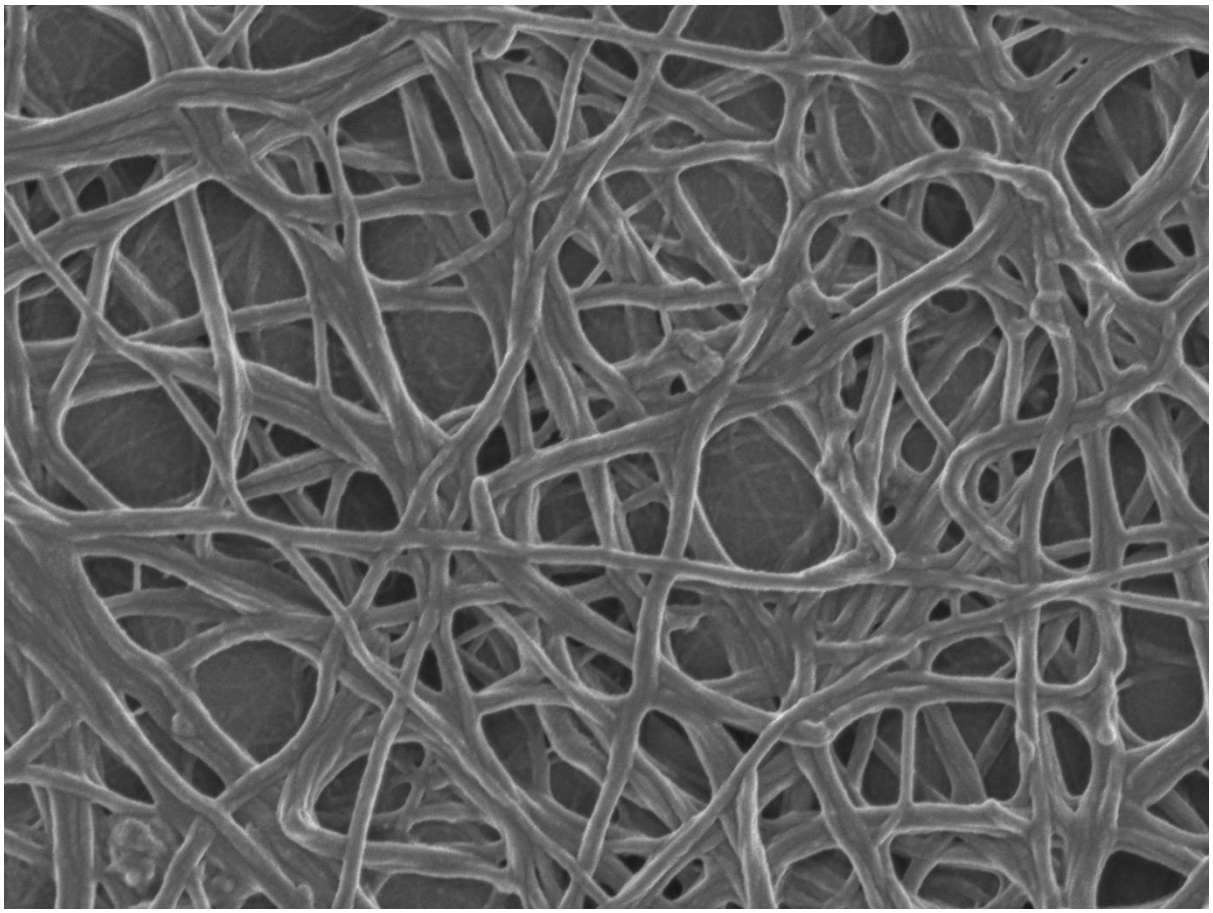

Figure 7.5.1.1 Healthy control PPP clot. One can clearly see the cooked spaghetti-like appearance of the thick and thin fibrin fibres typical of healthy individuals.

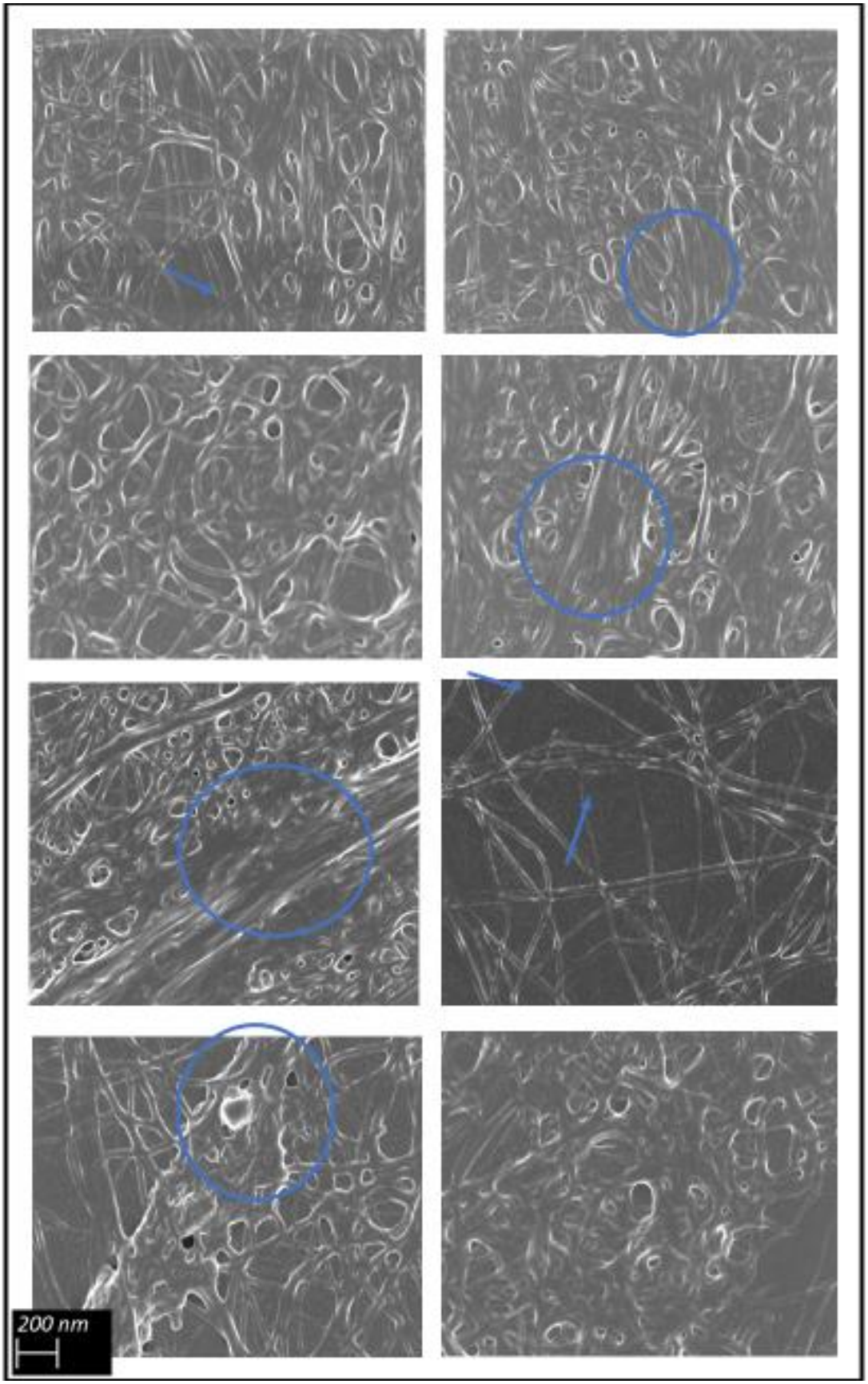

Figure 7.5.1.2 Naïve T2DM PPP. Once can see that the cooked spaghetti like appearance of thick and thin fibrin fibres is absent. Instead fibres are fused together as shown by blue circles, giving more of a mesh-like appearance. Blue arrows show DMD.

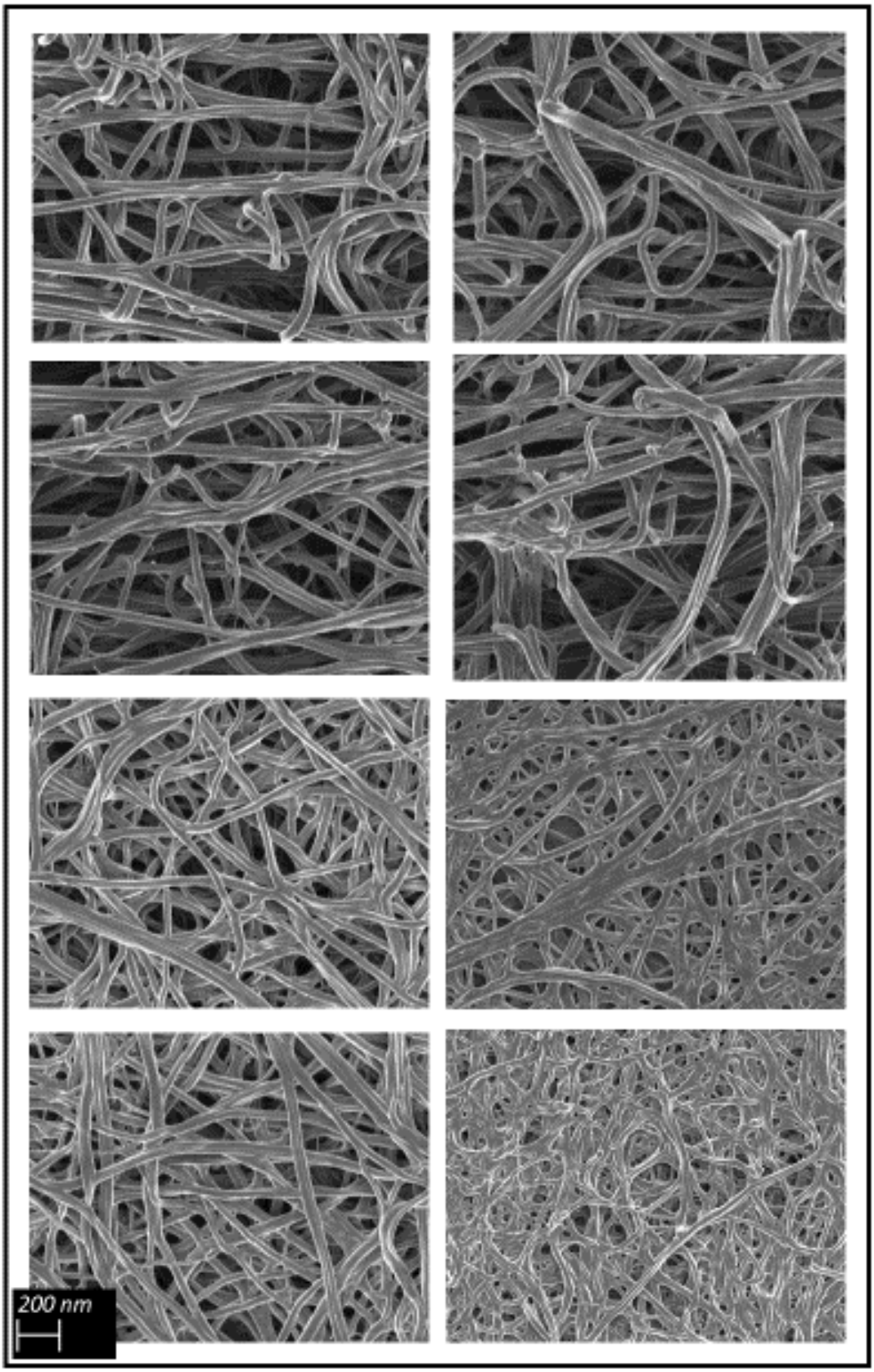

Figure 7.5.1.3 Type two diabetes mellitus PPP with added ApoA1 treatment. One can see the spaghetti like appearance typically seen in healthy individuals is once again visible once ApoA1 is added.

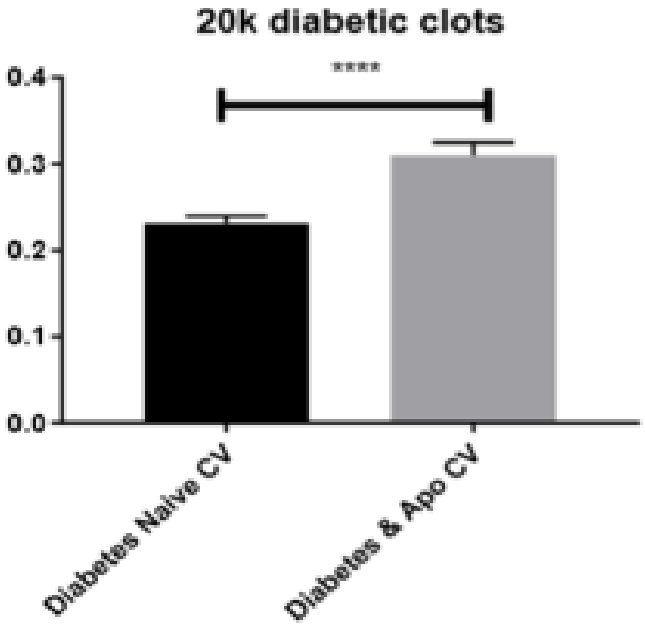

Figure 7.5.1.4 Graph representing statistical analysis performed between naive PPP and ApoA1 treated PPP. Statistical significance p>0.0001. ***= p>0.0001

7.5.2. Confocal

For the T2DM model, confocal analysis was used to investigate whether the addition of ApoA1 to T2DM PPP will attenuate the amyloidogenesis typically observed in T2DM patients the following results were obtained:

In Figure 7.5.2.1, micrograph A it was observed that naïve T2DM PPP had more intense fluorescence compared to T2DM PPP that was treated with ApoA1 as seen in Figure B.

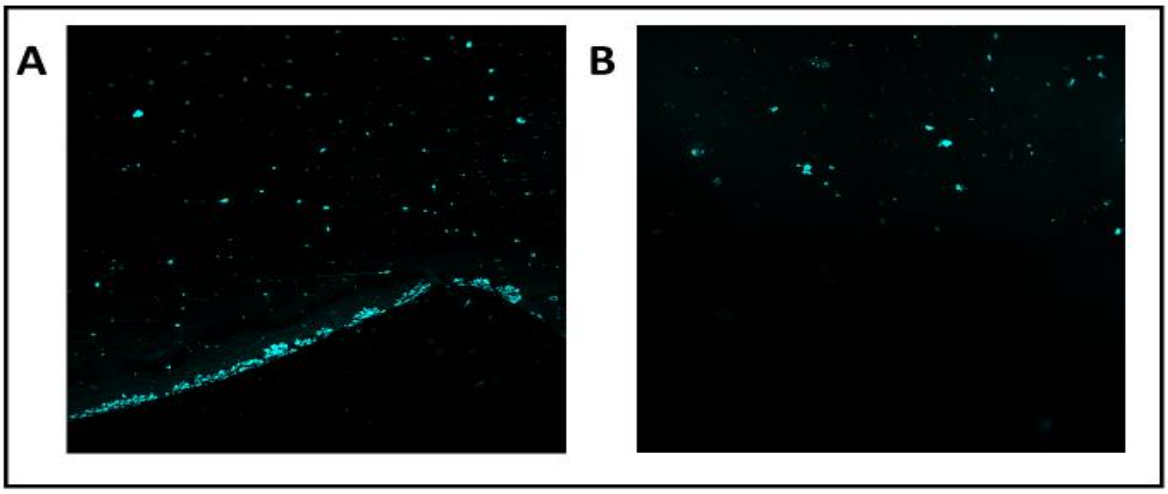

Figure 7.5.2.1 Type two diabetes mellitus PPP micrographs. Micrograph A) naive T2DM PPP B) is T2DM PPP that has been treated with ApoA1. B has less fluorescence compared to A as expected.

The images obtained in Figure were analysed further by splitting the image into different channels. From this it was noticed that the three markers bind to different regions on the amyloid protein. This is clearly demonstrated in Figure 7.5.2.2. Here, Amytracker™ 480 fluoresces blue, Amytracker™ 680 fluoresces red and Tht fluoresces green. It is observed that Amytracker ™ 480 illuminates the most, indicating that it binds to amyloid protein the more than Amytracker™ 680 and Tht. Furthermore, in Figure 7.5.2.2, it was observed that fluorescence was more intense in naïve micrographs A-C compared to ApoA1 treated micrographs D-F, thus verifying what was observed in Figure above. To determine whether the difference in fluorescence intensity was significant, statistical analysis was performed and the difference in intensity was found to not be significant as seen in Figure 7.5.2.3.

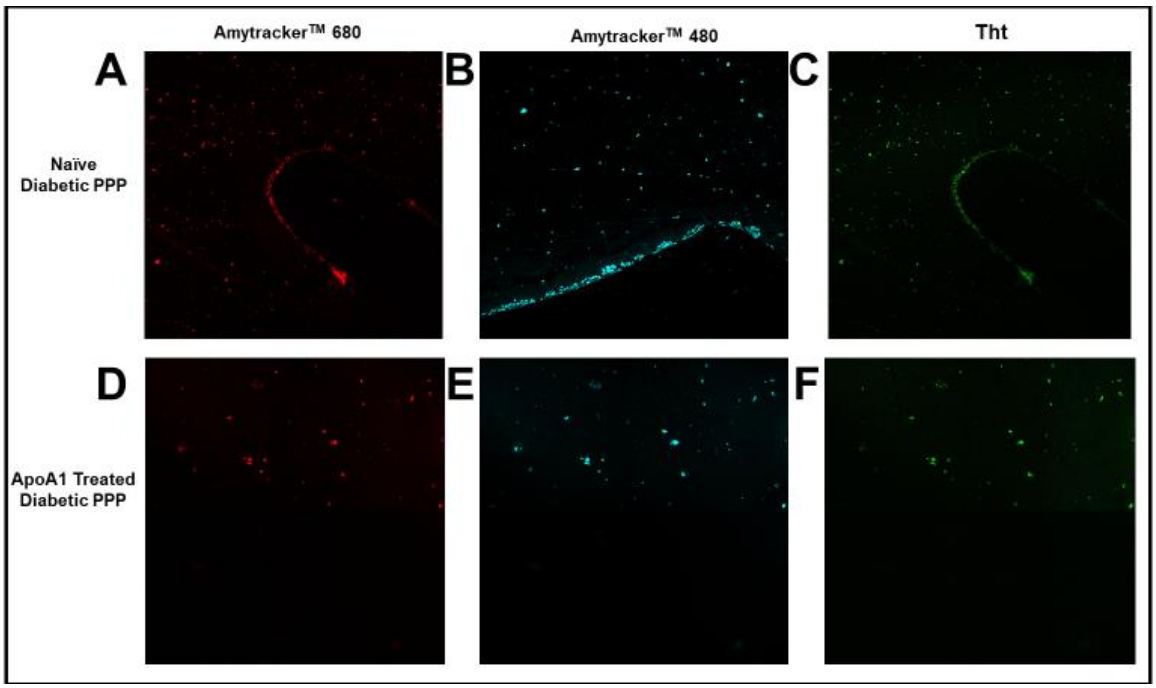

Figure 7.5.2.2 Type two diabetes mellitus PPP split channels before (A-C) and after (D-F) the addition of ApoA1.

As previously mentioned, statistical analysis showed the difference in intensity between the two T2DM PPP groups was not significant. However, from the graphs below in Figure 7.5.2.33, a common trend developed across all the groups. One observed that the intensity of the fluorescence decreased in the presence of ApoA1 treated T2DM PPP compared to the untreated T2DM PPP.

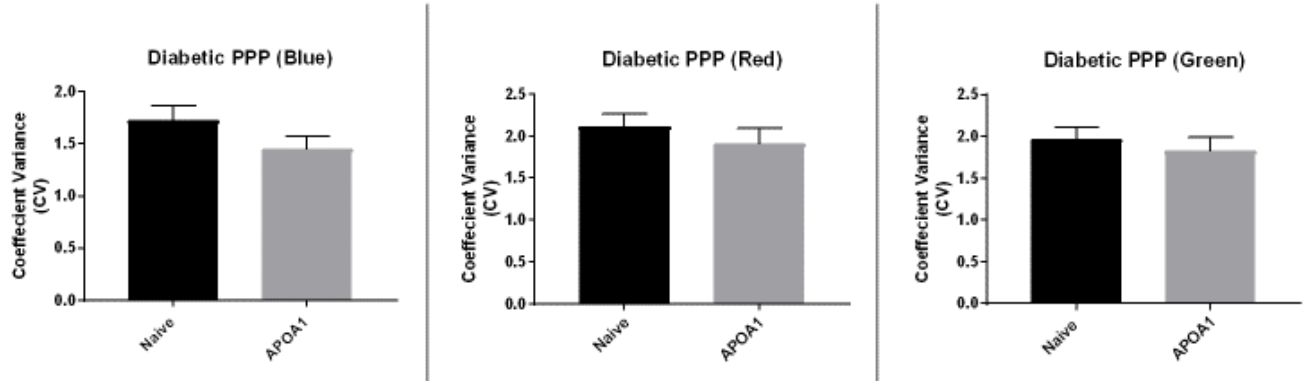

Figure 7.5.2.3 Graph representing statistical analysis performed between naive PPP and ApoA1 with confocal microscopy. No statistical significance was found, but a trend is observed.

7.6. Discussion

As mentioned in the introduction, T2DM patients typically present with a hypercoagulable state due to chronic inflammation that is present (Pretorius *et al.*, 2018). It is also mentioned in the introduction that ApoA1 may have anti-inflammatory properties that may be beneficial to T2DM patients. In this chapter the hypercoagulable state of T2DM PPP and the potential anti-inflammatory effects of ApoA1 was shown. This was achieved by viewing T2DM PPP with confocal microscopy and SEM before and after the addition of ApoA1 to the T2DM PPP.

The results showed that there was a significant difference in amyloid signal between naïve T2DM PPP and T2DM PPP treated with ApoA1 as seen from Figure 7.5.1.1 to Figure 7.5.2.3. With SEM, it was found that there was a significant decrease in the hypercoagulable and amyloidogenic state seen in T2DM PPP once ApoA1 is added. This suggests that ApoA1 has anti-inflammatory properties that could be beneficial in an inflammatory environment such as the one seen in T2DM patients. Healthy individuals' fibres consist of thick major fibres, which make up most of the clot and thin minor fibres that are clearly visible and resemble that of cooked spaghetti (van Rooy *et al.*, 2015). These results are in accordance with literature which states that there is a relationship between T2DM and ApoA1. Studies found that with T2DM there is an inverse relationship between serum HDL and serum ApoA1 levels concentrations and cardiovascular events (Mooradian, Haas and Wong, 2004). The exact mechanism that ApoA1 uses to assert its anti-inflammatory effects is not yet known. However, proposed potential mechanisms of how ApoA1 asserts its cardioprotective effects include ApoA1's ability to enhance the reverse cholesterol transport system, attenuate oxidative stress, increase peroxonase activity, and enhance anti-coagulant activity (Mooradian, Haas and Wong, 2004).

7.7. Conclusion

To determine whether the addition of ApoA1 to T2DM PPP would attenuate the hypercoagulation seen and attenuate amyloid seen SEM and confocal microscopy was used respectively. We found that in the presence of ApoA1 there was significantly less hypercoagulation and amyloid occurrence in T2DM PPP. These are significant findings because they show that a direct relationship exists between T2DM PPP and ApoA1. And that in T2DM patients there is less ApoA1 which contributes to the severe effects of T2DM.

Chapter 8: Conclusion and Future Recommendations

8.1. Conclusion

It is a well-established fact that various diseases are accompanied by inflammation and hypercoagulability. In this thesis, it was shown that the addition of low concentrations of LTA to healthy WB and healthy fibrin(ogen) leads to significant changes of plasma proteins and the coagulation cascade. For this thesis, it was hypothesised that the addition of LTA to healthy WB and fibrinogen will cause amyloidogenesis and hypercoagulation of WB and fibrin(ogen) and that this damage will be amyloidogenic in nature. It was also hypothesised that LTA will cause platelets to become hyperactivated and that RBC will become eryptotic. Furthermore, it was hypothesised that by adding 'mopping' agent ApoA1 to healthy WB and fibrin(ogen) spiked with LTA, it will decrease hypercoagulation and amyloid signal observed with confocal microscopy in the above-mentioned entities. The final hypothesis of this thesis is that by adding mopping agent ApoA1 to T2DM PPP, ApoA1 will decrease the coagulopathies and amyloid signal seen in the PPP of T2DM patients.

To investigate these hypotheses, TEG was used to analyse parameters of the coagulation cascade and it was found that in the presence of LTA, WB shows a trend towards hypercoagulation even though the results were not statistically significant. Furthermore, it was found that by adding ApoA1 to WB already spiked with LTA, the WB became hypercoagulable and these results were significant. These results can be seen in full detail in Table 3.4.1 .and a summary of the results is seen in Table 8.1.1 below. As mentioned in chapter 4, these results were not expected. A trend towards the attenuation of hypercoagulation in the presence of ApoA1 was the expected result. We suggest that the reason for this is that either the concentration of ApoA1 used was too low or the exposure time of ApoA1 in WB was too short.

Table 8.1.1 TEG results displayed as arrows showing where parameters increased and decreased as well as significant values (including multiple comparison). p is significant when p>0.005 and significant p values are displayed in bold and in purple text.

TEG Parameters	Control	LTA	LTA and ApoA1	p value	Significant difference		
					Control vs LTA	Control vs. LTA & ApoA1	LTA vs. LTA&ApoA1
Whole blood analysis (10ng/ml ApoA1)							
R-time	↔	↓	↓	0.1864	-	-	-
K-time	↔	↑	↑	**0.0148**	Yes		-
α angle	↔	↓	↓	0.1002	-	-	-
MA	↔	↓	↓	**0.0212**	-	Yes	-
MRTG	↔	↓	↓	0.1352	-	-	-
TMRTG	↔	↓	↓	0.1338	-	-	Yes
TTG	↔	↓	↓	0.0937*	-	Yes	-
Whole blood analysis (30ng/ml ApoA1)							
R-time	↔	↓	↓	0.3202	-	-	-
K-time	↔	↑	↑	0.8620	-	-	-
α angle	↔	↑	=	0.9943	-	-	-
MA	↔	↓	↓	0.3171	-	-	-
MRTG	↔	↓	↓	0.1873	-	-	-
TMRTG	↔	↓	↓	0.2716	-	-	-
TTG	↔	↓	↓	0.1720	-	-	-
Whole blood analysis (164µg/ml ApoA1)							
R-time	↔	↓	↓	**0.0202**	-	Yes	-
K-time	↔	↓	↓	0.6992	-	-	-
α angle	↔	↓	↑	0.1590	-	-	-
MA	↔	↑	↓	0.6034	-	-	-
MRTG	↔	↓	↑	0.8302	-	-	-
TMRTG	↔	↓	↓	**0.0276**	-	Yes	-
TTG	↔	↓	↓	0.3908	-	-	-

After TEG analysis, it was investigated whether any morphological and ultrastructural changes occurred in platelets and RBC in the presence of LTA and LTA with ApoA1 using SEM. We found that in the presence of LTA platelets became hyperactivated and RBC agglutinated as seen in Figure 5.3.1 and Figure 5.3.2. Furthermore, by adding ApoA1 to WB spiked with LTA, platelet hyperactivation and RBC agglutination was significantly reduced. A detailed summary

of all changes observed is given in Table 8.1.2. below, one can also refer to Figure 5.3.1 and Figure 5.3.2 for more detailed images. Therefore, we conclude that ApoA1 attenuated the hypercoagulable effects of LTA, thus confirming this hypothesis.

Table 8.1.2 A summary of all SEM results for both healthy control and type two diabetes Mellitus controls

SEM SUMMARY						
	CONTROL	**CONTROL+ LTA**	**CONTROL+ LTA + APOA1**	**HEATHY CONTROL**	**NAÏVE T2DM PPP**	**ApoA1 TREATED T2DM PPP**
PLATELET SPREADING	-	✓	✓	-	-	-
PLATELET AGGREGATION	-	✓	-	-	-	-
Platelet Pseudopodia	-	✓	-	-	-	-
Platelet OCS		✓		-	-	-
RBC discoid shape	✓	✓	✓	-	-	-
RBC agglutination	-	✓	-	-	-	-
Thick and Thin fibrin fibres	-	-	-	✓	-	✓
DMD	-	-	-	-	✓	-

Subsequently, amyloidogenesis in the presence of LTA with the use of confocal microscopy was investigated. It was found that in the presence of LTA, fibrin(ogen) clots that formed appeared to be amyloid in nature as fluorescence intensity was enhanced compared to the naïve samples. Even though the amount of amyloid formed was not statistically significant. It was also found that when ApoA1 was added along with LTA, the amyloid like fibrin(ogen) clots formed are reduced as the fluorescence intensity decreased. A summary of fluorescence intensity is shown in Table 8.1.3 below. Although not statistically significant, these results are trending towards significance.

Table 8.1.3 A summary of all Confocal results for both healthy control and type two diabetes mellitus controls

Confocal Summary					
Fluorescence Intensity	**CONTROL**	**CONTROL+ LTA**	**CONTROL+ LTA + APOA1**	**NAÏVE T2DM PPP**	**ApoA1 TREATED T2DM PPP**
Low	✓	-	-	-	✓
Medium	-	-	✓	-	-
High	-	✓	-	✓	-

Observing the potential of ApoA1 towards attenuating the effects of LTA, it was decided to investigate the effectiveness of ApoA1 on an inflammatory condition like T2DM. Here, it was found that by adding ApoA1 to T2DM PPP, there is a significant improvement in the clots formed. With SEM we found that T2DM PPP formed anomalous clots and DMD, however with the addition of ApoA1 the T2DM PPP clots resembled that of healthy controls PPP. Here, individual fibrin fibres are visible compared to the DMD seen prior to the addition of ApoA1 in T2DM PPP as seen in Table 8.1.2. With confocal microscopy it was found that there is significantly less fluorescence in T2DM patients PPP when ApoA1 is added, thus inferring that fewer amyloid proteins are formed in the presence of ApoA1 as seen in Table 8.1.3. From these results it was deduced that ApoA1 provides some level of protection against hypercoagulation and amyloidogenesis, thus confirming our hypothesis that ApoA1 attenuates amyloidogenesis.

The findings of this thesis are clinically significant because, in the presence of bacterial components such as LTA via leaky gut etc., inflammation is brought about. And in the case of pathophysiological inflammatory conditions, inflammation could be further perpetuated by bacteria and their components. Additionally, in the presence of bacterial components such as LTA, the haematological system is negatively affected as it becomes hypercoagulable. Platelets become hyperactivated and RBC agglutinate. This is problematic because hyperactivated platelets and agglutinated RBC are increased risk factors for CVD like strokes, heart attacks and thrombotic diseases, as hyperactivated platelets and agglutinated RBC are inclined to attach to blood vessel walls and forming clots. Thus, in the presence of LTA these conditions could be exacerbated. Another reason these findings are significant is because by adding ApoA1 with LTA, it was found that ApoA1 attenuates the negative effects of LTA. Platelets became less hyperactive, RBC no longer agglutinated and when added to T2DM PPP healthier clots were formed i.e., the clots form in the presence of ApoA1 resemble those of healthy PPP clots, therefore, they looked more like a bowl of cooked spaghetti than DMD.

Thus, suggesting that ApoA1 has protective effects on the haematological system against LTA and in T2DM.

8.2. Future Recommendations and Limitations

For future recommendations it is recommend that higher concentrations of ApoA1 be used. Especially with confocal analysis (confocal imaging was only done with 30ug/ml) ApoA1, as trends were observed here, and no significance was found.

We also recommend that sphingosine-1-phosphate (S1p) or HDL be used in conjunction with ApoA1 as literature has shown that the have the potential to enhance ApoA1 anti-inflammatory effects.

Use American ApoA1 rather that Sigma ApoA1 as Sigma A1 showed enhanced fluorescence compared to American ApoA1. This may be due to the stabilizers used.

Finding the mechanism that ApoA1 uses to provide it protection against LTA.

8.3. Limitations

The amount of LTA that was available was limited.

The T2DM population is (n=7) which is a very small population for the study.

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Chapter 10: Addenda

Addendum 1: Consent Form

Information leaflet and Informed consent form (Healthy Participants)

Title of study: *The role of Lipoteichoic acid on blood coagulation and amyloid formation*

Principal investigators: Prof E (Resia) Pretorius; Department of Physiological Sciences, Stellenbosch University (2017)

Dr Janette Bester; **Department** of Physiology: University of Pretoria

Ethical clearance number: 298/2016

Introduction

You are invited to participate in research study conducted by Dr Janette Bester and 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 3 blood tubes will be a part of research study. **First tube** will be sent to pathology laboratory to determine your iron levels (because the increased or decreased iron levels will determine how its influences the shape of the red blood cells, platelets and fibrin) and **second tube** will be used for haematological (blood) analysis (to determine blood count) and gene regulation studies (to determine platelet receptor expression) and **third tube** will be used by the research team at the University of Pretoria for microscopic work (to find out any alteration in blood clotting mechanism). All the result of this investigation will be sent to your medical practitioner, who will discuss the results with you if necessary.

Purpose of the study

The researcher is investigating the healthy clotting of blood, consisting of red blood cells, platelets and fibrin. This will be done by using specialized microscopes (that can magnify up to 100 000x (called an electron microscope) and a fluorescent microscope called a confocal microscope); we will also use equipment that tests clotting (called a thromboelastograph). Your sample will be used as healthy control blood.

Who will draw the blood?

Either your medical practitioner, a qualified phlebotomist or a staff member of a pathology laboratory (Ampath) will draw the blood.

Procedures

3 tubes of blood will be drawn.

Tube 1: 5 ml blood (one teaspoon which is 5 ml) in a clot activator tube.

Tube 2: 5 ml blood (one teaspoon which is 5 ml) in a citrate tube.

Tube 3: 4 ml blood (a little less than one teaspoon which is 5 ml) will be drawn to determine your iron levels.

The total amount of blood drawn will be less than a table spoon (which is 15ml).

Medical practitioner will fill the table at the bottom of this informed consent form, regarding information that is needed by the research team.

Has the trial received ethical approval?

The Faculty of Health Sciences Research Ethics Committee, University of Pretoria, approved this protocol and written approval has been granted by that committee. The study has been structured in accordance with the Declaration of Helsinki, which deals with the recommendations guiding doctors in biomedical research involving human/subjects.

Research knowledge obtained in this study

Results generated from this study will allow researchers to determine the physiology of healthy clotting and red blood cell structure.

May the procedures result in discomfort or inconvenience or side effects?

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 pathology laboratory nurse (Ampath), and will be taken during the same time as your routine blood tests.

Insurance and financial arrangements

Neither you nor your medical scheme will be expected to pay for the study. During a study-related injury, the Department of Physiology or Dr Bester /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 Ampath, who have insurance for injuries that might occur during routine blood drawing practices.

Confidentiality

All information obtained during the course of this study is strictly confidential. Data that may be reported in scientific journals will not include any information, which identifies you as a patient in this study.

Informed consent

I hereby confirm that I have been informed by my doctor about the nature, conduct, benefits and risks of study. I have also received, read and understood the above written information (Patient Information Leaflet and Informed Consent) regarding research. I am aware that the results of the study, including personal details regarding my sex, age, date of birth, initials and diagnosis will be anonymously processed into a study report. I may, at any stage, without prejudice, withdraw my consent and participation in the study. I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.

Patient's name (Please print)

……..

Cell phone/e-mail:

……..

Patient's signature ……..**Date**……...

Medical Practitioner's signature…….

Principal investigator's signature …….

Information filled by medical practitioner

Age	Gender
Blood group	Education /occupation
Height	Weight BMI
Allergy/Inflammatory condition	**Y / N**
Does the patient smoke?	**Y / N**
Is the patient on the pill if female	**Y / N**
Any other chronic condition (including anaemia)?	**Y / N**
Pills that the patient uses?	**Y / N**
Pills that the patient used 2 weeks prior?	**Y / N**

Addendum 2: Exposure Concentrations

LTA Preparation

Stock 1 LTA= 10ng/ml

Take 500µL LTA into 3.5 ml Distilled H_2O

Stock 2 LTA = 1.25 ng/L^{-1} (working stock)

Exposure concentrations:

Working solution: 20µL of stock 2 LTA into 340µL WB and 20 µL Cacl (20ng/L), resulting in a final exposure concentration of 5ng/L^{-1} of LTA.

Sigma Apolipoprotein A1

Stock 1 ApoA1= 1.3 mg/ml

4µL of ApoA1 into 496µL working solution

Resulting in a final ApoA1 exposure concentration of 10µg/ml

American Apolipoprotein A1 30µg/ml ApoA1

Stock 1 ApoA1= 2.133mg/ml

Add 1.4µl into 98.6µl of WB OR

Add 7µl into 493µl WB for a final exposure of 30µg/ml ApoA1

American Apolipoprotein A1 164ug/ml ApoA1

Stock 1 ApoA1= 2.133mg/ml

Add 38.5µl into 491.5µl of WB OR

Add 9.6µl into 114.4µl WB for a final exposure of 164ug/ml ApoA1

10µL of this left-over working solution is used for SEM

Fibrinogen or PPP

Add 4µL of 125ng/L^{-1} LTA into 96µL fibrinogen/PPP

Add 2.3µL American ApoA1 into 97.7µL fibrinogen/PPP

Add 4µL 125ng/L^{-1} LTA and 2.3µL American ApoA1 into 93.7µL fibrinogen/PPP

Congo Red

Molecular weight of 696,66g/mol

Working solution of 0.5 mM

Final exposure concentration 5µM to µL PPP

0.5.2mg Congo Red into 7.5ml Distilled H_2O

Amytraker™ Preparation

Add 1µL Amytracker to 9µL PBS

Addendum 3: Thromboelastogram

Stellenbosch University: Haematology Lab

Before using the TEG please make a booking in the pink folder on the computer's CPU. Please bring all samples and reagents to room temperature before use.

Start-up

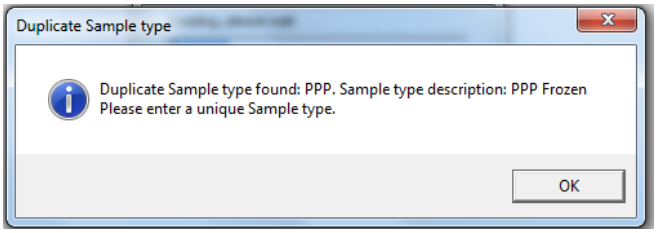
1. Turn on the CPU and log in to Windows.

2. Take the cover off the TEG and turn it on.

3. Check the spirit level on the top of the TEG by looking directly down at it from a 180° angle. If the air bubble is not in the centre adjust the TEG pedestals by turning them.

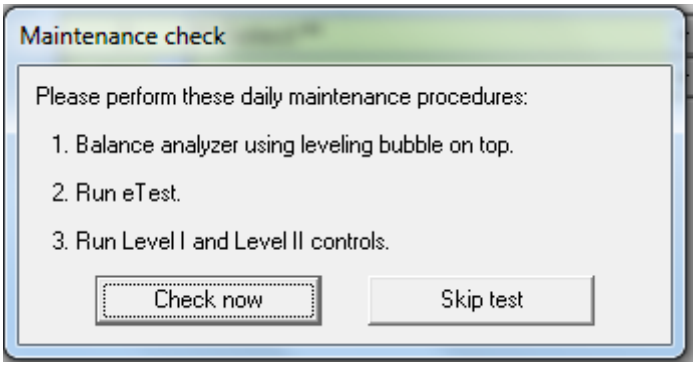
4. Open the TEG programme and log-in by selecting "Site administrator" as the username and typing in "teg" as the password. Once logged in a second window will appear, select "Temporary operator" and click "logon". The "Patients" database will automatically open in the main window.

5. If at any point a "duplicate sample type" error window appears, simply click "OK" to continue



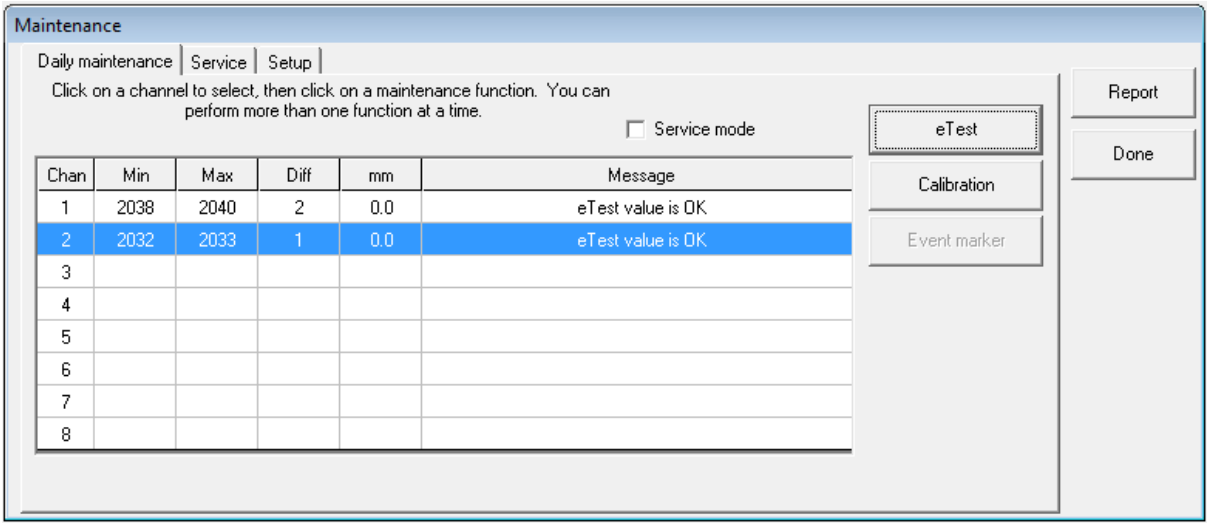
Maintenance and Quality Control

1. A small window will appear with the message "it is time to run a QC sample, please run one now". Click exit. A second window will appear prompting you to perform the maintenance checks. Select "Check now" and continue to the eTest.

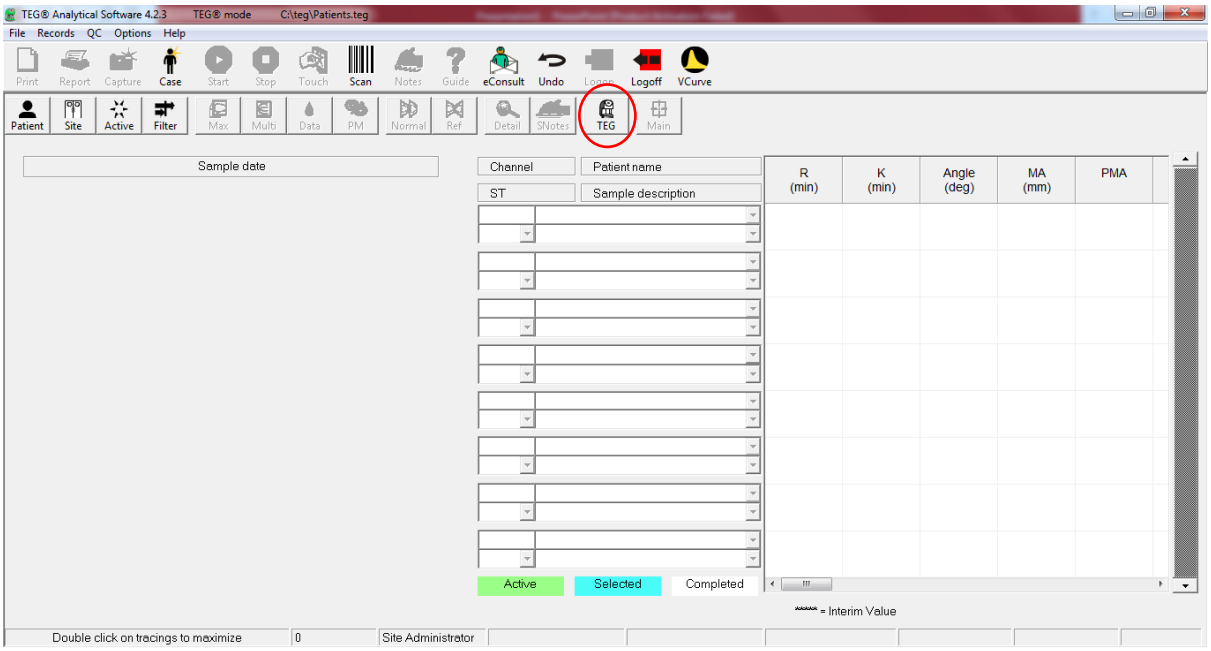


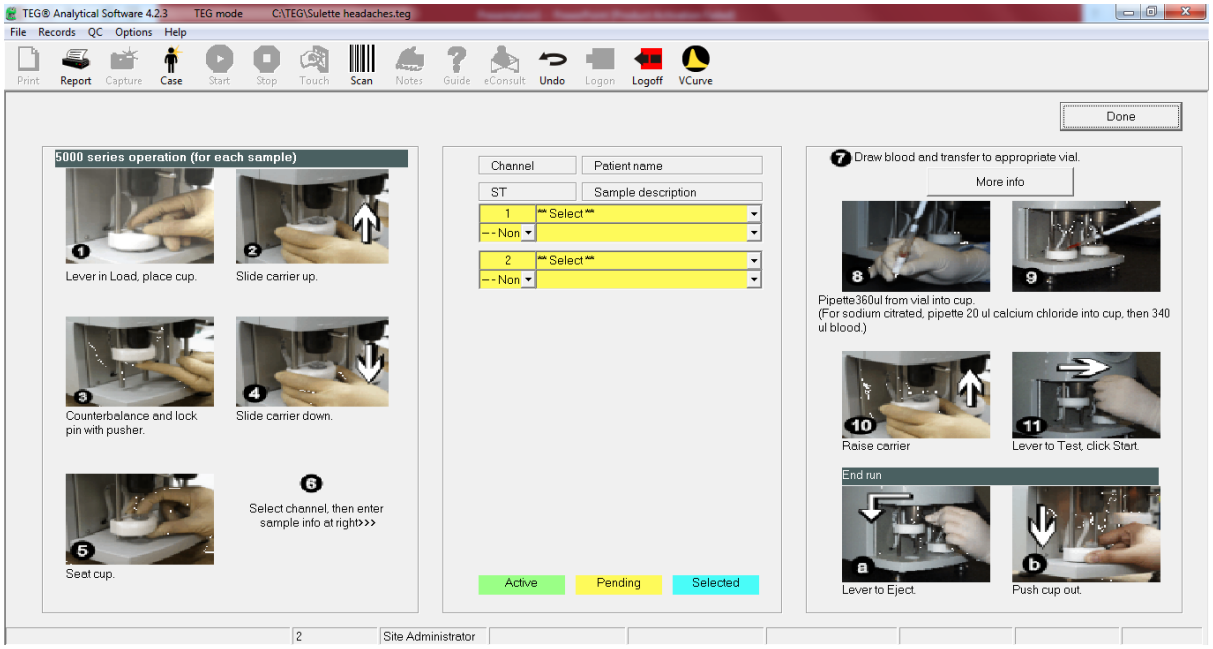
2. Channel one will automatically be highlighted in the eTest window. Click the "eTest" button and move the channel one lever into 'test' position. Select channel two and repeat. Should the eTest be off centre or the channel not in equilibrium, repeat the ETest for both channels. If the error persists, the technician needs to be contacted. If the "eTest value OK" message

is displayed close the window and return both channels to load position. You will be returned to the TEG main window.



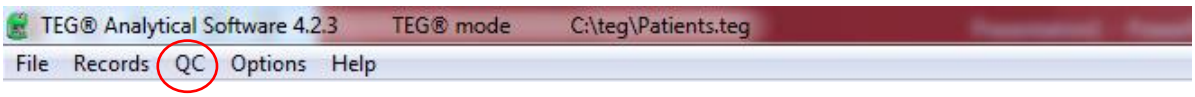
3. Prepare the level I and level II controls by adding one green capped vial to each powdered control vial. Agitate the vials slightly so that all the powder is controlled. The level I control needs to rest for 15 – 20 mins; the level II control needs to rest for 40 – 60 mins. Do not leave the dissolved QC vials outside for more than 2 hours. The QC requires each level to be run in both channels simultaneously. In the main window click on the “TEG” button.





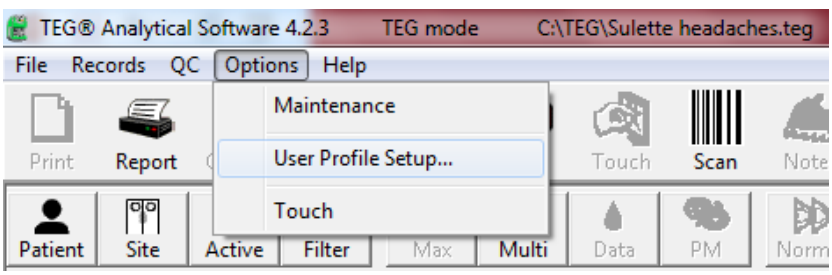
4. Start with the level I QC. From the sample type (ST) drop-down menu, select level I control. In the patient name window select the number range that appears on the vial label. Do this for both channels. No sample description is required for QCs. Load a cup and pin into each channel and pipette 20 µL **(be very precise!)** calcium chloride ($CaCl_2$) into each cup. Make sure that channel 1 is selected. Add 340 µL QC solution into each channel sequentially; immediately load the cup, move the lever into test position and click start (or press F10 on the keyboard) as soon as the solution is added to the $CaCl_2$. Click on “Done” to monitor the tracing. Allow the QC to finish running in both channels. If an error is detected, repeat the QC. If the error persists, the technician needs to be contacted. If the QC runs without any errors, eject the cup and pin and place in a washing beaker. Repeat the process for the level II control.

5. A QC needs to be run once a week of every 50 samples. To check when the last QC was run and view the results, click “QC” on the toolbar and select “GoTo”. Deselect “GoTo” to return to the main window.

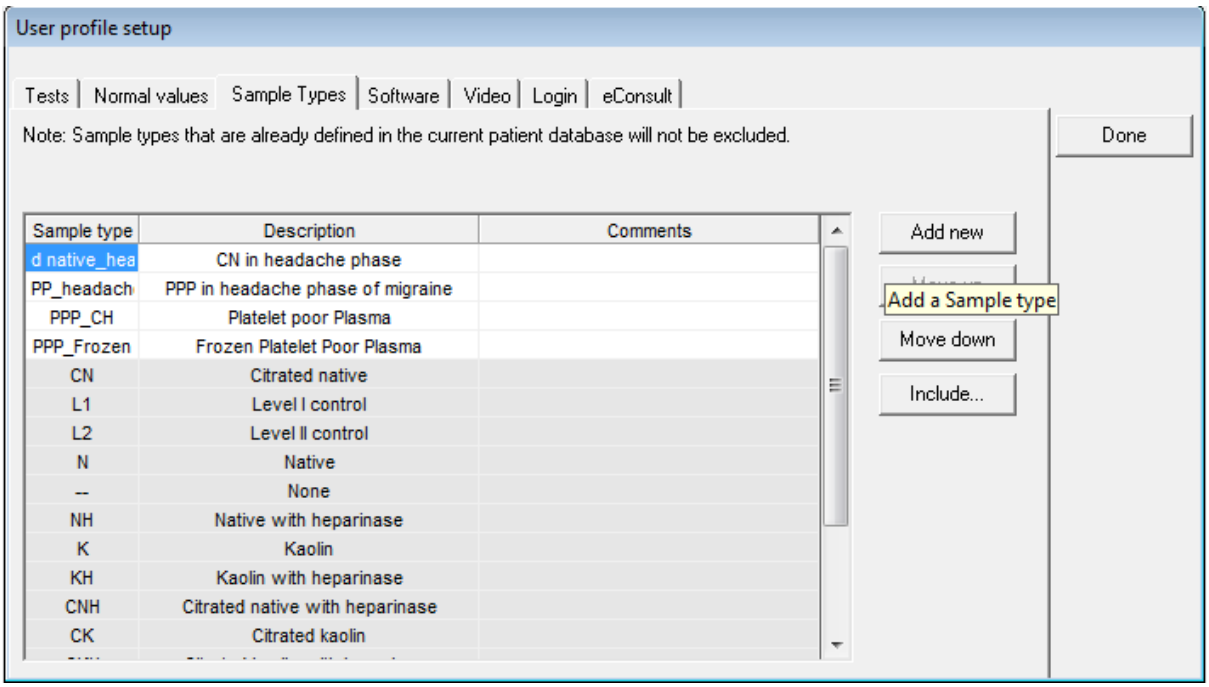


<u>Creating a Database</u>

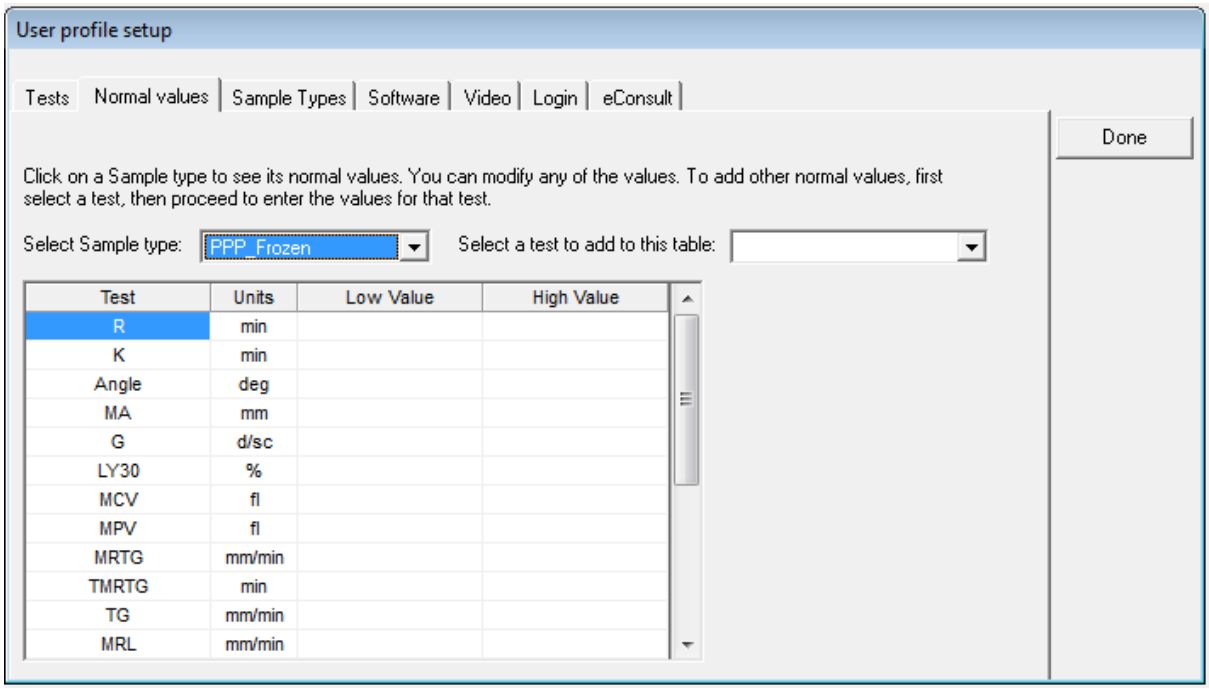
1. Select “file” and “create database”. Define database name and save.
2. Click on the “Options” tab and select “User Profile Setup…”
3. If you want to create a new sample type



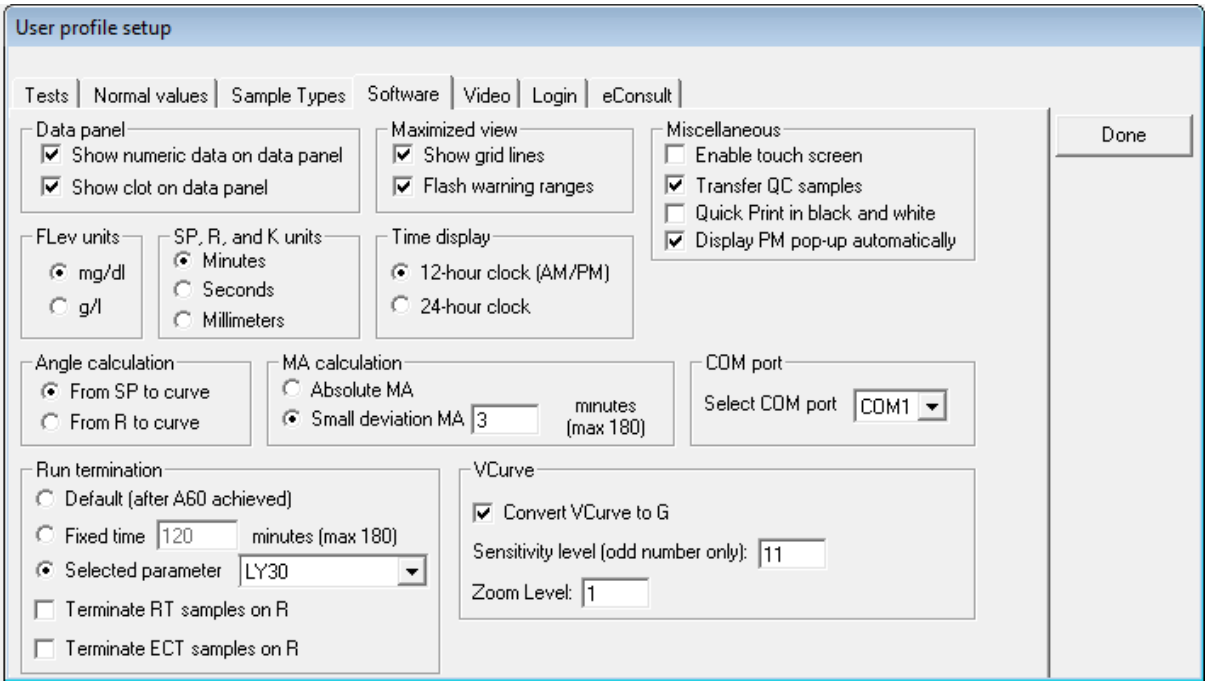
specific to your study click on the “Sample types” tab >>> “add new” >>> define your new sample type, e.g. PPP_diabetes



4. To set the parameters for your new sample type click on the “Normal values” tab >>> Select sample type >>> select a test to add to this table >>> and choose parameters from the drop down menu. Be sure to add MRTTG, TMRTTG and TTG to the table.



5. Lastly, click on the “Software” tab. In the “Run Termination” block select “LY30” from the drop-down menu. In the “VCurve” block tick “Convert VCurve to G”. Click “Done”.



PPP and citrated native TEG

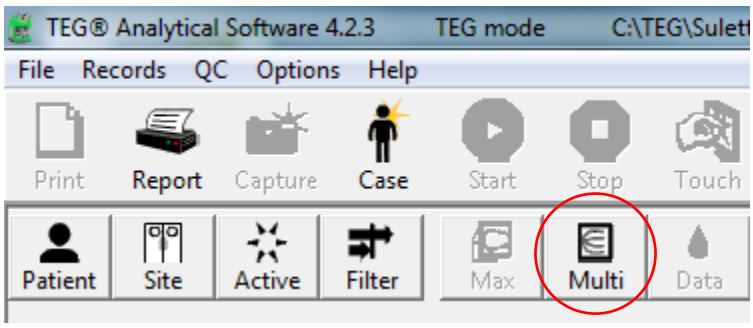
1. Allow sample to come to room temperature. Click “File” and “Open database”.

2. Select “TEG” and enter sample details for each channel. If a new patient or control sample is being run, a prompt will appear to define the new patient. Enter the patient’s/control’s details.

3. Once the sample types have been defined load a cup and pin into each channel.

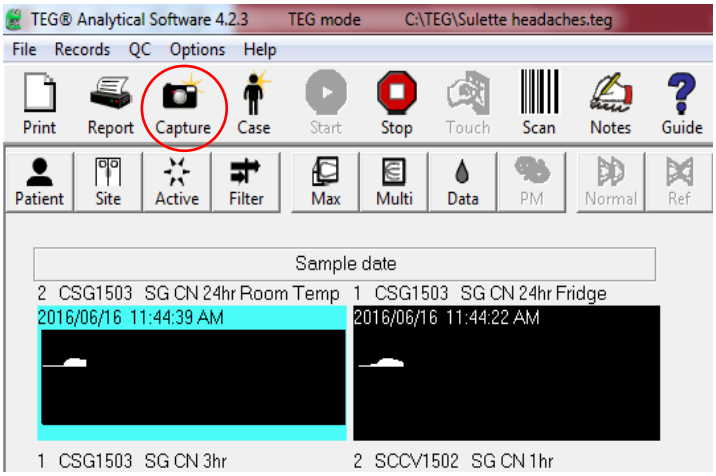
4. Add 20 µL $CaCl_2$ into each cup, followed by 340 µL of the sample. Once again, as soon as the sample has been added to the $CaCl_2$ load the pin, move the lever to test position and start the tracing. **For native blood (directly out of the vein), do not add $CaCl_2$, instead pipette 360 µL into the cup and immediately start the tracing.**

5. To monitor a sample double click on the tracing in the main window. You can monitor several samples simultaneously by first clicking “Multi”, selecting the tracings, and clicking “done”, the tracings can be viewed offset or superimposed.

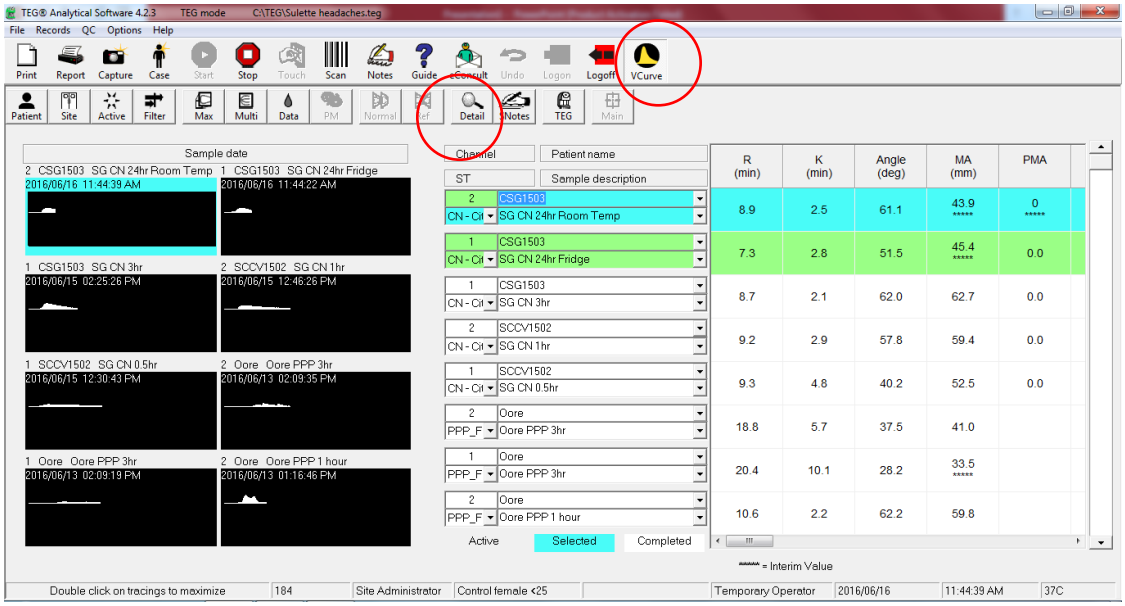


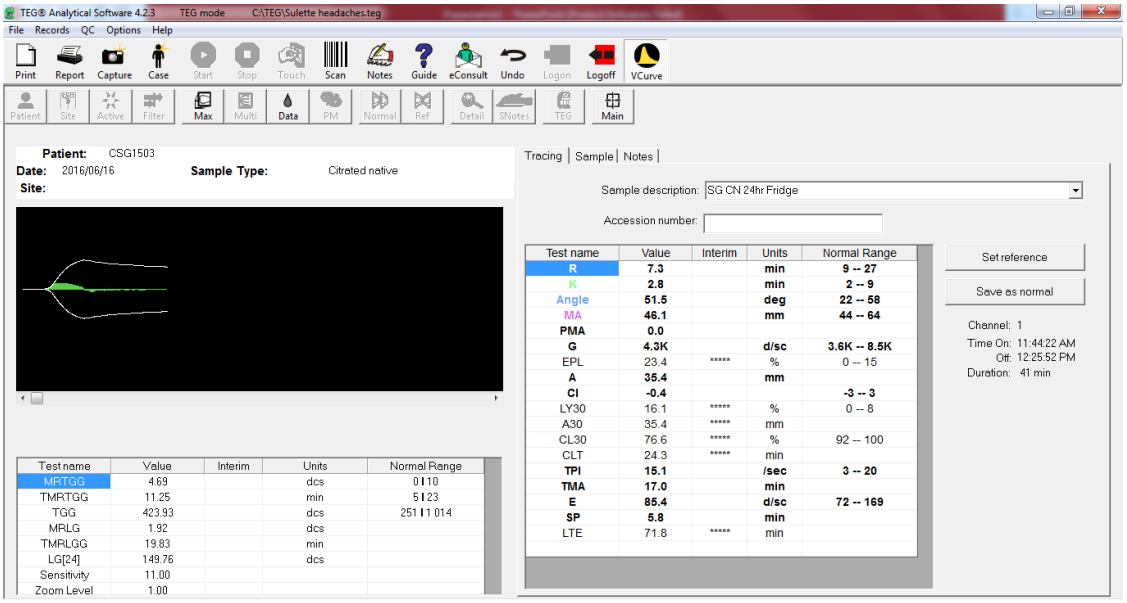
Data Capture

1. To export a tracing: in the main window select a tracing and click “Capture”, select your preferred format and click “Done”. Open a blank presentation in Microsoft PowerPoint and paste the tracing into a blank slide.

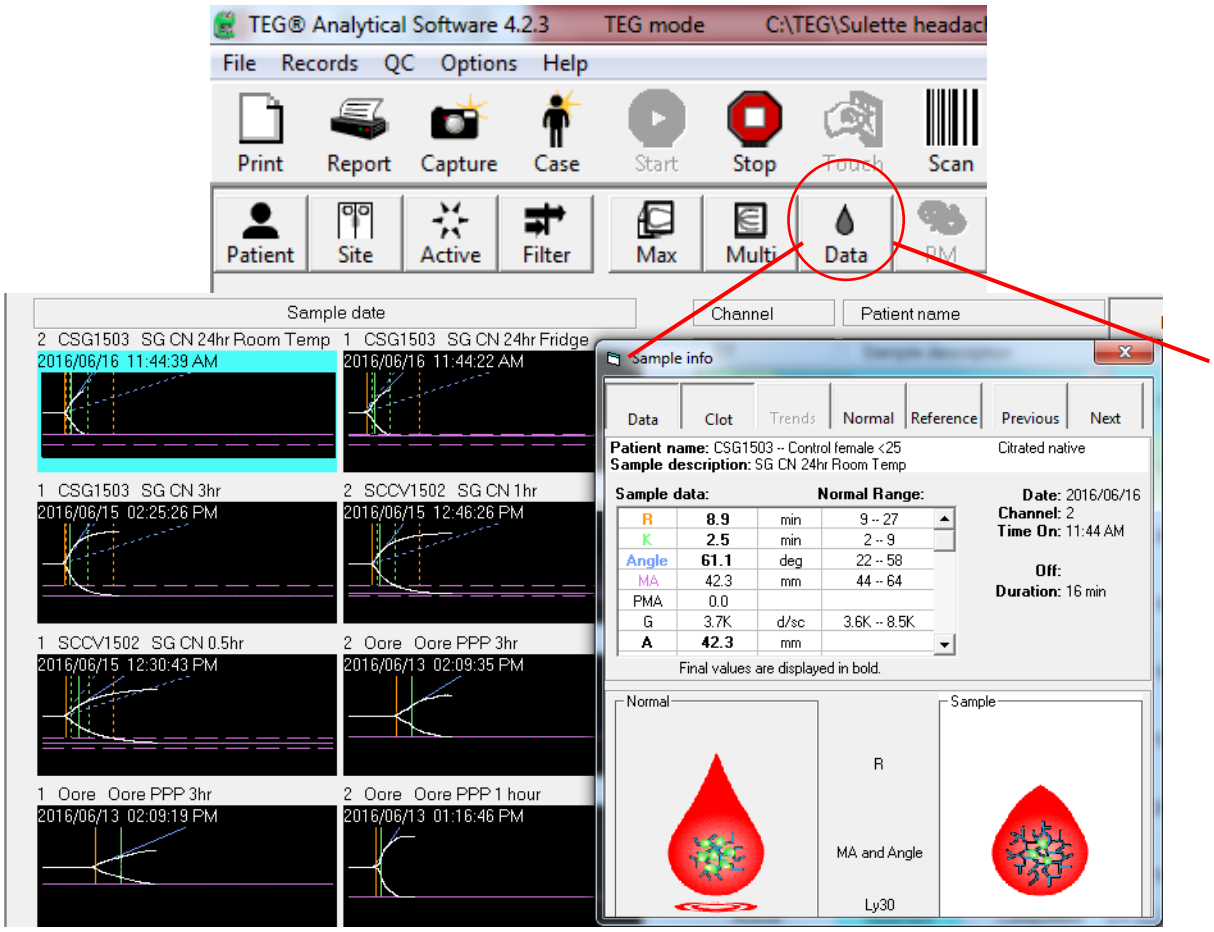


2. VCurve: To observe parameters such as MRTTG, TMRTTG and TTG select “VCurve”. Click on a tracing and then select “Detail”. The tracing, it’s VCurve and all measured parameters will be shown in the detail view. You can once again capture and export this data.





3. To get an overview of the sample vs the normal range, click “Data” in the main window. This will show a summary of the clot formed by the sample.



When you are done using the TEG make sure all cups and pins have been ejected and washed. Do not re-use cups and pins more than three times. Press the power button on the TEG to turn it off and cover it. Close the TEG programme and shutdown the computer if no-one else is using it.

Addendum 4: Scanning Electron Microscopy

AMRC LAB SOP: SEM PREPARATION

Tube preparation:

1. All citrate tubes have to stand for 30 min after blood draw before any work can be done. This is to allow for complete binding of calcium. DO NOT REFRIGERATE YOUR BLOOD TUBE – keep it at room temperature until you can process it – which must be done within 4 hours of blood draw.

2. Blood can be spun down in two ways (platelet poor plasma (PPP):
   - Centrifuge for 30 mins in the ultracentrifuge, OR
   - Centrifuge for 20 min, pipette the plasma into an Eppendorf tube and then centrifuge the Eppendorf tube for 10 min – check you centrifuge's RPM – you want to create PPP with no platelets left in plasma
   - Pipette the PPP into a clean Eppendorf tube and freeze in the -80 °C

Notes on pipetting:

- Take care to not touch the buffy coat with the pipette tip
- Do not reverse pipette: pipette the plasma fraction up to first stop, pipette down to second stop into the new Eppendorf tube

Reagent preparation:

Buffer: mix equal parts 0.075 M PBS (fridge stock) and distilled water (dH_20)

Fixative: prepare 25 ml of 4 % formaldehyde by mixing 4 ml 25 % formaldehyde (fridge stock) and 21 ml of your prepared buffer

SEM sample preparation for PPP, PRP or WB

1. Pipette 10 µl sample (PPP, PRP or whole blood) onto a 10 mm round cover slip. If preparing with thrombin, pipette 5 µl onto the cover slip. Thrombin is added to prepare extensive fibrin fibre clots either with PPP, PRP or WB. If WB is used, RBC will be seen in clot.

2. To create extensive fibrin fibres when thrombin is added:
   Using a bent pipette tip carefully spread the sample droplet (PPP, PRP or WB with added thrombin) over the cover slip – take care to only touch the droplet, not the glass. Smear in a 8 cycle, and with the final movement remove the top layer of the gel. Place directly in an 8-well marked holder

3. Let dry at room temperature for 45 seconds for PPP and a bit longer 1 minute for WB.. If a gel-like layer has formed wash immediately.
4. For only WB smears leave drop a bit longer as it washes of more easily.
5. Add buffer to cover smears and leave for 15 min.
6. After 15 minutes, remove buffer and add just cover the sample with fixative, cover plate for 30 min. **Samples can be left in fixative for max 7 days – but not advisable.**

7. Discard fixative, 3 x 3 min buffer wash.

8. In the fume cupboard discard the third buffer, wash and place one drop of osmium tetra-oxide (OT) directly onto the cover slip. Just cover sample with dH_2O, cover 6-wells plate and leave in the fume cupboard for 15 min.

9. Discard the OT/dH_2O into the OT waste. 3 x 3 min Buffer wash, discarding the first buffer wash into OT waste.

10. Ethanol series dehydration: wash the samples in 30 %, 50 %, 70 %, 90 % and 100 % ethanol for 3 mins each. Repeat the 100 % ethanol wash twice more. **At this stage, the closed well plate can be sealed with parafilm and the samples stored for a maximum of 28 days.**

11. In the **fume cupboard**, discard the ethanol and cover the samples with HMDS for 30 min. Discard the HMDS and place one drop directly onto the sample. Tilt the 6-well plate and draw off any excess. Carefully lift the edge of the cover slip for a few seconds, allowing the bottom to dry. Put the cover slip down and leave the well plate in the **flow cabinet** overnight for the HMDS to evaporate. Please make sure your well plate has your name and the date on it. Seal the dry well plate with parafilm until you are ready to mount and coat your samples.

<u>SEM preparation for TEG clots</u>

1. The clot will either be inside the TEG cup or adhered to the pin. Use fine-tipped tweezers to remove the clot. PPP clots will collapse into a small clot; whole blood clots are much larger and can be separated into smaller pieces.

2. Place the clot onto a 10 mm glass cover slip, DO NOT LET TOO MUCH OF THE FLUID RUN OFF OF THE COVER SLIP.

3. Transfer the cover slip with the clot directly into a 6-well plate.

4. Place 1-2 drops of fixative on the clot. The fixative should form a bubble over the cover slip but not run off and fill the well. Leave for 10 min for a PPP clot, and 20-30 min for a whole blood clot.

5. Fill the well plate with enough fixative to cover the entire sample. Leave overnight.

6. Proceed with SEM preparation as above.

<u>Mounting and coating</u>

1. Label a steel SEM plate with your name, the date and the sample type.

2. Place one or two strips of double-sided carbon tape of the steel plate.

3. Check that your cover slips are loose in their wells before attempting to lift them out. Lift the individual cover slips out using fine-tipped tweezers and a small piece of prestik adhered firmly to the edge of the cover slip, keeping the cover slip at 90° to the workbench surface. Gently place the cover slip on the carbon tape.

4. Once all your cover slips are mounted use the carbon coater to coat your SEM plate once in the middle, and once each tilted to the left and right of the coater chamber.

5. For a very thick sample (e.g. a prepared clot from the TEG), place a small droplet of conductive carbon paint on either side of the thick area, as close to the edge of the coated cover slip as possible. Allow to dry.

6. Store SEM plates in a container and complete the lab register book. Check number of samples against ethical number to trace sample to filled in ethics document.

Addendum 5: Confocal Microscopy

Amytraker™ Preparation

Add 1µL Amytracker to 9µL PBS

LTA Preparation

5mg into 1L into 10mg/L

Take 10µL of stock A into 500ml final volume

Stock B= 100ng/L

Stock C (working stock) is 20ng/L LTA

From stock B (100ng/L) take 2 ml and 8mL H_2O therefore take 20ng/L in 10ml

Exposure concentrations:

0.5µL= stock C (20ng/L) into 975µLWB/ PPP

Or

1ng/L= 5µL of stock C (20ng/L) into 95µL WB/ PPP

Or

0.2ng/L= 1µL of stock C (20ng/L) into 99µL WB/ PPP

Platelet Poor Plasma

From the whole blood sample of the volunteer:

1. Centrifuge the blood from the patient in its citrated tube for 15 minutes in the centrifuge at 3000xg.
2. Decant platelet poor plasma epi tubes and store at -80^{o} C until the day of analysis.
3. On the day of analysis, thaw the PPP for approximately 30-40 minutes at room temperature.
4. Once the PPP has thawed, incubate the naïve control with the following 3 markers with the following concentration: 5µM Tht and 0.1uL of Amytracker™ 480 and 680 into 100µLPPP for 30 minutes. For the treated incubate it with 1ng.L^{-1} LTA.
5. For the naïve control: pipette PPP that was only incubated with the 3 markers onto a glass cover slip and thrombin to a 1:2 ratio of thrombin to PPP therefore we added 5µL thrombin and 10µL PPP.

For the treated group: pipette PPP that was incubated with the 3 markers and LTA onto a glass cover slip and thrombin to a 1:2 ratio of thrombin to PPP therefore we added 5µL thrombin and 10µL PPP

6. Wait about 30 seconds for clot formation to then place a coverslip over the newly formed clot.
7. Analyse the newly formed clot using Zeiss LSM 510 META confocal microscope with a Plan-Apochromat 63x/1.4 Oil DIC objective.
8. For Tht, excitation laser used was 488nm and emission measured at 508 to 570 nm. For Amytracker ™ 480 the 405nm laser was used with the emission measured at 478 to 539 nm and for Amytracker ™ 680 the 561 nm was used for the excitation with emission measure at 597 to 695 nm.
9. Prepare a selection of micrographs of the clots with and without LTA.
10. Gain settings were kept the same during all data capture and used for statistical analyses. However, brightness and contrast were slightly adjusted for figure preparation.

Addendum 6: Image Analysis

ZEN Black:

1. Select image that you want to analyse from the selection of prepared micrographs
2. Select processing method select copy>delete image>select image to be deleted>change numbers to 3:3 > save as new .czi file.

ImageJ Fiji:

1. File, open .czi file and select split channel option
2. Three different images appear in different colour
3. Analyse one image at a time: select analyse on the strip
4. Under analyse select histograms use these number to calculate coefficient of variation as SD/mean.
5. Enter these values on GraphPad Prism version 5.00 software and analyse the data using one-way ANOVA.