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The effect of the neurotoxin lipopolysaccharide on the coagulability and red blood cell ultrastructure of blood from healthy individuals

Dissertation submitted in partial fulfillment of the requirements for the degree,

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The effect of the neurotoxin lipopolysaccharide on the coagulability and red blood cell ultrastructure of blood from healthy individuals

By

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Abstract

Lipopolysaccharide (LPS) is an endotoxin, which is found on the outer membrane of gram negative bacteria and is shed off during infections. This membrane contains a rich network of endotoxins that may circulate in the blood. Chronic inflammatory diseases are accompanied by hypercoagulable phenotypes. It is known that inflammation is typical initiated by pro-inflamatory cytokines but the origin of inflammation is still unclear.

The study's hypothesises is the LPS molecules from dormant microbes are resuscitated and bring about inflammatory changes seen in chronic inflammatory diseases, by changing red blood cells (RBCs) ultrastructure affecting coagulations.

The following objectives direct the study: to investigate the effects of LPS on the ultrastructure plasma (fibrin networks) and RBCs, using scanning electron microscope (SEM) a high resolution CrioCrossbeam 540 from ZEISS; to investigate the effect of LPS on RBCs shape and morphology using light microscope (LM); to investigate possible damages to RBCs' elasticity using the atomic force microscope (AFM). For this investigation 30 healthy individuals were used, each person served as their own control. A miniature concentration of LPS (0.2 $ng.L^{-1}$) was added. To study coagulation parameters blood was drawn into citrate tubes and aliquoted into two separate tubes one tube LPS was added to whole blood (WB) and PPP. To study the clotting properties of blood, Thromboelestograph (TEG) was conducted with and without added LPS.

From TEG results it was observed that the initiation time of a clot in minutes and the kinetics of the clot decreased with the addition of LPS in both whole blood and platelets poor plasma. It was observed that when LPS added to blood an increase in clots strength was evident in treated blood compared to untreated blood. There was an increase in the angle of the clot given with variable strength size of the clot, making it difficult for the clot to be dissolved. Clot hypercoagulability and increased clot strength are common characteristics found in inflammation.

From this investigation it was found that LPS changed the fibrin fibres forming matted mass deposits affecting coagulation. When viewing with SEM it was seen that LPS causes agglutination of plasma proteins on RBCs' surface changing the ultrastructure. The elasticity of RBCs decreased according to AFM results justified the shape changes of RBCs that have been exposed to LPS. These are also principal characteristics of hypercoagulation. Based on these results it was concluded that LPS could in part be the cause of physiological changes seen in inflammatory conditions where an underlying bacterial component is present.

Keywords: Lipopolysaccharides, Fibrinogen, Hypercoagulation, Light microscope, Platelets poor plasma, Red blood cells, Scanning electron microscope, Thromboelastograph

SIGNIFICANCE STATEMENT

Almost all chronic diseases (including those classified as cardiovascular, neurodegenerative, or autoimmune) are coupled by long-term inflammation. Typically these types of diseases are mediated by pro-inflammatory cytokines. However, the source of this inflammation is unclear. In this thesis it has been suggested that a dormant microbiome that has been resuscitated, sheds off the highly inflammatory lipopolysaccharide (LPS). Such inflammatory diseases are also accompanied by a hypercoagulable phenotype. I have shown that very low concentrations of LPS can affect the coagulation properties of blood and plasma significantly, and that this may be mediated by a direct binding of LPS to fibrinogen monomers as assessed with Isothermal titration calorimetry (ITC). In this thesis I have shown signs of LPS affecting RBCs visible with LM, SEM, AFM changing the membrane ultrastructure, elasticity of RBCs. It is still unclear as to where the LPS binds to RBCs but the effects are prominent and visible.

DECLARATION

I Sthembile Valencia Mbotwe , hereby declare that this research dissertation is my own work has not been presented for any degree at another University.

Signed.....

Date 30/04/2016

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My parents Mr and Mrs Nyambiyo, thank you for your endless love and support that you always give me. Your guidance and constant nurturing has modelled me into being the woman I am today. My siblings Menzi and Wandile, thank you for your continuous support. Finally but not the least in my heart , Dr Mthokozisi Sibanda thank you for always being by my side, guiding me and helping me through life and research. One could not ask for a better partner.

There is no tree that grows without it roots, to all my family and friends whose names I did not mention, thank you for nurturing and supporting me to grow and blossom at all times.

LIST OF ABBREVIATIONS

AD	Alzheimer's disease
AFM	Atomic Force Microscopy
Calcium	Ca^{2+}
Calcium Chloride	$CaCl_2$
CD	Cytokine dependent
DAMP	Damage associated molecular pattern
DM	Diabetes Mellitus
DMT	Derjan-Muller-Toporov
E.coli	*Escherichia coli*
Hb	Hemoglobin
HMDS	1,1,1,3,3,3-Hexamethyldisilazane
HIV	Human Immunodeficiency Virus
HR	Hydroxyl Radicals
IL1,3,6	Interleukens
ITC	Isothermal Titration Calorimetry
LAL	*Limulus* amebocyte lysate
LBP	Lipopolysaccharide Binding Protein
LM	Light microscopy
LPS	Lipopolysaccharides
MP	Microparticles
NSA	Nanoscope analysis
OsO_4	Osmium tetraoxide
PAF	Platelets Activation Factors
PAMPs	Pathogen Associated Molecular Pattern
PD	Parkinson's Disease
PPP	Platelets Poor Plasma
PMNs	Polymorphonuclear
PRP	Platelets Rich Plasma
QNM™	Quantitative Nanomechanical Property Mappping

RBCs	Red Blood Cells
RIAS	Radioimmunoassay
ROS	Reactive Oxygen Species
SEM	Scanning Electron Microscopy
SF	Serum ferritin
sTfR	Serum Transferrin receptor
S1P	Sphingosine 1-Phosphate
SD	Standard deviation
TEG	Thromboelastography®
TF	Tissue Factor
TLR-4	Toll- Like Receptor-4
TMN	Tumor necrosis factor
WB	Whole blood

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CHAPTER 1: Introduction

Lipopolysaccharide (LPS) is as an endotoxin found in the outer membrane of Gram-negative bacteria (Diacovich and Gorvel, 2010, Parija, 2009). It is shed off during infections and illnesses by invading bacteria or dormant microbe that has been resuscitated (Marshall et al., 2002). This neurotoxin is well known as a potent inflammatory agent (De Castro et al., 2010, Płóciennikowska et al., 2015a). Continuous production and replenishment of LPS increases the production of inflammatory cytokine causing damages from low grade inflammation to sepsis shock (death) (Kitchens and Thompson, 2003, Marshall et al., 2002).

Lipopolysaccharide is a lipoglycan (Polanowska-Grabowska et al.) composed of large molecules of lipids (fats) and polysaccharides (sugars) (Kell and Pretorius, 2015b). This structure of the LPS molecule is important in bacterial survival and the pathogenesis of Gram-negative bacteria (Tzeng et al., 2002). See **Figure 1** for a comprehensive schematic diagram of LPS. The sugar component is referred to as the O-antigen/Polisaccharide. It consists of recurring glycan polymer (Holst, 2007). This region is located in the outermost domain of the LPS molecule. It serves as a target for recognition by host antibodies (De Castro et al., 2010, Rittig et al., 2003). However, the composition of the O-chain varies from strain to strain. The presence or absence of O-chains determines whether the LPS is considered rough or smooth. Full-length O-chains would render the LPS smooth whereas the absence or reduction of O-chains would make the LPS rough (Rittig et al., 2003). Bacteria with rough LPS usually have more penetrable cell membranes to hydrophobic antibiotics since a rough LPS is more hydrophobic (Rittig et al., 2003, Kell and Pretorius, 2015b).

Lipid-A segment is the latter component of the LPS molecule. It is a phosphorylated glucosamine disaccharide decorated with multiple fatty acids (Ding et al., 2013, Müller-Loennies et al., 2003, Holst, 2007, De Castro et al., 2010). These hydrophobic fatty acids chains anchor the LPS into the bacterial membrane (De Castro et al., 2010). The lipid A domain is responsible for much of the toxicity of Gram-negative bacteria (Kell and Pretorius, 2015b, Holst, 2007). The core domain of Lipid-A contains an oligosaccharide component composed of sugars such as heptose and 3-deoxy-D-mannooctulosonic acid (also known as KDO, keto-deoxyoctulosonate).

When bacterial cells are lysed by the immune system, fragments of membrane containing Lipid-A are released into the circulation causing fever, diarrhoea and possible fatal endotoxic shock (also called septic shock) (Ding et al., 2013, Diacovich and Gorvel, 2010, Van Oosten et al., 2001).

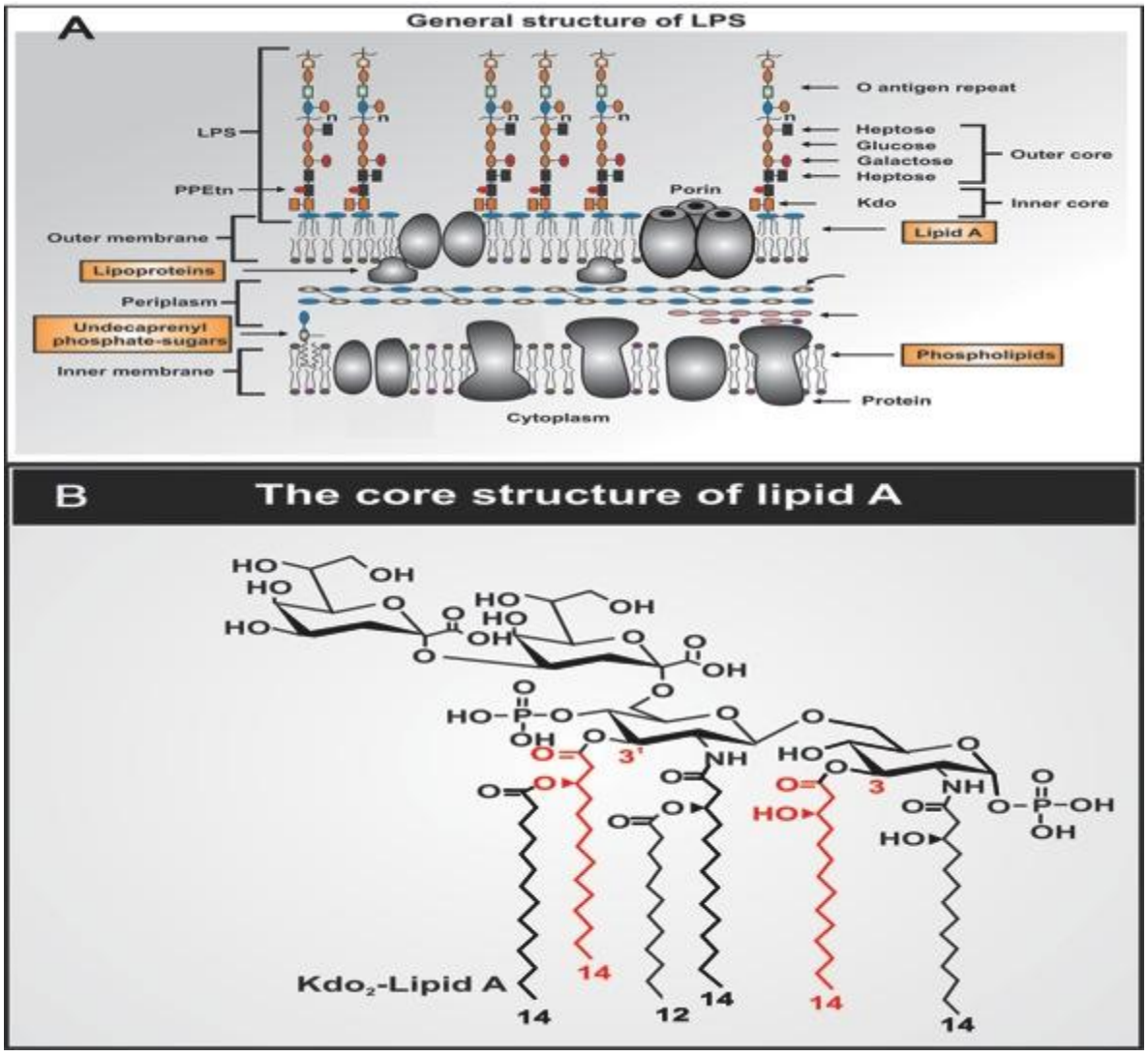


Figure 1.1: The structure of LPS. Section **A** represents the overall view of the LPS structure; **B** is Lipid A core domain the main toxic region of the LPS molecule. This figure was adapted from (Kell and Pretorius, 2015b)

Subsequent work showed that release of LPS from Gram-negative microbes does not necessarily require the destruction of the bacterial cell wall but rather, LPS is secreted as part of the normal physiological activity of membrane vesicle trafficking in the form of bacterial outer membrane vesicles (OMVs), which may also contain other virulence factors and proteins (Kell and Pretorius, 2015b, McBroom et al., 2006).

Lipolysaccharides constitute a major section of the outer membrane of Gram-negative bacteria. It increases the negative charge of the cell membrane and helps stabilize the overall membrane structure (Abrahamsson et al., 2014, Cuaz-Pérolin et al., 2008, McBroom et al., 2006). This is primarily for structural integrity of bacteria and protection of the bacterial membrane from certain kinds of chemical attack (Moran et al., 1996). However, LPS in host cells acts as the archetypal endotoxin. It binds the CD14/TLR4/MD2 receptor complex to immune cells such as monocytes, dendritic cells, macrophages and B cells. This promotes the secretion of pro-inflammatory cytokines, nitric oxide and eicosanoids (O'Neill, 2014, Park et al., 2009, Poltorak et al., 2000, Muroi and Tanamoto, 2002). An increase in pro-inflammatory agents can lead to chronic inflammatory reactions such as autoimmune diseases (harmful reaction to host tissues) and lower grade inflammation seen in Alzheimer patients (Bester et al., 2015b, Park et al., 2009). As part of the cellular stress response, superoxide is one of the products that forms and is a major reactive oxygen symptom (ROS) *i.e.* harmful chemical reactive molecules containing oxygen. They are by-products of normal metabolism of oxygen. These species are induced by LPS in various TLR4 expressing cell types (Beutler et al., 2001, Termeer et al., 2002, Płóciennikowska et al., 2015a).

A large part responsible for the LPS is dramatic clinical manifestations of infections with pathogenic Gram-negative bacteria such as *Neisseria meningitides*. These are the pathogens that cause meningococcal disease including meningococcemia, Waterhouse-Friderichsen syndrome, Haemophilus influenzae and meningitis ((Tzeng et al., 2002, Moran et al., 1996, Van Oosten et al., 2001). The core oligosaccharides of low molecular weight LPS of pathogenic *Neisseria* spp. mimic the carbohydrate moieties of glycosphingolipids present in human cells. Such mimicry may serve to camouflage the bacterial surface from the host (Moran et al., 1996).

The problem that we are facing across the world is chronic inflammatory diseases such as cardiovascular, neurodegenerative and autoimmune diseases. Most of these diseases are known to be accompanied by long term inflammation, an increase in serum ferritin (SF) levels and hypercoagulable phenotype (Bester et al., 2015b, Kell and Pretorius, 2015b, Schumann and Zweigner, 1999). Typically they are mediated by pro-inflammatory cytokines, however, we still do not know the origin of this inflammation.

Previously it was suggested that a primary explanation for systemic inflammation in these typical non-communicable diseases, is a dormant microbiome accompanied by a constant and chronic dysbioses of the gut (well-known to be present in most if not all inflammatory condition) that can shed the highly inflammatory LPS. This was first discussed in extensive literature reviewed in papers by our group (Kell et al., 2015, Kell and Pretorius, 2015c, Potgieter et al., 2015b). Due to the known fact that all inflammatory conditions show a pathologic haematological and coagulation system, it is believed that LPS may play a prominent role in the pathology of these systems. Therefore our aim was to investigate the effects of LPS on the haematological system, by looking at the ultrastructure using scanning electron microscopy (SEM) of RBCs, fibrin networks, platelets, as well as the coagulation parameters as measured with thromboelastography (Moreno-Navarrete et al.) (Moreno-Navarrete et al.) of healthy individuals, where we added physiological low levels of LPS *ex vivo*.

The hypothesis was therefore: LPS affects coagulation, by changing RBCs ultrastructure and membrane elasticity, and causing hypercoagulation, with a resulting fibrin fiber ultrastructure change.

Therefore, in the current thesis I will show how very low concentrations of LPS can affect the coagulation properties of blood and plasma significantly using SEM, AFM and TEG. We believe that LPS causes hypercoagulation by binding to fibrinogen and affecting fibrinolysis. I will also show evidence of LPS binding to fibrinogen by using commercialised fibrinogen monomers. This was conducted to eliminate patients' variability. Researchers have previously shown that LPS binds to blood via LPS binding protein (LBP) which is a glycoprotein or to the lipoprotein ApoE that is defensive against LPS (Schumann and Zweigner, 1999, Kitchens and Thompson, 2003, Schumann, 2011, Van Oosten et al., 2001). Binding of LPS to blood show signs of low grade inflammation (Bester et al., 2015b). The membrane integrity and elasticity of RBCs' membrane might also be affected and the damage can then be visible through SEM, LM and AFM. Impaired RBCs can contribute to inflammatory conditions and in the presence of hydroxyl radicals, RBCs lose their discoid shape (Pretorius, 2013b).

Therefore the following research objectives and hypothesis direct this thesis:

1. To study the general morphology and structure of RBCs after WB is exposed to LPS using LM. We have used the Cell-analyzer program to analyze the shape changes of RBCs.
2. To investigate the fine ultrastructural changes of RBCs using whole blood and SEM.
3. To investigate RBCs' membrane elasticity after addition of LPS using the AFM.
4. To investigate fibrin fiber formation after addition of LPS to PPP using the SEM by measuring fibrin fiber thickness.
5. To investigate the efficiency of blood coagulation using the TEG technique using PPP and WB. This was to determine the activity of the plasma coagulation system and fibrinolysis.

See **Figure 1.2** for visual layout of this thesis.

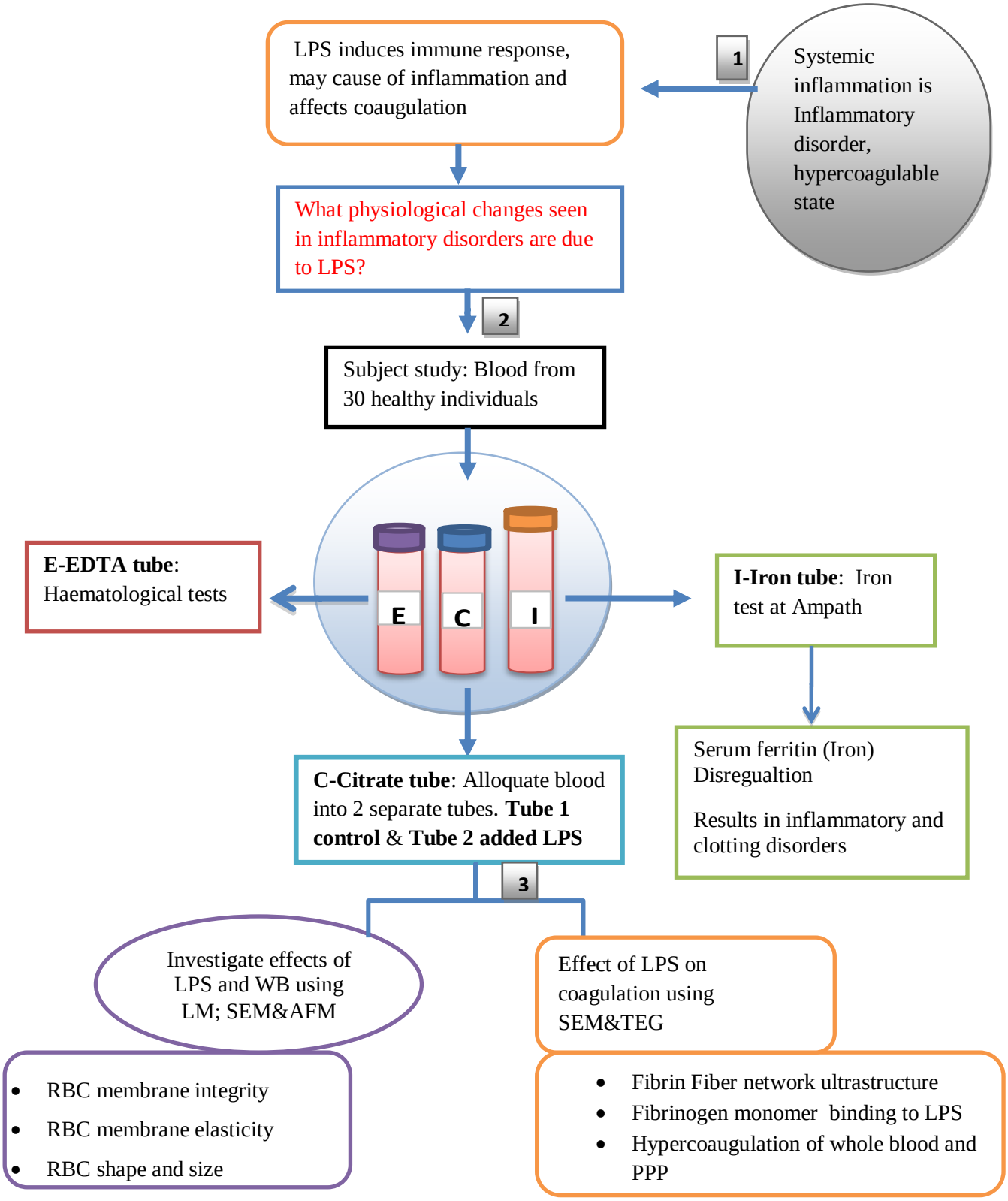


Figure 1.2: An overview diagram summarizing the content of this thesis. **1**. Systemic inflammatory disorders. LPS induces pro-inflammatory cytokines. **2** Study population 30 healthy individuals, to induce inflammation, 0.2ng.L^{-1} LPS was added to each sample. Techniques used (**3)** Atomic force microscopy (AFM), Light Microscopy (LM) used to study the effect LPS on red blood cells (RBC) membrane. Thromboelastography (Moreno-Navarrete et al.), Scanning electron microscopy (SEM) used to study ultrastructure.
PPP-Platelets poor plasma

CHAPTER 2: Literature review

2.1. Chapter Aim and objectives:

Inflammation and LPS is associated with the pathogenesis of severe chronic diseases. In this chapter I will review literature about systemic inflammation effect on RBCs and plasma. The aim of this chapter is to show the causal link on how the presence of LPS and its cytotoxic effect correlates with the changes in the haematological system that persists, and seen in (systemic) inflammatory conditions. Inflammation is both a biological response and a pathologic process consisting of a dynamic complex cascade of events. During inflammation unliganded (free) iron levels and serum ferritin levels are also increased. Central to this response is the role of plasma protein- fibrinogen and its interactions with both RBCs.

See Figure 2.1 for a flow diagram that shows the main points discussed in this literature review chapter.

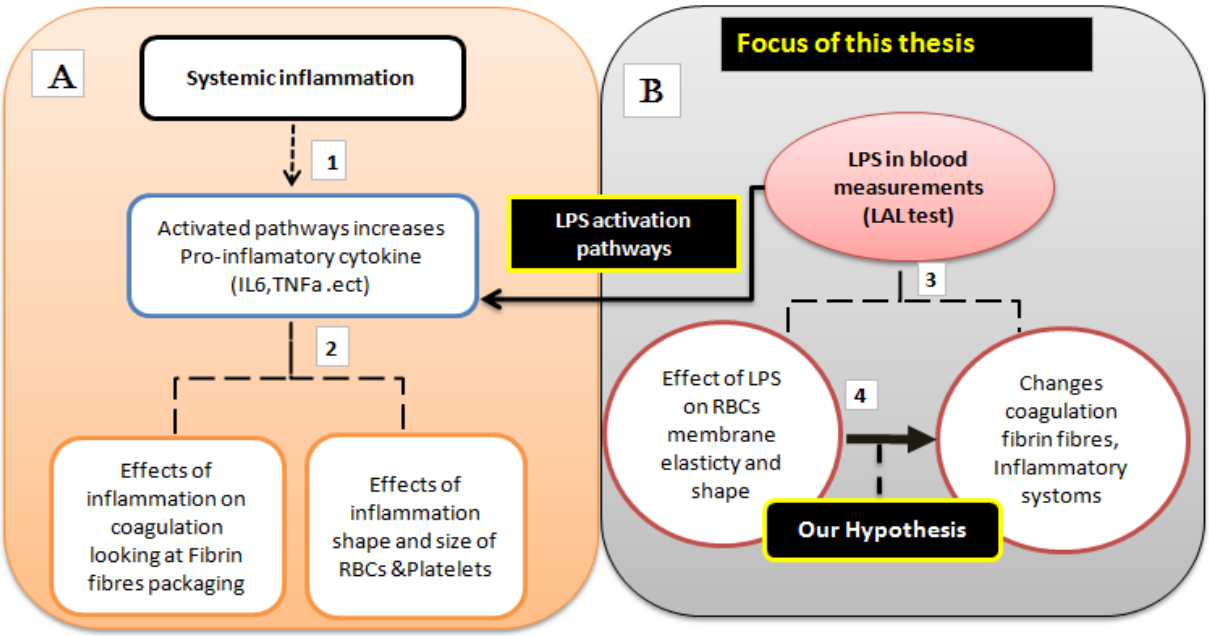
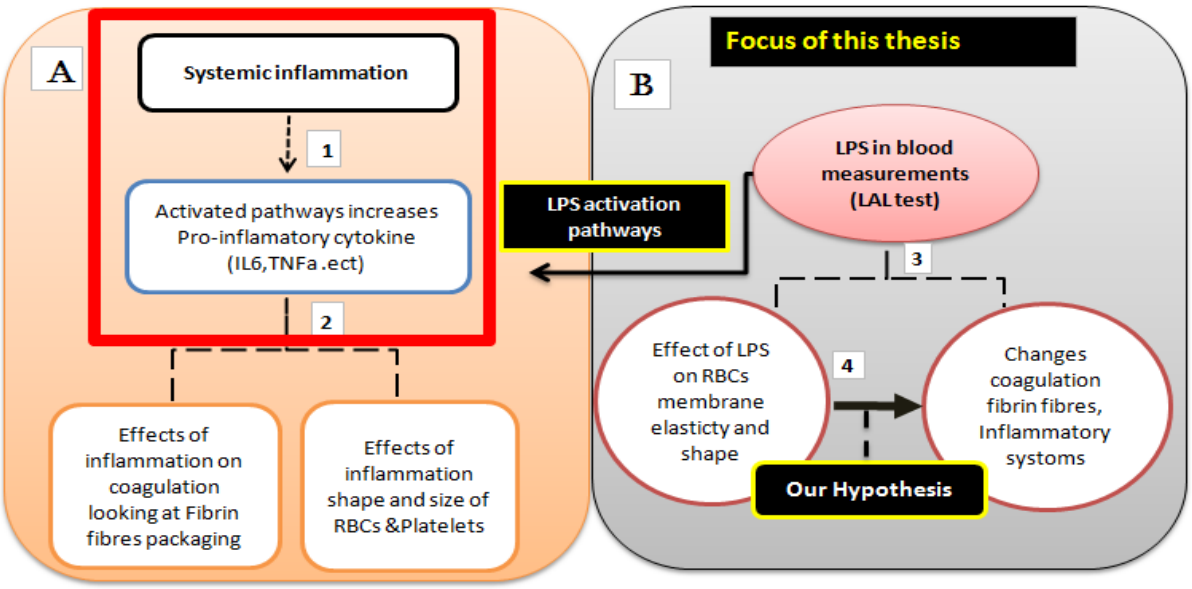


Figure 2.1: Flow diagram of the main points discussed in the literature review. (**A**) Systemic inflammation and inflammatory pathways that are activated in the presence of LPS (**1**) increase cytokine production; (**2**) adverse effect on RBC membrane and function, and affects fibrin formation 'clotting process' (**B**) Main focus of literature review is to review and reveal the effect of LPS **(3)** concentrations of LPS in blood measured by LAL assay; (**4**) How RBCs are affected by LPS, therefore changing coagulation parameters.

To understand the role of cytokine activation and how LPS affects the coagulation and haematological system, I must first review literature regarding systemic inflammation, followed by the role of LPS in activating pro-inflammatory cytokines, and effects on RBCs and fibrinogen. The specific area of focus as shown in Figure 2.1 will be shown in a red block before the relevant literature is discussed.

Systemic inflammation activates pathways that lead to activating cytokines: the role of increased levels of fibrinogen

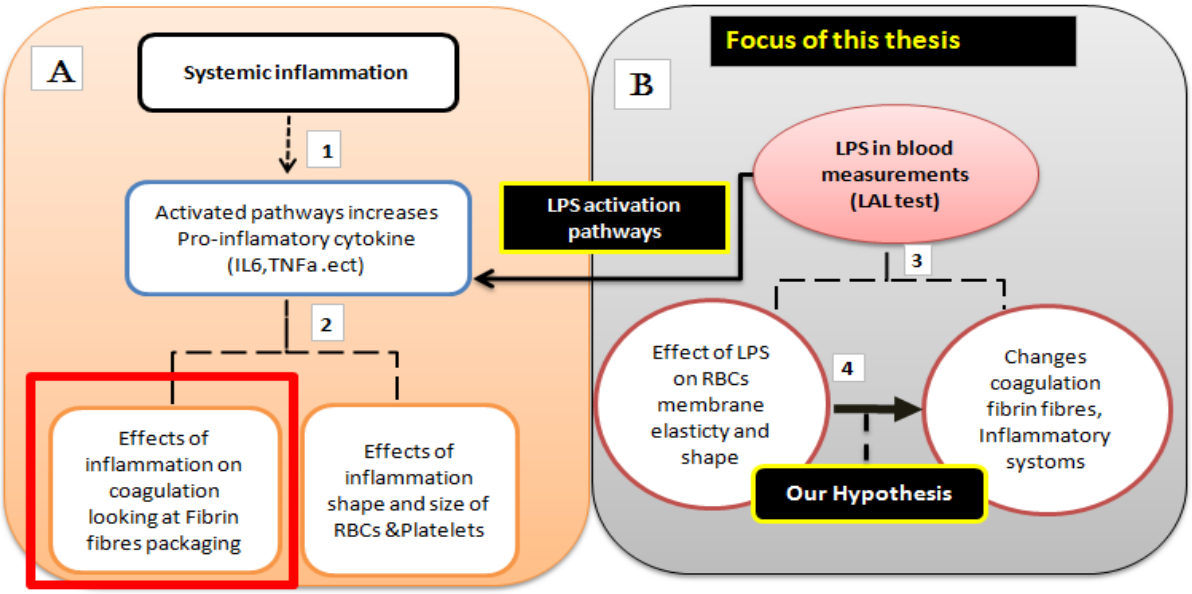


Chronic systemic inflammation (SI) is the result of release of pro-inflammatory cytokines from immune related cells and LPS, the chronic activation of the innate immune system (primary defence mechanism), contributing to the development and progression of certain conditions such as cardio vascular disorders (Miao et al. (2014). Researchers found that saturated fatty acid and LPS are associated with systemic or tissue inflammation and pro-inflammatory cytokines. As a consequence they overproduce ROS (reactive oxygen species) to potentiate secretion of pro-inflammatory cytokines (Miao et al.) More researchers found that up-regulation of C- reactive protein (CRP) and IL-6 (pro-inflammatory cytokines) levels may amplify activation of coagulation through up-regulation of tissue factors (TF) on innate immune cells (Ezzelarab et al., 2015, Miao et al., 2014). This is an indirect root of activated coagulation causing hypercaugulation.

Researchers reported that this extreme manifestation of increased pro-inflammatory cytokines response is systemic development of life-threatening coagulopathy (a condition in which the blood's ability to clot is impaired) (Heemskerk et al., 1997).

Coagulopathu is associated with prolonged or excessive bleeding, which may occur spontaneously or following an injury or medical and dental procedures. Researchers Miao and colleagues reported that the contributing mechanisms for coagulopathy includes inflammation, vascular injury, innate, humoral and cellular immune responses and molecular incompatibilities affecting the vital regulation of coagulation (Miao et al., 2014). As it may be known plasma proteins are essential for clotting process, for us to have a complete look at coagulation dysfunctions we must first look at one of the most vital plasma protein – fibrinogen which is essential for formation of clots. I will review how it is activated during inflammation and defense or abundance during systemic inflammation(De Moerloose et al., 2010)

Plasma Protein fibronogen activated during inflammation affects coagulation



One of plasma protein that is known for having the most affects on coagulopathy is fibrinogen. This highly molecular weight (340 kda) soluble protein is from human blood plasma and it plays a major role in hemostasis and thrombosis (Heemskerk et al., 1997). An insoluble fibrin is produced by the action of the enzyme thrombin it maintains and stabilizes the blood clots (De Moerloose et al., 2010).

Fibrin clot formation is part of the natural process of wound healing and tissue repair it forms a fibrous mesh that impedes the flow of blood in that area (van Rooy et al., 2015).

During fibrin formation there is a crucial role that is played by thrombin in initiating the polymerisation of fibrinogen **see Figure 2.2** for the normal activation process and role of thrombin in coagulation. Along with the normal degradation pathway of the fibrin which is catalysed by plasmin formed from the tissue plasminogen activator-catalysed activation of plasminogen.

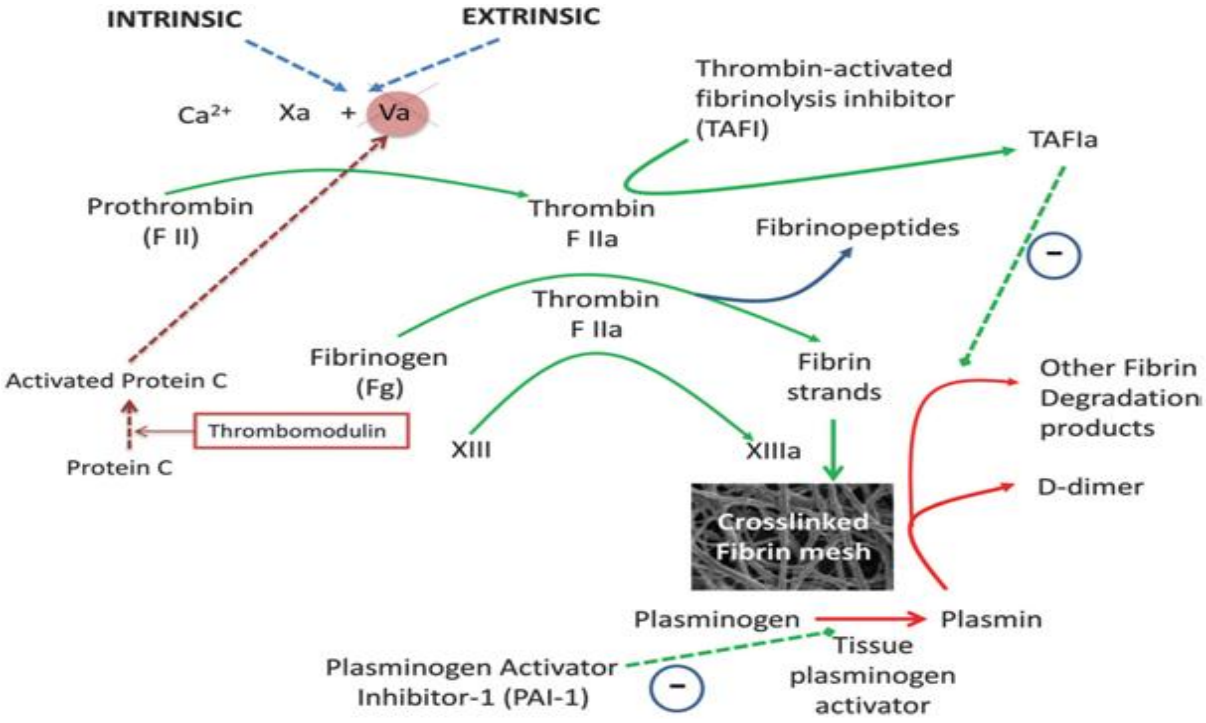


Figure 2.2: Coagulation pathways (intrinsic and extrinsic), showing the crucial role played by thrombin in plasmogen adapted from (Kell and Pretorius, 2015d)

An increased in fibrinogen levels has been marked in inflammatory response (Spencer et al., 2007). Hypercoagulability goes together with inflammation, and is strongly influenced by the fibrinogen concentration (Kell and Pretorius, 2015d). The increase in fibrinogen formation can also contribute to both acute and chronic inflammatory conditions (De Moerloose et al., 2010, Pretorius et al., 2015). Hence it is essential to understand the regulation of fibrinogen to get a clear vision how this plasma protein plays a role in hypercaugulation.

Leading research has shown that a change in fibrinogen levels associated with hypercoagulability results in a pathological change in clotting profile (Lipinski and Pretorius, 2013, Casini et al., 2014). One of the factors that is known to affect fibrinogen activity and formation is thrombin and plasmogen (Kell and Pretorius, 2015d).

Furthermore researchers (Undas et al., 2008) reported that increased levels of fibrinogen are associated with cardiovascular events. During a thrombotic event, the increased levels of fibrinogen cause abnormal fibrin fiber formation, visible as a denser coagulated mass or dense matted deposits (DMDs) and the resulting coagulum causes RBCs to change shape and to be trapped in the abnormal network (Lipinski et al., 2012a).

It is visible now that the combination of the formed tighter fibrin mesh or otherwise known as the clot and impaired fibrinolysis is a feature of hypercoagulation and is common in conditions like ischemic stroke (Pretorius and Lipinski, 2013c, Swanepoel and Pretorius, 2015) diabetes type II (Pretorius et al., 2015) along with numerous inflammatory diseases (Kell and Pretorius, 2015d, Casini et al., 2014). This tighter clot is due to a general phenomenon known as a prothrombotic state, this all justifies results obtained from researchers prio that an increased fibrinogen level is associated with hypercoagulability (Lipinski and Pretorius, 2012a).

As seen from the previous Figure 2.2 where I showed by means of a diagram how fibrin is formed and how a typical fibrin fiber network looks like versus fibrin fibers resembling inflammation and the pathologic formation of DMDs. The question that arises is what the difference in width or size of these fibrin fibers found a healthy versus chronic systemic inflammation patients. There is evidence that the individual fibers seen in normal healthy people have a much smaller diameter than that of normal fibrin fibers, the difference was statistically significantly and changed during thrombotic stroke (Pretorius and Lipinski, 2013a). Therefore typically there are thick and thin fibers in a healthy fibrin clot, with the thick fibers being the prevailing fibers, the formation of DMDs that may be the cause of an enhanced prevalence of thrombotic events (De Moerloose et al., 2010, Lipinski and Pretorius, 2012a). These researchers (Lipinski et al., 2012b) agreed on saying that DMDs reflect a hypercoagulability profile. **See Figure 2.3** for a diagram that illustrates how DMDs present in chronic inflammatory disorders. During normal coagulation, thrombin acts on fibrinogen to form fibrin polymer fibers, and these fibers will dissociate easily under fibrinolytic conditions (break down of fibrin clot).

An increase in fibrinogen level can lead to abnormal clotting, hence formation of DMDs by unusual agglutination of plasma proteins, which are resistant to typical fibrinolysis.When fibrin clots form abnormally, RBCs are entrapped more tightly inside the clot (Pretorius and Lipinski, 2013a). The involvement of RBCs is directly linked to hypercoagulability of fibrin (Pretorius et al., 2013c, Pretorius, 2013b, Pretorius and Lipinski, 2013c).

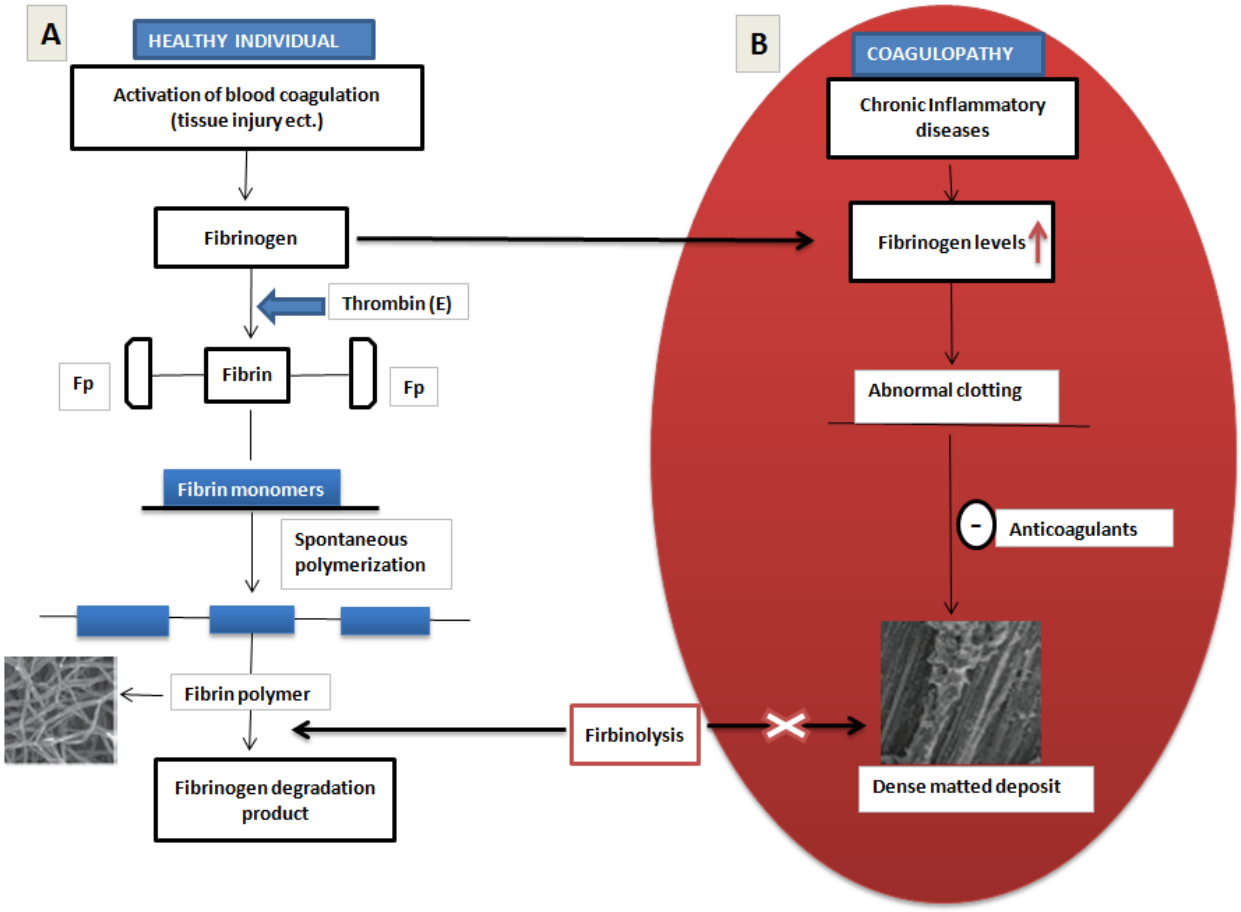
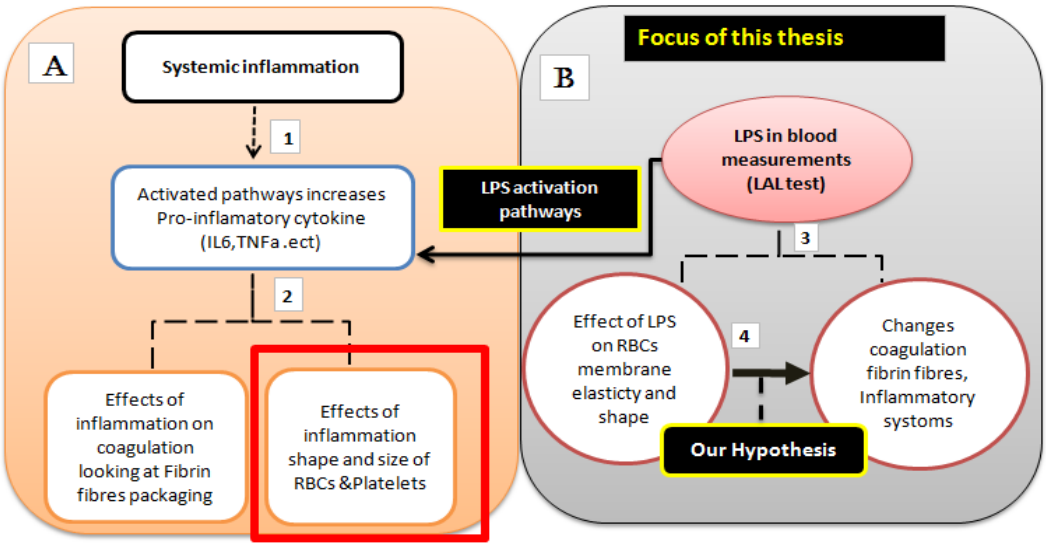


Figure 2.3: Flow diagram of coagulation pattern in (**A**) normal healthy individual versus (**B**) coagulation pattern in people with chronic inflammation diseases. Adjusted from (Lipinski et al., 2012a) **Fp= Fibrinopeptide**

The next paragraph I will review literature about how fibrin abnormalities affect the membrane and shape of RBCS.

Systemic inflammation and it effects on RBCs



Red blood cells have recently been marked cells that may marker if there is a presence of inflammation (Pretorius, 2013b) and the changes seen in membranes elasticity and shapes of RBCs are known to be elicited by inflammation (Bester et al., 2013a, Kuznetsova et al., 2007, Pretorius et al., 2014a). These RBCs changes are present in conditions such as those seen in Alzheimer and stroke patients (Bester et al., 2013a, Pretorius and Lipinski, 2013c). The general flow dynamics of blood is closely associated with RBC shape and deformability(Pretorius et al., 2016b). Red blood cells deformability refer to the ability of RBCs to change shape under a given level of applied stress (moving through small vessels) without rupturing, during inflammation reduction in RBCs deformability is seen (Pretorius, 2013b).

The role of iron in decreased RBCs' deformability during inflammation

One of the major leaders of a decrease in RBCs' deformability is the presence of iron and its role in the production of hydroxyl radicals (HR) (Undas et al., 2008, Olinescu and Kummerow, 2001, Feng and Hart, 1995). The question that arises now is what is the link between fibrin fibers, RBCs and iron levels. Pretorius and co-workers have hypothesized that fibrinogen, as well as plasma proteins present in healthy individuals can be converted into an insoluble, fibrin-like polymer by a non-enzymatic action of HR, following the addition of physiological levels of ferric iron (Lipinski et al., 2012a) this fibrin-like polymer entraps the RBCs causing hypercaogubility (Pretorius and Lipinski, 2013a). Therefore RBCs trapped in fibrin leads to the pulling of more RBCs , thickening blood (viscosity increases).

This form of reaction is presented in chronic inflammation. Hence the extent of both leukocyte and RBCs aggregation correlated with the concentration of fibrinogen (Rogowski et al., 2000). During inflammation unliganded (free) iron levels and serum ferritin (SF) levels are also increased, as seen in many inflammatory conditions such as atherosclerosis, diabetes type II, stroke, Alzheimer's disease and Parkinson's disease (Kell, 2009, Bester et al., 2013a, Pretorius and Lipinski, 2013c). It is also shown that, iron molecules leave ferritin and increase production of HR. Unfortunately with the accumulation SF levels along with increase in fibrinogen levels inflammatory conditions can aggregate and escalate mortality across the world. Scientists with growing technology established that iron chelating and/or hydroxyl trapping agents are able to reverse the formation of aberrant fibrin clot structure, even in the presence of iron overload (Bester et al., 2013a, Pretorius et al., 2013c, Pretorius et al., 2014a).

However the possibility of the reversal of the changed fibrin clotting is consistent with the view that the aberrant fibrin morphology resulting in trapped RBCs in the presence of increased iron (particularly serum ferritin) is caused, at least in part, by 'free' iron (Kell, 2010). In contrast to thrombin-generated fibrin the iron-induced parafibrin is totally resistance to the fibrinolytic degradation. This is due to the fact that paraffin has different tertiary structure than fibrin formed with thrombin (Lipinski et al., 2012b). The cleavage of disulfide bonds is induced by biologically reactive hydroxyl radicals (HR) chemical formulae (HO•) formed in the reaction between trivalent iron with hydroxyl groups of water according to the following reaction equation:

$$Fe^{3+} + HO^{-} \longrightarrow Fe^{2+} + HO^{\bullet}$$

As the consequence of the HR interaction with the fibrinogen a huge protease resistance polymer is formed that remains in the circulation for a long time resulting in a state of chronic inflammation due to the attraction of cytotoxic albeit ineffective T cell (Lipinski and Pretorius, 2013). On the basis of these findings we can conclude that the spontaneous formation of fibrin-like dense deposits in patients' **blood** may be a consequence of what is known as iron overload (Pretorius and Lipinski, 2013a). Red blood cells are easily deformed to a pointed shape and it is seen in smears and with the addition of thrombin they are entrapped in the fibrin mesh of dense matted fibrin deposits/ dense matted deposits (DMDs).

This entrapping causes severe shape changes due to the pressure of the fibrin onto the stressed cells. The entrapment of RBCs during a prothrombotic state, is associated with increased iron levels in diabetes (Pretorius et al., 2015). The most important observation of the current research is therefore how fast RBC can adapt in a changed environment and that the pressure of fibrin fibers may trap the RBC tightly in the resulting clot (Pretorius, 2013b). **See Figure 2.4** for a full comprehensive diagram of RBCs entrapped in fibrin. In this figure we see that RBC undergo a shape change during inflammation which may be due to increased iron levels (Pretorius et al., 2013c, Rogowski et al., 2000) this is associated with a change in the hematocrit, aggregation and viscosity of RBCs and an abnormal clotting profile. It is known that RBCs are characterized by a shape change in many disease states (Minetti et al., 2013, Shin et al., 2007, Piagnerelli et al., 2007).

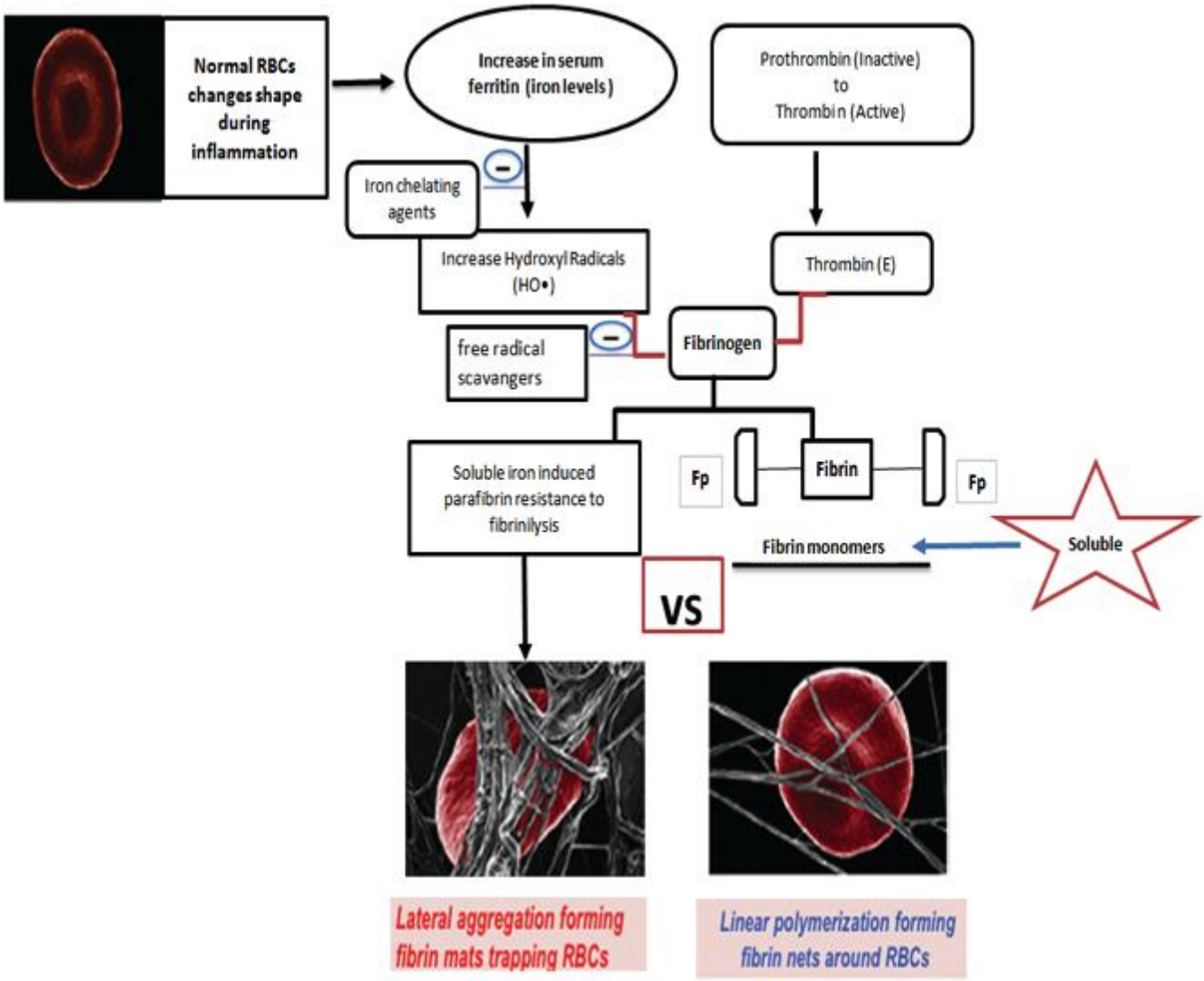


Figure 2.4: Diagram of red blood cells entrapped in fibrin. In chronic systemic inflammation lateral aggregation formation of fibrin mats would be visible which will entrap RBCs redrawn from (Lipinski et al., 2012a)

Under normal physiological conditions, RBCs are able to pass through narrow capillaries only as single cells rather than as aggregates (Tripette et al., 2009). The protein fibrinogen is an important determinant of RBC aggregation, with an almost direct relationship between aggregate size and plasma fibrinogen concentration (Baskurt and Meiselman, 2013). Fibrinogen molecules adhere readily to the membrane surface of RBCs (Cairncross et al., 1969, Lipinski et al., 2012a) this encourages RBCs aggregation (Tripette et al., 2009). Research conducted by (Rogowski et al., 2000, Steinvil et al., 2010) reveal that an increase in RBC aggregation (under the control of increased fibrinogen levels in the plasma) is a well-known clinical manifestation in acute circulatory failure as well as acute coronary syndromes and stroke (Simmons and Pittet, 2015, Tikhomirova et al., 2011, Ami et al., 2001, Justo et al., 2009, Bolokadze et al., 2006). In agreement, researchers (Baskurt and Meiselman, 2013) reported that the plasma protein fibrinogen attachment to the RBC membrane, affects not only aggregation but also RBC viscosity. This increased viscosity will ultimately cause the RBCs to struggle to pass through narrow capillaries (Baskurt and Meiselman, 2013) hence decreasing blood flow in several clinical states (Irace et al., 2014, Baskurt and Meiselman, 2012, Cairncross et al., 1969, Ju et al., 2013). Hence researchers further established that an increase in blood viscosity and an increased fibrin concentration correlate are strong predictors of cardiovascular diseases and are important factors in the development of atherosclerosis (Baskurt and Meiselman, 2013, Lipinski and Pretorius, 2013, DePalma et al., 2010).

From the above paragraphs, it is clear that aggregation and viscosity are closely associated with a changed fibrinogen profile as well as inflammation, and that these parameters will ultimately influence blood flow and circulation flow dynamics (Dupire et al., 2012). Red blood cells deformability is the ability to change shape, but to return to the original shape this is more helpful when RBCs have to move through small blood vessels. Often in cases of chronic inflammation, the shape of the RBC cannot reform to its discoid shape therefore RBCs aggregate in one section of the blood vessel and causing a disturbance in the flow of blood an increase in the blood viscosity can result (Rogowski et al., 2000, Dupire et al., 2012).

We then see that deformability is an important determinant of blood viscosity (Minetti et al., 2013, Ju et al., 2013, Shin et al., 2007). Therefore deformability of RBCs, while they remain viable, is an important mechanical property of cells (Minetti et al., 2013, Pöschl et al., 2003, Ju et al., 2013).

A reduced deformability is known to be present in conditions such as stroke (Pollock and Harrison, 1982, Tikhomirova et al., 2011) it is linked directly to the RBC membrane properties including stiffness, rigidity and elasticity (Shin et al., 2007, Chang et al., 2007, Johnson et al., 1994, Ju et al., 2013).One of the important properties used to look at membrane stiffness (not only restricted to RBCs) is the Young's modulus, which is a particular measure of stiffness and elasticity (Sokolov et al., 2013, Zhou et al., 2013). This can be measured using atomic force microscopy (AFM) technology. This is a measure of the stiffness of an elastic material (Chiou et al., 2013) and can generally be defined as stress divided by the corresponding strain, with greater values indicating increased stiffness or decreased deformability(Bester et al., 2013a).

I will discuss this more in details later in (chapter 6) when I look closer at the effect of LPS on elasticity of RBCs using AFM. All of the above properties will reflect and be reflected in the dynamics of RBC function and their capability to deform naturally under shear forces. During inflammation, RBCs have a changed shape (losing their biconcave shape), as well as inadequate flow properties, and this is thought to have major influences on the development of cardiovascular disease (AlMomani et al., 2012). In addition to causing the biochemical changes inflammation also moderately affects, and the mechanical properties of RBCs mainly the intrinsic cellular properties of the RBC membrane as well as the interactions of the RBCs with fibrin (Lipinski et al., 2012a, Pretorius et al., 2014a, DePalma et al., 2010). We see, however, in research conducted by (Bellary et al., 1994) that leukocytes and their factors are responsible for the rearrangements seen in the cytoskeletal proteins of the RBCs membrane.

Microparticles activation in systemic inflammatory diseases

Platelets are important in the haemostasis (stopping of blood flow) process. They are involved in the formation of a procoagulant surface (Salem et al., 2015). Two essential steps of the coagulation cascade, the formation of both factor Xa and thrombin, require a catalytic surface on which the enzyme complexes can be assembled. I have reviewed and discussed in detail the formation and function of thrombin. Also we looked at how the effect of inflammation can affect the role of thrombin in the hyper formation of fibrin presented in coagulopathy. If we take a look at factor Xa and its formation, the catalytic surface is provided by the phospholipids of the platelet plasma membrane (Salem et al., 2015). However, in the inactive platelet, the negatively charged phospholipids which are essential to the catalytic properties of the surface, are located in the cytoplasmic leaflet of the membrane.

Dependent on the activator, the normal asymmetric distribution of phospholipids is lost, resulting in the formation of a procoagulant surface (Massberg et al., 2003). Although platelets are primarily bound to exhibit this function, certain pathological conditions can lead to exposure of a procoagulant surface in other cells as well (Kolowos et al., 2005). However to explain the mechanisms of exposure of a procoagulant surface include the role of the cytoskeleton, the formation of micro vesicles from the plasma membrane, as well as the contribution of a membrane protein, which actively transports specific phospholipids from the outer to inner leaflet of the membrane bilayer (McBroom et al., 2006, Sims et al., 1989).

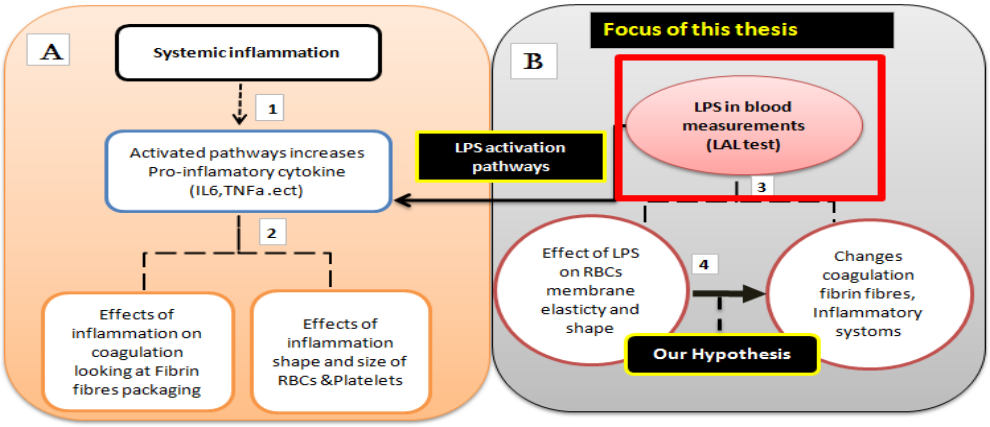
Platelets interact with several coagulation factors, while the coagulation product thrombin is a potent platelet-activating agonist(Palumbo et al., 2005). Activated platelets come in a procoagulant state after a prolonged elevation in cytosolic calcium (Ca^{2+}). These platelets adhere to collagen via glycoprotein VI, expose phosphatidylserine (PS) at their outer surface and produce (PS-exposing) membrane blebs and microvesicles (Salem et al., 2015). Inhibition of aminophospholipid translocase and activation of phospholipid scramblase mediate the exposure of PS, whereas calpain-mediated protein cleavage leads to membrane blebbing and vesiculation. Surface-exposed PS robustly promotes the coagulation process by facilitating the assembly and activation of tenase and prothrombinase complexes (Camus et al., 2015). Factor IXa and platelet-bound factor Va support these activities (Salem et al., 2015). Much to our interest platelets can support the initiation phase of coagulation by providing binding sites for prothrombin and factor XI. They thereby take over the initiating role of tissue factor (TF) and factor VIIa in coagulation activation (Massberg et al., 2003).

Activation of human platelets by complement proteins C5b-9 is accompanied by the release of small plasma membrane vesicles (microparticles) that are highly enriched in binding sites for coagulation factor Va and exhibit prothrombinase activity (Sims et al., 1989). Intracellular Ca^{2+} is increased in the platelets of hypertensive individuals (Heemskerk et al., 1997).

Researchers have demonstrated that platelet plasma membrane Ca^{2+}-ATPase (PMCA) activity inversely correlates with diastolic blood pressure and that inhibition of this Ca^{2+} pump could explain the elevation of cytosolic Ca^{2+} in hypertension (Blankenship et al., 2000). More recently, it was discovered that PMCA is phosphorylated on tyrosine residues during thrombin-stimulated platelet aggregation and that this phosphorylation causes inhibition of PMCA activity.

Results suggest that PMCA in platelets of hypertensive individuals is inhibited because of tyrosine phosphorylation, resulting in increased platelet intracellular Ca^{2+}, hyperactive platelets, and increased risk of heart attack and stroke (Blankenship et al., 2000). Platelet activation and blood coagulation are complementary, mutually dependent processes in haemostasis and thrombosis (Palumbo et al., 2005). Researchers and his team found that it is collagen but not fibrinogen matrix that acts as a potent activator of the procoagulant response through activation of tyrosine kinases and subsequent generation of sustained intracellular Ca^{2+} signals. Their results provided a molecular basis for collagen activation of platelets. Here we see that collagen is the first example of a nonimmune receptor stimulus to signal through a pathway closely related to signalling by immune receptors (Heemskerk et al., 1997). As seen from the above literature review, (SI) affects all cells of the coagulation system as well as fibrin formation, platelets activation and even collagen. One of the key elements, and the focus of this thesis is the role of LPS in causing inflammation and ultimately its effect on blood clotting. In the following paragraphs I will focus on LPS and review methods used by researchers, to get a quantitative measure of LPS in blood.

Testing for Endotoxaemia in blood



Gram-negative infection and the clinical syndrome of sepsis have been difficult to establish, in part because of the limitations of available endotoxin assays (Morrison and Ulevitch, 1978). Researchers have used critically ill patients from medical/surgical intensive care unit (ICU). To measure the amount of LPS present in their WB. On the day of ICU admission they measured endotoxin levels using a novel chemiluminescent assay - the endotoxin activity assay (Al-Attas et al., 2009) it is the chromogenic modification of the limulus amoebocyte lysate assay. They found that endotoxin levels were higher in patients with a diagnosis of sepsis than in patients admitted with a diagnosis other than sepsis.

Endotoxaemia was significantly associated with Gram-negative infection; white blood cell counts of patients with EAA-detected endotoxaemia were significantly higher (Zaman and Zaman, 2015). These correlations were not apparent using the LAL method. These researchers suggested that EAA may be a useful diagnostic tool for the investigation of invasive Gram-negative infection and incipient sepsis. Insight that researchers found on the LAL assay was good enough to render it as inadequate. The concentrations are typically assayed using the (Jiang et al., 2009, Harte et al., 2010a, Su and Ding, 2015).

However, although satisfactory in simple matrices, this test is not considered very reliable in blood, due to inconsistent results, even between controls and patients (Andrä et al., 2004, Ketchum and Novitsky, 2000, Mattsby-Baltzer et al., 1991, Novitsky, 1998) Indeed, because it is so hydrophobic, little or no LPS is actually free (unbound) and so it is not even obvious what its concentration in blood might mean see (Kell and Pretorius, 2015a). However it is essential for us to know in which diseases LPS is most prominently found and what are the concentrations of LPS found. **See Table 2.1** for a full comprehensive list of all diseases that have been associated with LPS and the measured concentrations of LPS found respectively.

Table 2.1: Diseases associated with LPS and quantified LPS concentrations based on ref.(Kell and Pretorius, 2015b)

Disease	LPS in disease	LPS in controls for study	Tissue type	Reference
Acute coronary syndrome	0.61 μg.mL^{-1}	1.87 μg.mL^{-1}	Serum chlamydial lipopolysaccharide (cLPS)	(Tiirola et al., 2007)
Healthy individuals	-	0.15 to 0.35 EU/mL	Plasma	(Ghanim et al., 2009)
Inflammatory bowel disease	12.6 pg.mL^{-1} (5·9−16·2)	12·2 pg.mL^{-1} (3·8−26·3)	Serum	(Funderburg et al., 2013)
Non-alcoholic fatty liver disease	7.8 -14.8 EU/mL	3.2 - 5.2 EU/mL	Serum	(Harte et al., 2010b)
Sepsis	300 pg.mL^{-1}	7.3 pg.mL^{-1}	Plasma	(Opal et al., 1999)
Type 1 diabetes				
Microalbuminuria group	31–60 EU/mL	-	Plasma LAL assay	(Nymark et al., 2009)
Normoalbuminuric group	38–74 EU/mL	-	Plasma LAL assay	
Type 2 diabetes				
Non-obese Postmemoposal Women	-	0.37±0.02 EU/mL	Plasma LAL assay	(Zaman and Zaman, 2015)
Diabetic non-obeseb Postmenoposal women	0.39±0.03 EU/Ml	-	Plasma LAL assay	
Insulin-treated diabetes	6.6–10.7 EU/ml	3.1–5.1 EU/mL	Serum	(Al-Attas et al., 2009)

The prospective sources of blood endotoxin may be derived from compromised leaky mucosal barriers and localized or chronic infections (Glaros et al., 2013). This is known to cause a non-resolving and low-grade inflammatory state (Maitra et al., 2011, Laugerette et al., 2011, Maitra et al., 2012). This may explain the pathogenesis of chronic inflammatory diseases associated with low dose endotoxemia (Glaros et al., 2013). **See Table 2.2** for list and quantified measurement of LPS found in tissues.

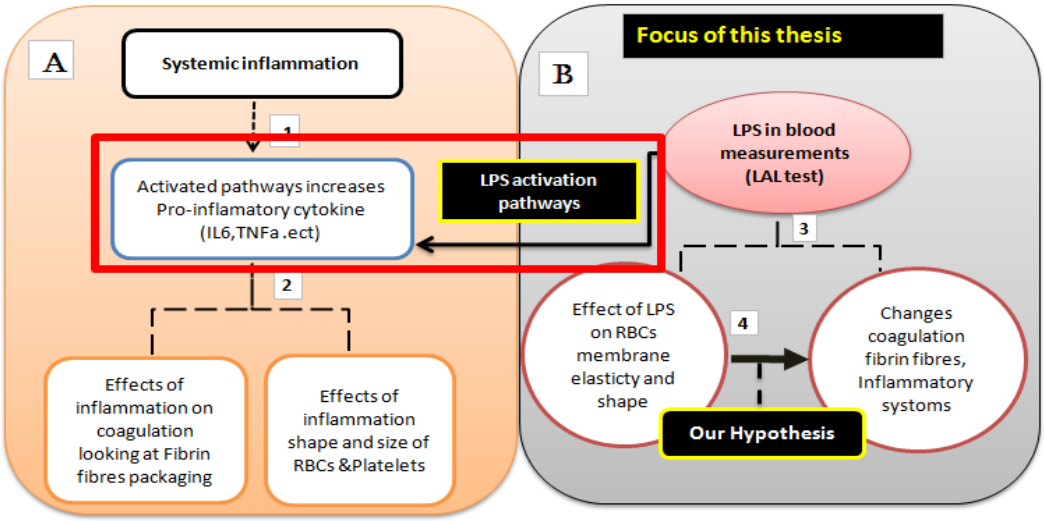
Table 2.2: Quantified concentrations of LPS found in different tissues adjusted from (Kell and Pretorius, 2015b)

Tissue type	(LBP) concentration	(LBP): control	REFERENCE
Bacterial gastrointestinal infections	28.5 ± 16.5 μg.mL^{-1}	-	(Elsing et al., 2011)
Crohn's disease (CD) and ulcerative colitis (UC)	57·11 μg.mL^{-1} (49·4−65·8)	50·01 μg.mL^{-1} (37·1−63·9)	(Funderburg et al., 2013)
Diabetes type 2	19.78 ± 6.40 μg.mL^{1}	20.53 ± 6.99 μg.mL^{-1}	(Zhou et al., 2015)
Endocarditis			
Infectious endocarditis	Median 33.41 mg.L^{-1}	Median 5.61 mg.L^{-1}	(Vollmer et al., 2009)
Noninfectious heart valve diseases	Median 6.67 mg.L^{-1}		
Inflammatory bowel disease	52·7 μg.mL^{-1} (45·4−64·6)	39·1 μg.mL^{-1} (32·1−43·7)	(Funderburg et al., 2013)

Although the quantitative assessment of LPS concentrations in WB can be problematic, its presence may have important and clinically relevant effects on the blood microenvironment, and may be central in the treatment of inflammatory conditions (Kell and Pretorius, 2015a, Potgieter et al., 2015b, Kell et al., 2015, de Punder and Pruimboom, 2015, Latta et al.).

I have reviewed prospective sources of blood endotoxin and localization of the LPS in blood and tissues. In the next paragraphs I will specifically focus on LPS and its role in pro-inflammatory cytokine production.

Lipopolysaccharide activating pro-inflammatory cytokines



Gram-negative bacteria endotoxin LPS is known to induce inflammatory cells, to express a number of proinflammatory cytokines such as IL-8, IL-6, IL-1β, IL-1, IL-12, and IFNγ (Ramachandran, 2014b, Tracey and Lowry, 1990, Beutler, 1993) .

In addition researchers have also included TNFα as it also plays a role during endotoxic shock and causes tissue damage (Ramachandran, 2014b, Beutler et al., 1985). Lipopolysaccharides in combination with TNFα, are known to induces apoptosis of the endothelium layer in several tissues including intestine, lungs, and thymus (Haimovitz-Friedman et al., 1997a). We see that LPS causes activation of pro-inflammatory cytokine with evidence provided, researchers observed that when LPS was injected in human volunteers, it would induced a cytokine response characteristic of inflammation disorder (Haimovitz-Friedman et al., 1997b, Ramachandran, 2014a). After an early and transient induction of the proinflammatory cytokines TNF- and IFN-, the acute-phase response was boosted by a dramatic increase in IL-6, which peaked at 3 to 4 hours after LPS injection (Verstrepen et al., 2008).

Through research LPS is recognised as a major pathogen associated molecular pattern (PAMP) that triggers the body's innate immune response to pathogens (Li et al., 2016, Zhang et al., 2016). The host's cells release damage-associated molecular pattern molecules (DAMPs) as signals that alert the innate immune system to unanticipated cell death and to microbial invasion (Li et al., 2016).

Due to growing technology and research we now known that the principal route by which LPS excites an inflammatory innate immune response is by binding to the toll-like receptor 4 (TLR4) these are single membrane spanning non catalytic receptors usually expressed macrophages or dendritic cells that recognise structurally conserved molecules derived from bacteria (Poltorak et al., 1998, Beutler et al., 2001, Park et al., 2009, O'Neill, 2014). Typically, the LPS is bound in blood to LBP, and the LPS is taken to the TLR4 receptor via a CD14 co-receptor. The CD14 co-receptor also links innate immunity with Alzheimer's disease (AD) (Reed-Geaghan et al., 2009, Heppner et al., 2015) as we had discussed in our introduction. This binding of LPS to TLR4 in turn activates the production of a variety of pro-inflammatory cytokines (Verstrepen et al., 2008, Tikhomirova et al., 2011, O'Neill, 2014). The extent of cytokine activation reflects in part the strength of binding to CD14/TLR4 (Tsutsumi-Ishii et al., 2008).This means the stronger the binding of LPS to CD14/TLR4 the stronger the more exciting and activation of cytokines.

These inflammatory cytokines are induced via a set of pathways illustrated in **Fig. 2.5**, with the transcription factor NF-kB playing a prominent role (Tak et al., 2001, Noort et al., 2015). It may be known that, NF-kB is normally held inactive in the cytoplasm by being bound to an inhibitor IkB protein, and the means by which extracellular signals such as LPS are transduced involve a series of kinases, one of which (Tiirola et al., 2007) in particular phosphorylates the IkB and thereby releases the NF-kB that can translocate to the blood to LBP, and the LPS is ferried to the TLR4 receptor via a CD14 co-receptor (Reed-Geaghan et al., 2009, Heppner et al., 2015, Liu et al., 2005).

Research has shown that NF-kB is normally held inactive in the cytoplasm by being bound to an inhibitor IkB protein, and the means by which extracellular signals such as LPS are transduced involve a series of kinases, one of which (Tiirola et al., 2007) in particular phosphorylates the IkB and thereby releasing the NF-kB that can translocate to the nucleus to turn on a large variety of other genes, including TNF-a and IL-6 (Kellum et al., 2007). **See Figure 2.5** for an overview of these inflammatory cytokines activities in the inflammatory pathway at low concentrations of LPS. There is also a 'non-canonical' inflammasome LPS activation pathway which is independent of TLR4 (Hagar et al., 2013, Kayagaki et al., 2002, Shi et al., 2014) that occurs at higher external concentrations of LPS. However we will not be looking into this pathway as we are only using small concentrations of LPS showing the effects of LPS at physiological concentrations.

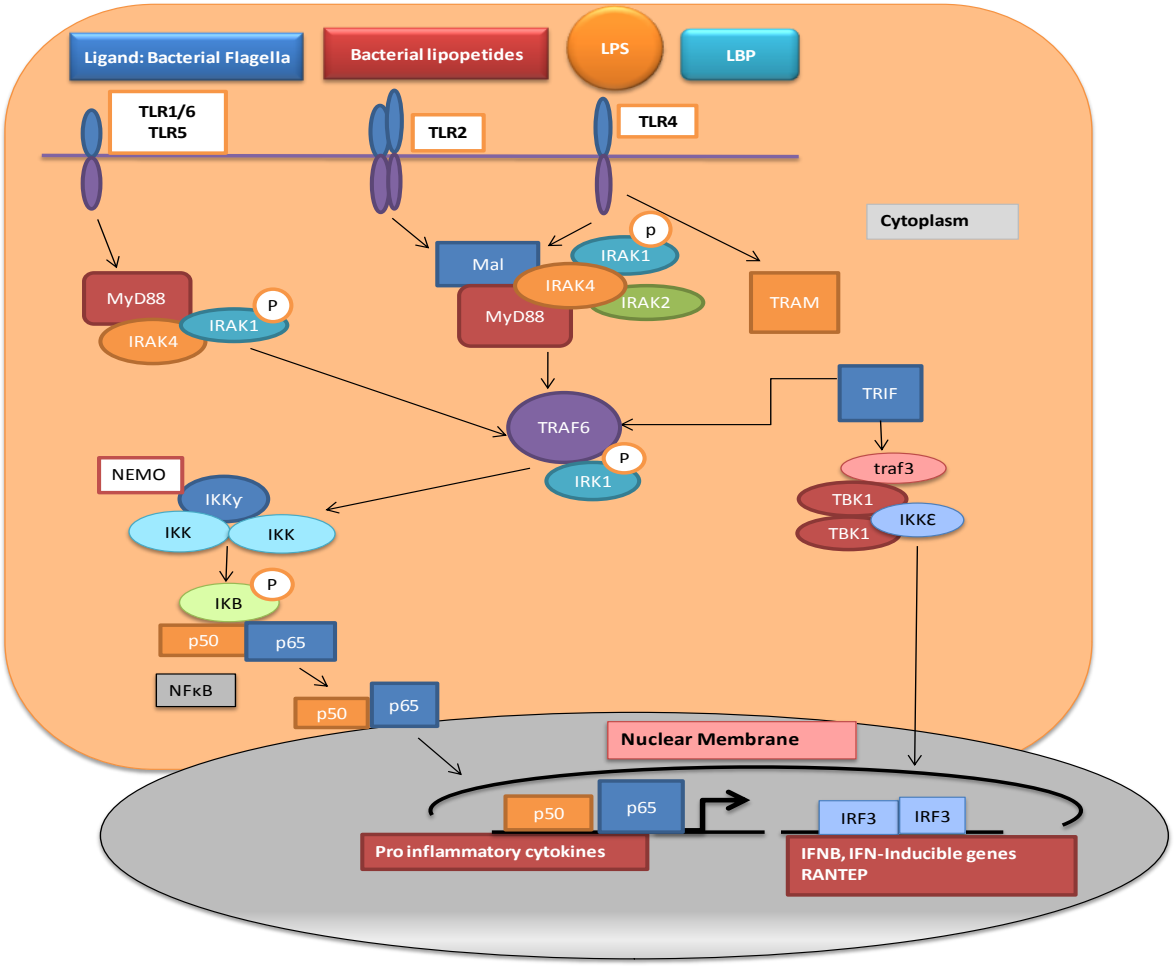
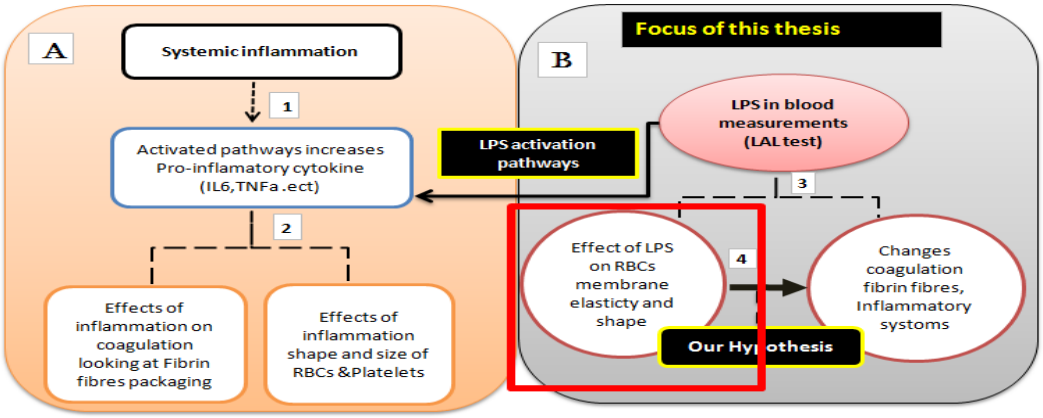


Figure 2.5: Inflammatory cytokines activated by LPS adapted from (Kell and Pretorius, 2015b)

In the following paragraphs I will revolve with the aid of literature, the existence of LPS in blood and how it might bind to RBCs, keeping in mind that RBCs do NOT have the regular LPS binding receptors present on other cells.

Effect of LPS on RBCs may Propagate Inflammation



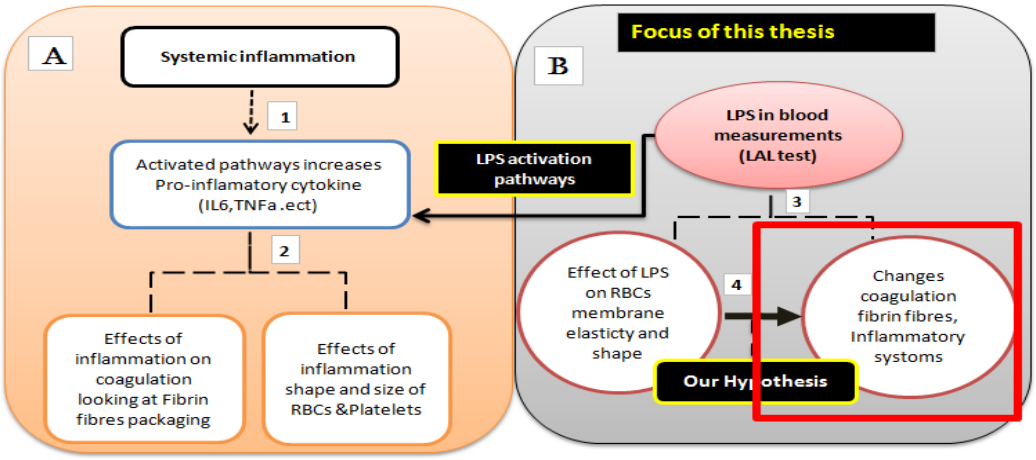
As we have seen from previous paragraphs the biologic availability of LPS is regulated by a number of serum proteins, including LBP, bactericidal/permeability-increasing protein and soluble CD14 (sCD14) which attaches to TLR4 and activates cytokines (Juffermans et al., 1998, Goldblum et al., 1994). Through research, scientists found that LBP in blood facilitates the binding of LPS to CD14, a glycoprotein which is expressed only on monocytes and neutrophils **BUT NOT ON RBCs**. This glycoprotein CD14 is known to be essential for the induction of an inflammatory response to LPS (Viriyakosol and Kirkland, 1995, Czerkies et al., 2013). It is believed that the carbohydrate portion of LPS binds to the CD14 protein (Pugin et al., 1994, Heumann et al., 2001). This is due to the glycoprotein CD14 structure which has hydrophilic regions that are located outside the N-terminal pocket that is in close proximity to the carbohydrate region of LPS (Kim et al., 2005, Liu et al., 2005). Researchers (Zanoni and Granucci, 2013) found that the flexible CD14 structure could explain why it is capable of binding different LPS species with similar affinity.

Although RBCs do not have the CD14 binding sites, it is believed that LPS DOES bind to the cell, but it is suspected that binding of LPS to RBCs membrane correlates with the presence of hydrophobic nature of both the LPS molecule, as well as the RBC membrane (Peters et al., 2009). Hydrophic LPS can potentially interact with lipoproteins, and LPS also has the ability to convert prions to their more toxic PrPSc form (De Castro et al., 2010). However, lipid A and LPS binding to RBC membranes was studied by measuring the amide-linked hydroxymyristic acid by gas chromatography. The detection rates of hydroxymyristic acid were 82% for LPS and 79% for lipid A in buffer solution. In membranes of washed RBCs, the detection rates of LPS and lipid A were 0.03% and 0.5% and in RBC membranes of WB the detection rates were 2.6%. This proves that LPS does bind to WB and perhaps RBCs. The decrease in RBCs deformability was related to the amount of hydroxymyristic acid measured in RBCs membranes, suggesting that endotoxins binding directly affects mechanical properties of RBCs (Pöschl et al., 2003).

Researchers (Camus et al., 2015) established that MPs from RBCs also carry heme and these heme-laden MPs are known to have a physiopathological impact on the rest of the haematological system. These types of MPs from RBCs are known to frequently elicit an immune response (Camus et al., 2015). However Kell and his co-workers in their review stated that MP may occur under conditions that lead to chronic inflammatory diseases that are normally considered to lack a microbial component (Kell and Pretorius, 2015b).

Inflammation and LPS is associated with the pathogenesis of severe chronic diseases such as atherosclerosis, diabetes, and aging-related neurological diseases, as a key culprit in provoking a non-resolving low-grade inflammation (Glaros et al., 2013). The term "low-grade" or "metabolic" endotoxemia is used to reflect an important clinical phenomenon of sub-clinically elevated levels of circulating blood endotoxin (Terawaki et al., 2010, Moreno-Navarrete et al., 2010). This has also been found to be true in chronic inflammatory diseases such as atherosclerosis and diabetes (Cani et al., 2008, Wiedermann et al., 1999). In the following paragraphs I will review how LPS plays a role altering coagulation, in comparison to some key factors altered in coagulation as seen in systemic inflammation.

LPS stimulates coagulation and formation of thrombosis



The hallmark of many chronic, inflammatory diseases is the fact that they simultaneously exhibit both hypercoagulability and hypofibrinolysis (Kell and Pretorius, 2015d). While great many biochemicals can influence both the kinetics and the end product structures of the clotting process, unliganded (unbounded) iron and the fibrin concentration itself (Kell and Pretorius, 2014a, Pretorius et al., 2014a, Pretorius, 2013b).

Through all the articles and books published by researchers, it is evidence that LPS is a strong procoagulant (Wu et al., 2014, Simmons and Pittet, 2015).The mechanisms are direct some mechanisms are still not yet established, although certainly it is known that LPS can bind to RBCs membranes from our previous paragraph (Bellary et al., 1995a, Pöschl et al., 2003) resulting in changes on the RBCs membrane. Due to the involvement of LPS in inflammation, it is suspected that it may be involved in a changed coagulation profile (Chaby, 2004).

When one investigates further the machinism of LPS, one comes across research conducted by Branger and team where they intravenous injected LPS and studied the changes. Their results showed that LPS is associated with activation of the coagulation system, which was seen by a rise in the plasma concentrations of the prothrombin fragment (Branger et al., 2003). Pretorius and coworkers have shown in numerous articles that inflammation affects the coagulation profile, the RBC shape and fibrin formation (Buys et al., 2013, Kell and Pretorius, 2014b, Kell and Pretorius, 2015e, Pretorius et al., 2014b, Pretorius and Kell, 2014, Pretorius and Lipinski, 2013b, Pretorius et al., 2014c, Pretorius et al., 2014d, Pretorius et al., 2014e, Pretorius et al., 2013d) A characteristic of systemic inflammation is a hypercoagulatory state it is known as a thrombotic state and is the common pathology underlying all thrombotic conditions including ischemic heart disease, ischemic stroke, and venous thromboembolism (Raskob et al., 2014). Furthermore, a hypercoagulable state is typically associated with pathological changes in the concentrations of fibrin(Nielsen) (Farrell, 2012, Danesh et al., 2005, Alexander et al., 2011), and particularly an increased D-dimer level (Halaby et al.) is seen as a reliable biomarker for cardiovascular risk (Tonkin et al., 2015, Halaby et al., 2015, Hou et al., 2012). **See Figure 2.6** that shows a flow diagram displaying the effects of LPS on coagulation, when it is present in blood. The risk of one to develop cardio vascular diseases and coagulation (formation of thrombin) due to increased levels of LPS.

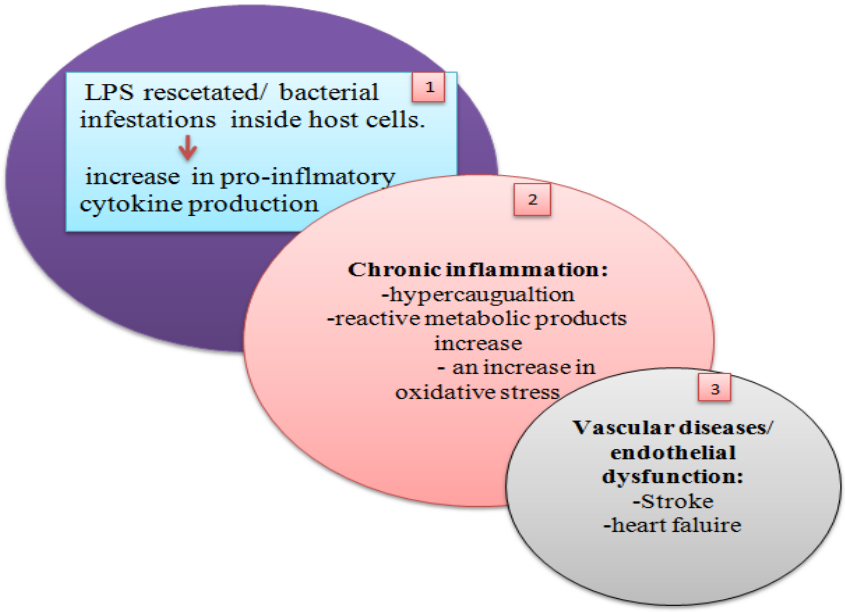


Figure 2.6: Flow diagram from when there is presence of LPS in blood. 1. Up-regulation of cytokines production. **2.** Chronic inflammatory disorders. **3.** Vascular diseases results.

We have seen that LPS has cytokine-dependent effects, the question then arises as to whether LPS can cause hypercoagulation by acting on the coagulation pathway more directly. One route is via tissue factor (TF) upregulation - TF is related to the cytokine receptor class II family, and is active early in the (extrinsic) coagulation cascade, where it is necessary to initiate thrombin formation from prothrombin (Rao and Pendurthi, 2005, Monroe and Key, 2007). Tissue factor is also known to be the most important activator of coagulation (Mackman, 2009), as its upregulation plays a central role in driving a thrombosis-inflammation circuit including hypercoagulability (Chu, 2006b, Chu, 2006a). This is known as coagulation-dependent inflammation (Strukova, 2006). Through research it was shown that 100 ng.mL^{-1} LPS added to healthy cord whole blood (WB) of newborns and the WB of healthy adults, induced TF-mediated activation of hemostasis (Koch et al., 2009). Lipopolysaccharide from *E.coli* (100 ng.mL^{-1}) also activated the coagulation system when added to WB, via a complement and CD14-dependent up-regulation of TF, leading to prothrombin activation and hypercoagulation (Landsem et al., 2013, Landsem et al., 2015b). Research conducted by Landsem and colleagues also revealed that WB incubated with LPS and *E. coli* enhanced TF surface expression on monocytes (Landsem et al., 2013) this resulted in the increased of TF expression, thus hypercoagulation followed.

However this was noted after 2 hours of the incubation, therefore it was not an acute process (Landsem et al., 2015b). Both the complement system *i.e* part of the immune system that enhances opsonization and the ability of phagocytic cells to clear pathogens from an organism, and TF play a key initiating role in coagulation and are activated in sepsis (Landsem et al., 2013, Lin et al., 2010). The complement system is also activated efficiently by whole Gram-negative bacteria, whereas pure LPS can activate the complement system only at high concentrations (Brekke et al., 2007, Landsem et al., 2013). However, in addition to changes in TF expression by LPS, the process may also involve the direct binding of the lipophilic LPS (Calabrese et al., 2015, Maeshima and Fernandez, 2013) to circulating plasma proteins, particularly fibrinogen, and this rapid binding is also known to cause pathological changes in the coagulation process(Lin et al., 2010). This would be independent of the slower TF activation, and thus an acute and relatively immediate process. Here we see direct and indirect pathways that LPS can affect the coagulation process. Thus, to summarize, **see Figure 2.7.** For the possible actions of LPS on hypercoagulation.

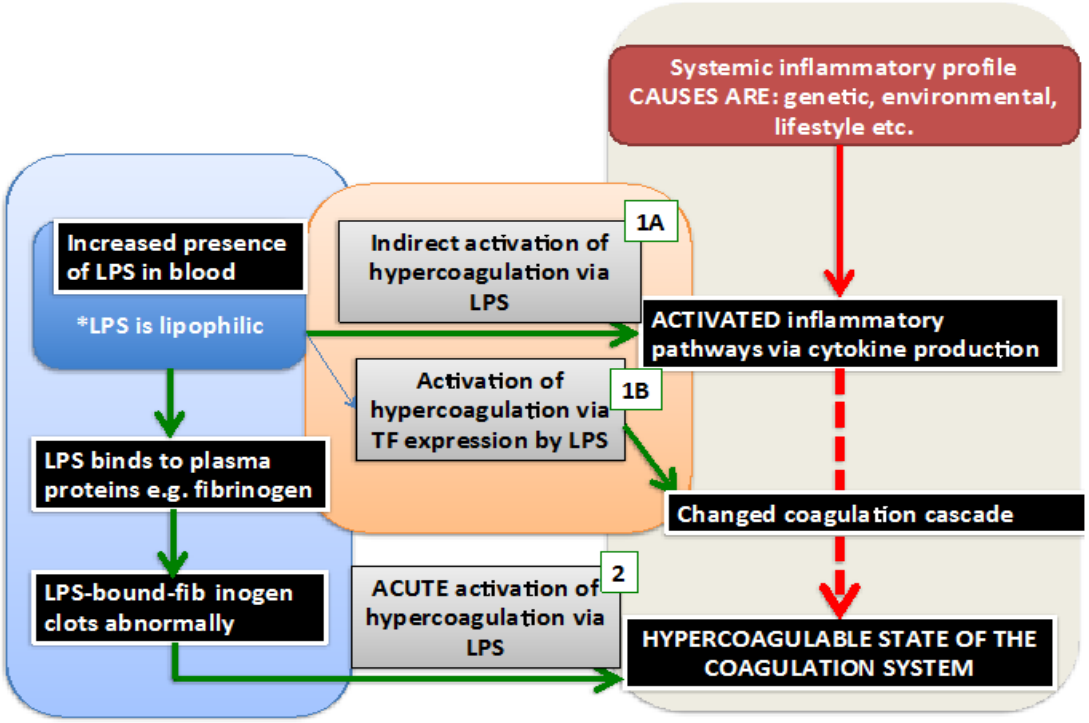


Figure 2.7: The effect of LPS on coagulability (1A) Indirect activation of hypercoagulation by the LPS **(1B)** reflect the direct activation of coagulation via LPS affecting TF direct approach **(2)** Acute activation of hypercaugulation can occur when the LPS binds to fibrinogen (plasma protein) adapted from (Pretorius 2016 Submitted Science).

The role of LPS in activating microparticles

There is very little literature available about the role that LPS plays in platelets activation. However, we acknowledge Morisson for his attempt to understand the role LPS plays in platelets activating factors (Morrison and Ulevitch, 1978). They found that LPS or TNF individually can directly activate Polymorphonuclear leukocytes (PMNs) and promote adhesion between PMNs and endothelial cells (Morrison and Ulevitch, 1978), thus enhancing the leukopenic effect of platelets activating factor (PAF). On the other hand in a study conducted by Sun, X and colleagues they showed that TNF caused PAF production in bowel tissue TNF and LPS are synergistic in inducing bowel necrosis they results also revealed that TNF-induced bowel necrosis is due to PAF release (Sun and Hsueh, 1988). However literature also reveals that TLR agonists LPS and Pam_3CSK_4 have no direct effect on platelet activation and that platelet TLRs may be a remnant from megakaryocytes.

They found that TLR2 and TLR4 agonists have a significant role in diseases such as atherosclerosis and DIC, but research suggests that this is through a mechanism other than direct platelet activation or by modification of platelet responses to other agonists (Ward et al., 2005). Therefore LPS indirectly binds to TLR4, thus causing inflammation. Since we have seen that LPS does not necessarily activate platelets inflammation directly, I will review pathways that involve general microparticles (MP) activating inflammation a more indirect pathway. Microparticles in general are very small vesicles released from the membrane of a cell into the circulation. They are immunologically unique to their cells origin and may play a role in antigen presentation, they are known to be present in many inflammatory diseases (Badran et al., 2015, Camus et al., 2015, Kolowos et al., 2005, Salem et al., 2015).

Various cells such as platelets, lymphocytes, endothelial cells, RBCs and monocytes do release surface derived MP (Kolowos et al., 2005) and these MP are seen as multi-purpose carriers (Kolowos et al., 2005, Salem et al., 2015). Microparticles generally carry proteins, lipids and nucleic acids and play a fundamental role in the pathogenesis of thrombosis and are known to modulate the properties of target cells (Salem et al., 2015). These tiny vesicles might develop via an external or internal stimulus on cells (e.g. RBCs and platelets), in Kell and Pretorius review they suggested that the presence of LPS might cause MP formation (Kell and Pretorius, 2015b).

While pure lipid systems are not always good membrane mimics (Kell and Pretorius, 2015b) LPS has been shown to insert spontaneously into lipid bilayers, and this insertion can lead to membrane breakdown, insertion of LPS into model membranes confirmed the preference for sphimgomyelin/cholesterol-containingsystems(Ciesielski et al., 2012, Ciesielski et al., 2013). Such MP formation is prominently associated with inflammatory conditions, MPs also can be associated with proinflammatory effects and autoimmune processes(Niccolai et al., 2015) . Indirectly the MP carrying heme may thus be a source of oxidant stress for the endothelium, linking haemolysis to vascular injury (Camus et al., 2015, Kolowos et al., 2005). This pathway could provide new targets for the therapeutic defence of vascular function in Sickle cell disease (Camus et al., 2015).

Concluding remarks

Diagram (**Figure 2.8**) provides a summary of the literature reviewed it explains how LPS may cause some of the changes seen in chronic (systemic) inflammation. From literature it was shown how the cells of the haematological system and fibrin formation might be involved and changed to a pathophysiological state due to the presence of LPS.

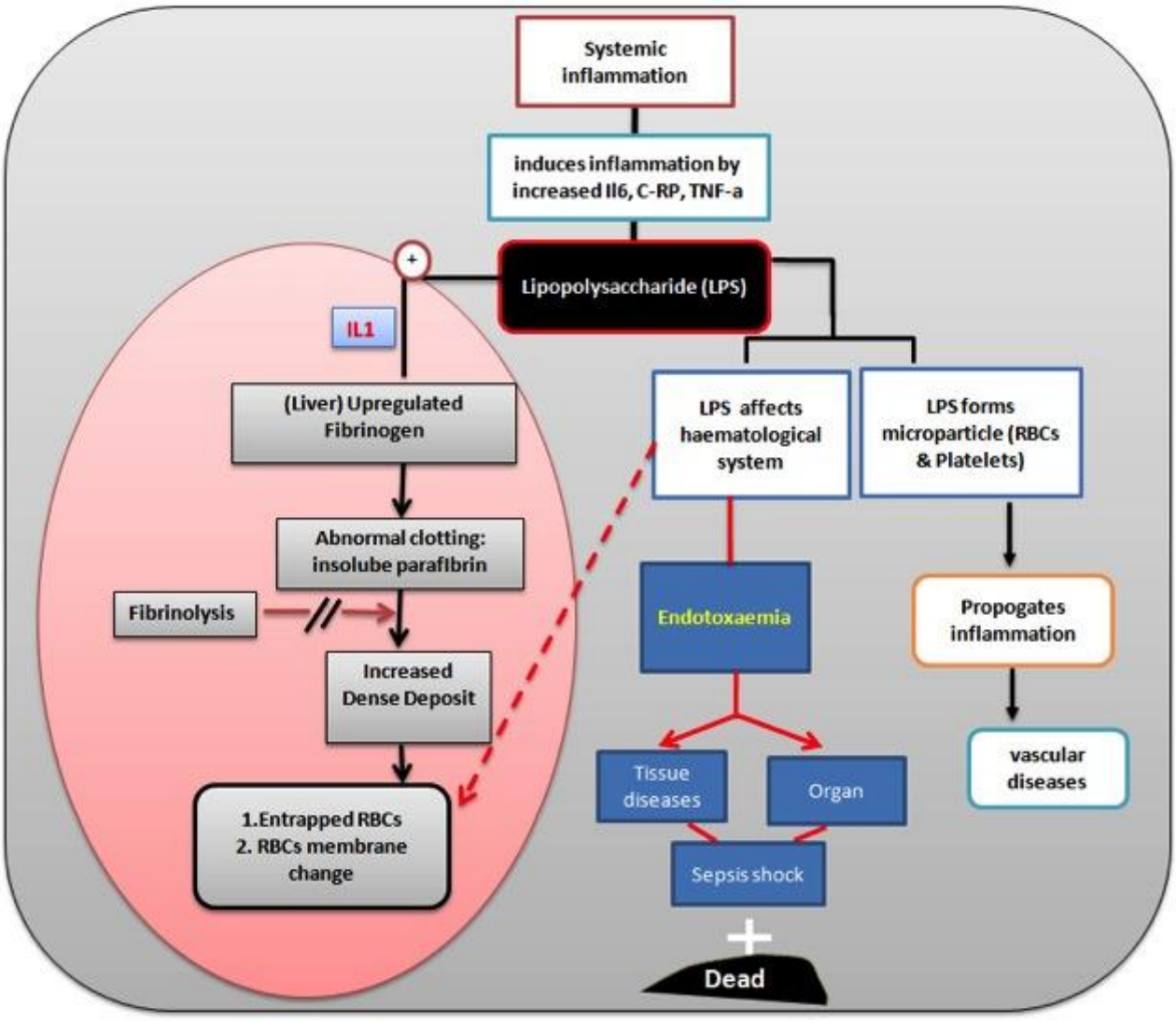


Figure 2.8: Literature over view

The following chapters will focus more specifically on the effect of added LPS to RBCs and fibrin formation to try to correlate the changes we see in inflammatory conditions where LPS presence (and therefore ultimately the presence of a bacterial blood microbiome component) may be linked to the inflammatory changes seen in the coagualtion system of these conditions (**See Table 2.1**).

CHAPTER 3: Choice of study population, sample size and tests to confirm the healthiness of the population

3.1. Chapter Objectives

In this chapter I will discuss recruitment and selection of the study population. For this study 30 healthy individuals were chosen who were between the ages of 18 to 65. As a measurement of healthiness, we used the full iron profile (we measured from AMPATH Free iron, transferring, percentage saturation and ferritin levels from all 30 individuals) as it is known that particularly serum ferritin (SF) is a pro-inflammatory marker, and high values are indicative of systemic inflammation (Kell, 2009) See **(Figure 3.1)** diagram for influence of iron on inflammation, an increase in free iron levels can lead to oxidative stress.

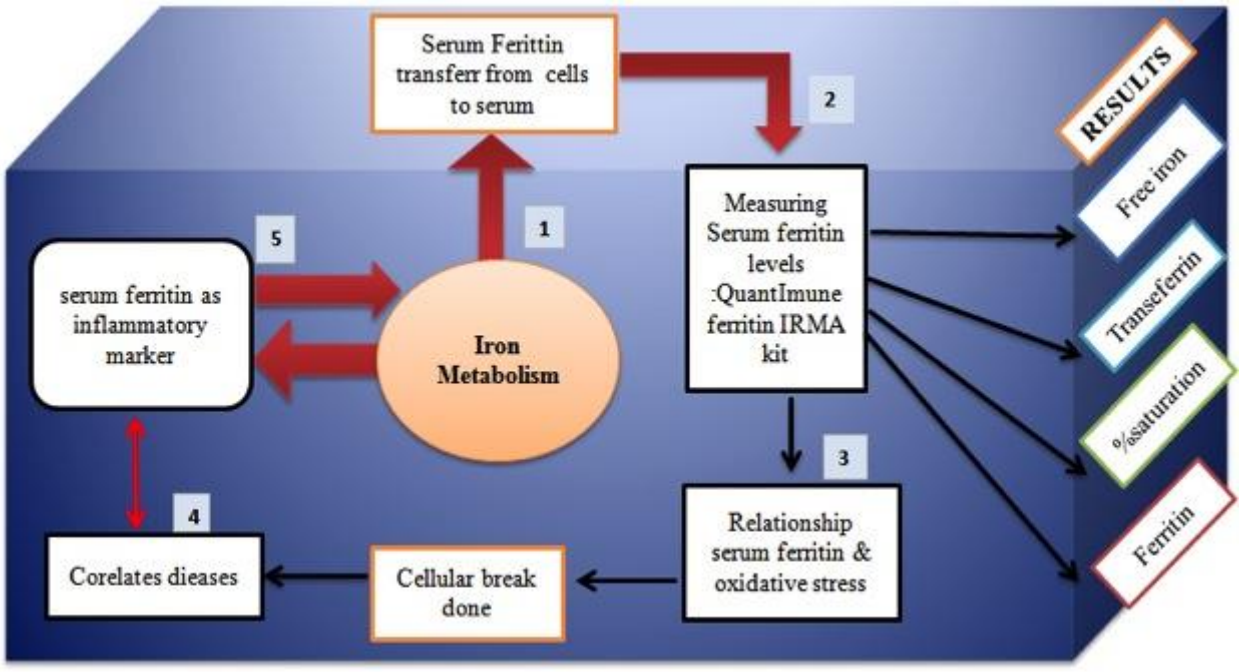


Figure 3.1: A diagram representing the systems biology of iron metabolism (**1**) the relationship between SF and body iron stores and (**2**) its measurement, the relationship between SF and (**3**) markers of oxidative stress and (**4**) disease, and finally (**5**) ferritin is transferred from cells to serum mainly via cell damage and leakage rather than by regulated secretion. Finally results will be from the four measurements of iron.

3.2. Introductory and literature review on Serum ferritin

The amount of ferritin in the blood serum ferritin (SF) is directly related to the amount of iron stored in the body. Scientist and researchers have studied the role of ferritin in the body and noticed a correlation between the levels of inflammation and increased SF levels (Bester et al., 2013a). To this day SF is well known as an inflammatory marker, many researchers have reported that SF arises from damaged cells. They go on to say it is a marker of damaged cells (Andrews, 2008). The protein present in SF is considered benign, but it has lost most of its normal complement of iron which when unliganded is highly toxic *i.e.* free iron levels leading to inflammatory diseases (Kell, 2009) **see Figure 3.1**. Iron is absorbed in the intestine as ferrous ions and transported in the serum bound (in the ferric form) to transferrin, where it can enter peripheral tissues via suitable receptors, being re-reduced in the process. Ferrous iron is incorporated into ferritin, simultaneously being oxidised at a di-iron centre (Ebrahimi et al., 2012) to ferric iron. Hence ferritin is made in cells this includes intestinal cells, and not in serum as most people confused. The ferritin molecule has two regions known which are known as the H and L form these are structurally compatible (Kell and Pretorius, 2014a). The facts that serum ferritin levels can correlate with presence and progressions of numerous diseases and with body iron stores, it is thus expected to participate on a number of chemical grounds such as the correlation that is seen and exists between SF with biomarkers of cell damage; biomarkers of HR formation and oxidative stress and lastly the studies revealed the existing correlation between (Pretorius et al., 2013c).

While iron is necessary in every metabolising tissue, a substantial amount of iron is held in the liver, these are known as liver iron stores. Customarily liver iron stores were measured in a biopsy, although this is not something that can be done with any frequency. Fortunately non-invasive measurement and imaging methods, such as neutron stimulated emission controlled tomography (Agasthya et al., 2012), SQUIDbiosusceptometry (Nielsen et al., 2000, Nielsen et al., 2002) and MRI (Dereure et al., 2008, Musallam et al., 2011), also are widely used for brain imaging (Zheng et al., 2013), and are among the few that are used more often. In some instances where there is no inflammation or if a specific iron-related disease state known, the liver iron content can show a relationship with serum ferritin (Kim et al., 2001, Olthof et al., 2007), although the correlation may be poor (Nielsen et al., 2000, Tony et al., 2012, Jensen et al., 1995).

As it is evidently provided that there is a correlation between serum ferritin and inflammatory disorders but in some instances the correlation is weak hence, scientist found something great to indicate and mark inflammation, Serum ('soluble') transferrin receptor (sTfR) (Punnonen et al., 1997). Transferrin is an iron binding blood plasma glycoprotein that controls the level of free ion in biological fluids (Crichton, 1987). It was established that sTfR can be used to distinguish anaemia of chronic disease from iron-deficiency anaemia (Punnonen et al., 1997, Cook, 2005) due it role during inflammatory response, today the sTfR Index is widely used as a prerequisite for inflammatory test and it is measured in $mg.L^{-1}$ (**see Table 3.1**). Serum transferring receptor is now considered to provide an estimate of body iron over a wide range of normal and depleted iron stores (Skikne et al., 2011).

It remains to be determined whether the lack of correlation between the urinary hepcidin and serum prohepcidin measurements is due to technical limitations of serum assays or if serum prohepcidin concentration does not reflect inflammation or iron metabolism changes. (Hower et al., 2009). The diagram **see Figure 3.2** serves to illustrate why there tend to be correlations between the amount of ferritin in cells, the rate of ferritin excretion by cell damage, involving liberation of unliganded iron posing harm and iron overload disorders, and the levels of serum ferritin. The serum ferritin correlates with disease but the cause is iron, with which it too can correlate. As with any systems biology network, multiple differences in dissimilar elements of the network can lead to the same overall effects, explaining the lack of a perfect correlation with any individual process.

Thus a first order rate of efflux of ferritin is the product of (and thus contains contributions from) both the internal ferritin concentration and the rate constant for efflux, which may vary independently. **See Figure 3.2** were it indicates the production of peroxide and superoxide is an inevitable consequence of aerobic metabolism, and while these particular 'reactive oxygen species' (ROSs) can exhibit a number of biological effects, they are not of themselves excessively reactive and thus they are not especially damaging at physiological concentrations (Ebrahimi et al., 2012). However, their reactions with poorly liganded iron species can lead to the catalytic production of the very reactive and dangerous HR, which is exceptionally damaging, and a major cause of chronic inflammation (Lipinski and Pretorius, 2012a).

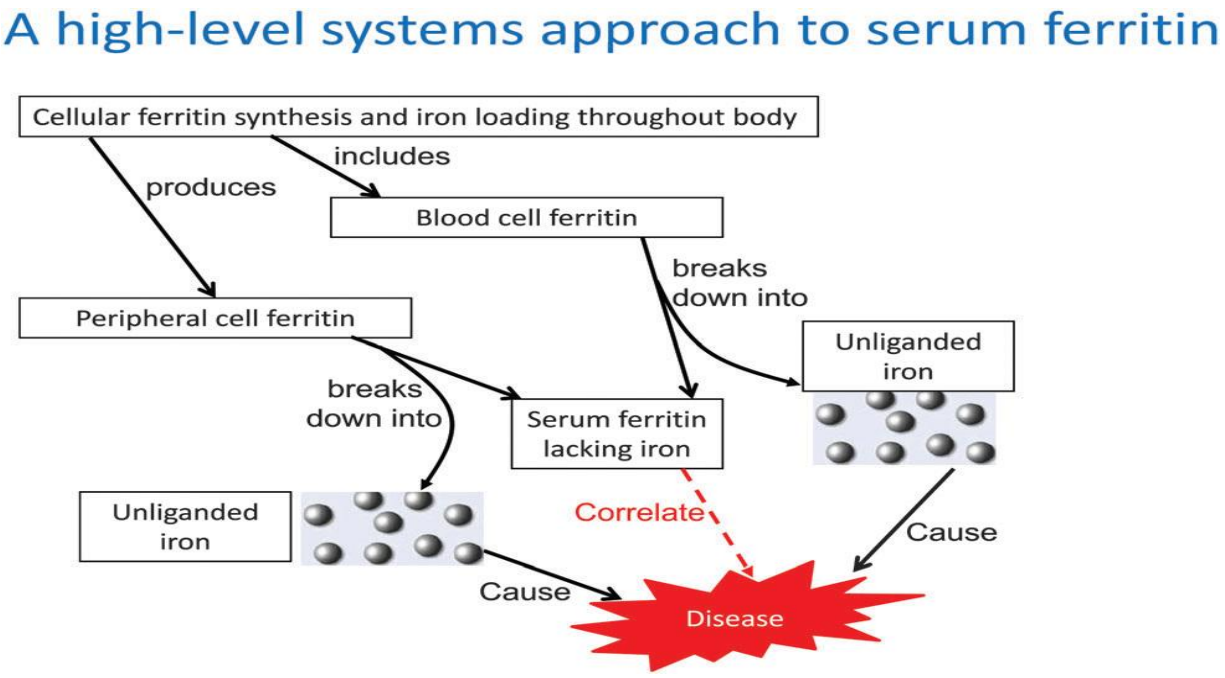


Figure 3.2: A high-level systems approach to serum ferritin. Diagram shows the correlations between the amount of ferritin in cells , the rate of its excretion by cell damage involving the liberation of unliganded iron and the levels of serum ferritin this was adapted from (Kell and Pretorius, 2014a).

Researchers have provided evidence that iron can be found at the sites of plaques and lesion showing the significance of iron to aging and longevity (Sezgi et al., 2014, Olthof et al., 2007, Zheng et al., 2013). The effective chelation of iron by natural or synthetic ligands is thus of major physiological and potentially therapeutic importance (Pretorius et al., 2013c). This explains the decidedly mixed effects of antioxidants that have been observed, since in some circumstances (especially the presence of poorly liganded iron) molecules that are nominally antioxidants can actually act as pro-oxidants. Therefore we see that understanding the exact speciation and liganding of iron in all its states is thus crucial to separating its various pro- and anti-inflammatory activities. Redox stress, innate immunity and pro- (and some anti-)inflammatory cytokines are linked in particular via signalling pathways involving NF-kappaB and p38, with the oxidative roles of iron here seemingly involved upstream of the IkappaB kinase (Tiirola et al.) reaction. In a number of cases it is possible to identify mechanisms by which ROSs and poorly liganded iron act synergistically and autocatalytically, leading to reactions that are hard to control reaction.

Lipinski and Pretorius did iron experiments that described show for the first time that ferric ions (Fe^{3+}) can generate hydroxyl radicals (HR) without participation of any redox agent, thus making it a special case of the Fenton reaction (Lipinski and Pretorius, 2012a).

Some molecules such as statins and erythropoietin, not traditionally associated with anti-inflammatory activity, do indeed have 'pleiotropic' anti-inflammatory effects that may be of benefit in these instances (Kell, 2009). Similiarly the effects of free iron was research further by Pretorius and colleagues, they suggested that the increase or rise of the HH and HF RBCs is caused, partly by unliganded iron (Pretorius et al., 2014a).

As it is evidently provided that iron plays a crucial role in the progression of inflammatory diseases, control subjects for this study had to be selected precisely under strict exclusion and inclusion criteria, I will briefly discuss subject recruitment in the following paragraph.

3.2. Sample size and collection

Together with the statistician, it was determined that at least 30 healthy individuals must be recruited for the study –see Appendix 2. A total of three blood tubes (a citrate tube, EDTA tube for hematology studies and iron tube for iron studies at AMPATH) were drawn from each control. The blood was drawn by a qualified phlebotomist and a medical practitioner. For the study to commence we obtain ethical clearance from the Human Ethics Committee from, Faculty of Health Sciences, University of Pretoria (Protocol number 2013/2015) see Appendix 3. All participants filled informed consent forms see Appendix 1(consent form leaflet), that gives us their consent to use them for the study. Blood was drawn in a good healthy clean environment. The participants were allowed to leave the study at any time they wished to, the main aim was to collect blood and make people feel at ease. For a direct comparative study we used each individual as their own control before and after LPS addition.

The tubes drawn for iron level analysis were tested and analysed at AMPATH laboratory. These results were used to ensure that our controls were indeed healthy, and that there are no underlying inflammatory diseases typically the serum ferritin, as high serum ferritin and low transferrin levels are acute phase inflammatory protein markers (Ritchie et al., 1999) and indicative of inflammation.It is known that iron levels if raised that could be an indication of inflammation see (Kell and Pretorius, 2014b, Pretorius and Kell, 2014).

3.2.1. The exclusion criteria were:

- Non smokers
- No chronic medication
- No known inflammatory chronic diseases (asthma, Tuberculosis, Human immunodeficiency virus)
- Females who were on hormonal contraceptives

3.2.2. Inclusion criteria were:

- Must be in or between the ages of 18-65 years old
- All races and genders were accepted

3.3. Serum ferritin, iron levels of volunteers participating in the study

Blood obtained from each participant was drawn via venepuncture into a tube and taken to Ampath laboratories for iron parameters tests. These are indicators of inflammation chronic present in individuals. The iron parameters that were tested were:

- Free iron
- Transferrin
- Percentage saturation
- Ferritin levels

3.3. Assays used to measure iron parameters

Serum ferritin is measured by using the Bio-Rad Laboratories' QuantImune ferritin IRMA kit which is a single-incubation two-site immunoradiometric assay. In this IRMA, which measures the most basic isoferritin, the highly purified I-labelled antibody to ferritin is the tracer and the ferritin antibodies are immobilized on polyacrylamide beads as the solid phase. Serum is mixed with the combined tracer/solid-phase antibody reagent, and the mixture is incubated. During incubation, both the immobilized and the I-labelled antibody bind to the ferritin antigen in the serum, thus creating a 'sandwich'.

After incubation, the beads are diluted with saline, centrifuged and decanted. The level of I-labeled ferritin found in the pellets is measured by using a gamma counter. There is a direct relationship between the radioactive levels of the pellets and the amount of endogenous ferritin in the serum, rather than the inverse relationship measured by most radio-immuno-assays (RIAs). **Table 3.1** reveals the iron parameters standard ranges used to assess the iron measurements obtained from each individual. I then plotted graphs according to the results and included limits used to visually show number of our volunteers that were within the ranges of all parameters see **Figures 3.3** which display iron test results that we obtained from AMPATH.

Table 3.1: Upper and lower limits for each iron parameter measured

Iron Parameters Ranges			
Free iron	**Transferrin**	**% saturation**	**Ferritin**
11.6 - 31.3 $\mu mol.L^{-1}$	2.2 - 3.7$g.L^{-1}$	20 - 50%	20 - 250$ng.ml^{-1}$

3.4. Results

Results were obtained from AMPATH laboratories the results from each participating individual are displayed in **Table 3.2**. Four iron parameters were the focus of the study that is (free iron, transferring {TF}, percentage saturation {%S} and ferritin {SF} levels). The iron parameters were used as inclusion or exclusion parameters for selection, high levels of serum ferritin were major exclusion criteria as this was an indication of inflammatory diseases (Kell and Pretorius, 2014a) presence of underlining diseases which could have hampered our results.

Table 3.2: Iron profiles of all participating individuals from AMPATH laboratories

Iron			
Iron	**TF**	**%S**	**SF**
15.3	3.3	19	24
10.9	3.5	12	15
15.9	3.2	20	19
12	3	16	12
12.9	2.7	19	71
21.8	2.1	42	57
19.8	2.2	36	20
14.5	2.5	23	16
11.9	2.4	20	66
16.8	3	22	13
8.2	4.1	8	4
17.2	2.8	25	37
16.1	2.6	25	24
20.8	2.8	30	11
10.7	2.3	19	109
24.9	2.5	40	44
9.6	2.6	15	35
23.4	2.3	41	251
25.8	3.2	32	21
16.9	2.8	24	41
25.7	3	34	342
32.5	2.3	57	218
10.8	3.5	12	12
29.1	3.1	38	141
16.9	2.8	24	164
18.1	2.5	29	277
15.3	2.4	26	44
24.9	3	33	121
18.6	2.3	32	70
24.5	2.4	41	224

All the values from Table 3.2 were used to plot scattered graphs seen in **Figure 3.3** with each spot representing each individual with their respective measured iron parameters. Each graph contains an upper and lower limit ranges **(**See Table 3.1**)** the red dotted lines across the graph in all four graphs. Individuals participating in the study had to be within the ranges to qualify. As it is visible from the graphs most of our controls fell within the upper and lower limits ranges from each category.

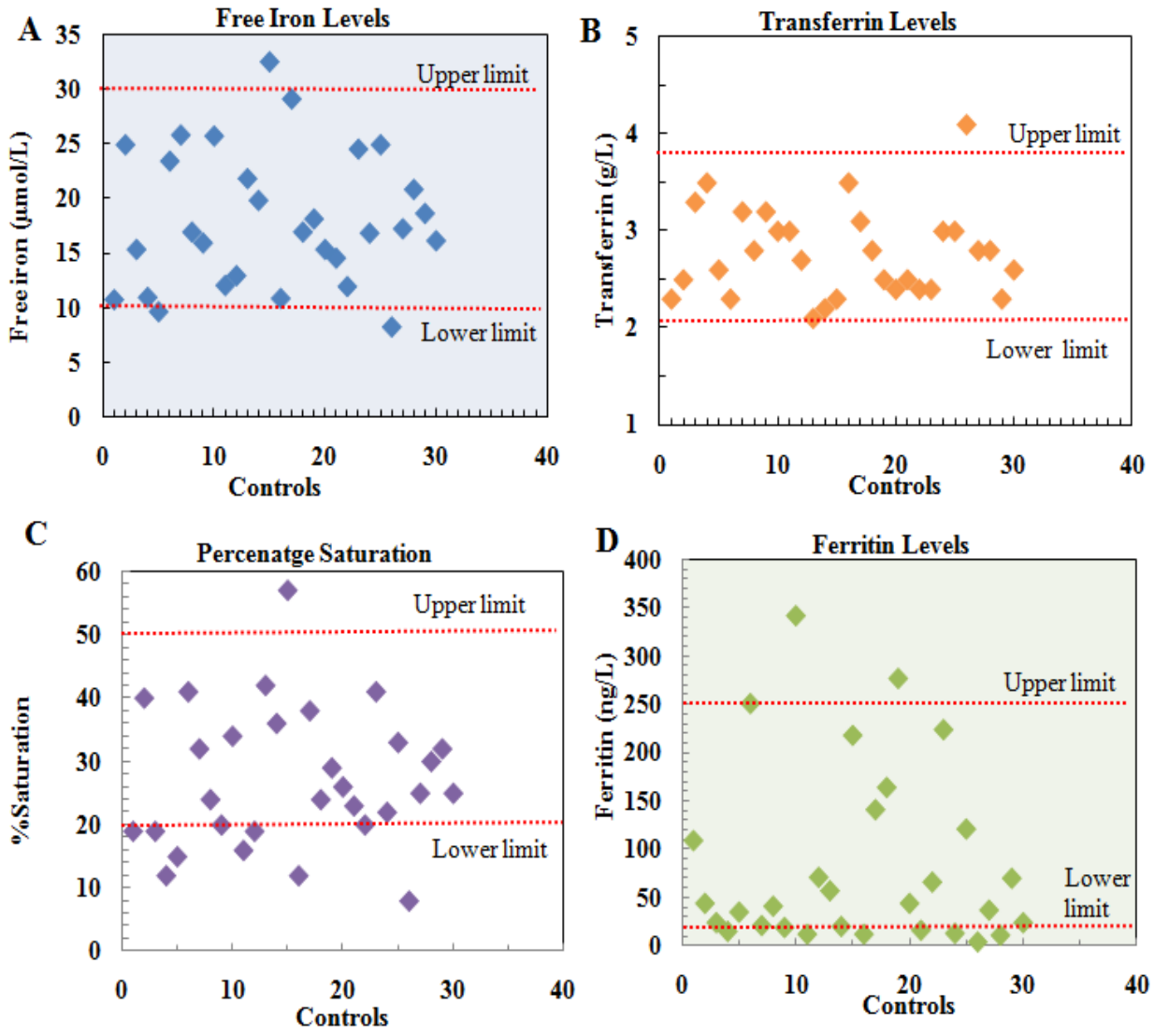


Figure 3.3: Iron parameters of all our controls from graph (**A**) Free iron levels (**B**) Transferrin levels (**C**) percentage saturation , and (**D**) Ferritin levels concentrations measured in serum.

3.5. Discussion

Like most things in life if we received anything in abundance especially unregulated can be of harm to the body. Hence when there is iron overload it may lead to formation of diseases. **See Figure 3.2.**for descriptive flow diagram unbounded iron leads to inflammatory reaction. This is because most chronic inflammatory diseases are associated with a microbial origin(Potgieter et al., 2015a). For this study it was crucial to obtain controls that were healthy, had no underlining inflammatory diseases due to that fact that the study.

Full iron tests were performed, as high SF and low transferrin levels are acute phase inflammatory protein markers (Sezgi et al., 2014) and indicative of inflammation. Therefore for the 30 individuals that were selected, the exclusion criteria were used to select adequate and healthy study population based on their iron results. Individuals whose iron parameters were out of range for all four measurements (**see Table 3.1 for normal iron ranges**) we excluded them from the study. Controls who had extremely low iron levels indicative of anaemia or high levels of iron in the case of haemochromatosis, a physician contacted us and the individuals were informed in a discrete manner. Subjects with known inflammatory conditions such as asthma, human immunodeficiency virus (HIV) or tuberculosis, and risk factors associated with metabolic syndrome, smoking, and if female, being on contraceptive or hormone replacement treatment were strictly excluded from the study. As these conditions are known to have elevated levels of serum ferritin (Christopoulou et al., 2002, Theil, 2013). See **Table 3.2** for a selection of individuals and their respective iron profiles from AMPATH laboratories.

It was justified from our obtained results that all individuals used for the study were indeed healthy, their iron parameters were in the at least three markers per individual were in range **see Figure 3.3**. Free iron levels are shown in graph (A) it is visible that on this category 2 out of 30 individual's free iron levels were slightly out of the ranges however this was not on extreme level of unbounded iron, Transferrin measured levels graph (B) transferring levels also our major inflammatory marker it seemed that impressively one person out of the 30 individuals had increased levels of tranferrin and this was insignificant. **Figure 3.3.C-D** both these categories iron is examined when bounded and fully saturated (**C**) and levels of functional ferritin levels (**D**) these controls can deviate slightly as they fluctuate often.

The free unbounded iron levels if they are not regulated tend to bind to HR as mentioned in the introductory paragraphs (Lipinski and Pretorius, 2012a).

The production of peroxide and superoxide is a predictable consequence of aerobic metabolism (Crichton, 1987). These ROSs can exhibit a number of biological effects, their reactions with poorly liganded iron species (if most of our controls free iron levels was beyond the optimal physiological levels) this can lead to the catalytic production of very reactive and dangerous HR damaging cells, and causing chronic inflammation (Kell, 2009, Lipinski and Pretorius, 2012a). This could have tempered with our results as we were inducing inflammation by adding LPS (endotoxin) and studying the effects there after. A patient with already existing chronic inflammation was not ideal for this study, hence proper iron levels tests of all volunteers was essential.

All participating individual are represented by a dot on the graph surrounded by the upper limits (top line) and lower limit (bottom line) range sin red line dots. As we can see within every range (the area between the top and bottom line dots) a large number of dot appear. This is an indication that indeed our controls were healthy and did not have underlying inflammatory diseases.

3.6. Conclusion remarks

This was a quality measurement of our subjects and based on the results revealed and discussed in this chapter I conclude by stating that the ‘controls’ healthy individuals that were used for this study were indeed healthy with no underlying diseases that may or could have cause deception of our results.

CHAPTER 4: Scanning electron microscopy of fibrin fiber network of controls with and without LPS analysis and fibrinogen activating in haemostasis analysis with Thromboelastograph

4.1. Chapter objectives

- To analyze the fibrin fiber formation after addition of LPS to PPP using the SEM, by measuring fibrin fiber thickness.
- To investigate the efficiency of blood coagulation using the Thrombo-Elasto-Graph (Moreno-Navarrete et al.) technique, using PPP and whole blood. This is to determine the activity of the plasma coagulation system.

See **Figure 4.1** for a visual lay out of this thesis. Main techniques discussed in this chapter (SEM and TEG). Spectometere was used to quantify and analyse the actual binding of LPS to fibrinogen and confical to justify and prove that LPS does indeed bind to fibrin monomers. Isothermal Titration Calorimetry (ITC) analysis of the LPS−fibrinogen interaction.

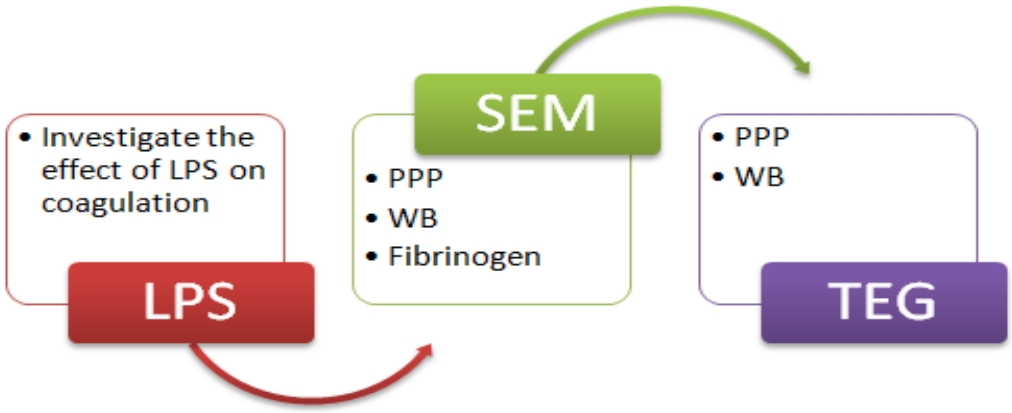


Figure 4.1: Schematic diagram representing our main focus on this chapter.
The main techniques used to study the effects of LPS on coagulation parameters in platelets poor plasma (PPP), whole blood (WB), fibrinogen levels and ultrastructure using SEM; the second major technique used in this chapter was Thromboelastograph ® which was used to study viscoelastic properties of PPP and WB before and after incubation with LPS for 10 minutes time intervals.

4.2. Introduction

Lipopolysaccharides describe a variety of cell wall shed by Gram-negative bacteria; they have been found in various fluids, including WB. The 'concentrations' are typically assayed using the *Limulus* amoebocyte lysate assay (Jiang et al., 2009, Harte et al., 2010a, Su and Ding, 2015).

However, although satisfactory in simple matrices, this test is not considered very reliable in blood (Novitsky, 1998, Kell and Pretorius, 2015c). Indeed, because it is so hydrophobic, little or no LPS is actually free (unbound), and so it is not even obvious what its 'concentration' in blood might mean (Kell and Pretorius, 2015c). Although the quantitative assessment of LPS concentrations in whole blood can be problematic, its presence in this fluid may have important and clinically relevant effects on the blood microenvironment, and may be central in the treatment of inflammatory conditions (Kell and Pretorius, 2015c, Potgieter et al., 2015b, Kell et al., 2015, de Punder and Pruimboom, 2015). LPS molecules are potent inflammagens (Tan and Kagan, 2014, Płóciennikowska et al., 2015b, Rutherford et al., 2015) and may be both cytotoxic and/or neurotoxic (Liu and Bing, 2011, Zielen et al., 2015, Frey et al., 2015, Kell and Pretorius, 2015c).

They are known to induce the production of a variety of pro-inflammatory cytokines (Fujimoto et al., 2012, Medzhitov, 2008, Rossol et al., 2011, Savage et al., 2012) that are involved in various apoptotic, programmed necrosis and pyroptotic pathways (Kell and Pretorius, 2015c, Bryant et al., 2010, Croker et al., 2014). Indeed, cytokine production (Fujimoto et al., 2012) is central to the development of inflammation (Pedraza-Alva et al., 2015). A characteristic of systemic inflammation is a hypercoagulatory state (Choi et al., 2006, Conway, 2012, Emsley and Tyrrell, 2002, Esmon, 2005, Kell and Pretorius, 2015e). We also discussed in the previous chapters that hypercoagulable state is typically associated with pathological changes in the concentrations of fibrin (Farrell, 2012, Alexander et al., 2011) in this chapter I aim to reveal exactly how fibrinogen concentration and fibrin ultrastructure is affected by inflammation, to help us understand whether LPS can cause hypercoagulation by acting on the coagulation pathway more directly or indirectly eg. one route is via tissue factor (TF) upregulation (see **Figure 2.2**).

4.3. Materials and methods

4.3.1.Sample population

For this study 30 healthy individuals were included. We included controls only if their iron levels were within normal ranges (**see Figure 3.3**). Whole blood of the participants was obtained in citrate tubes and either whole blood or platelet poor plasma was used for TEG®, confocal and SEM experiments.

LPS types, purified fibrinogen and thrombin concentration used

The LPS used was from *E. coli* O111:B4 (Sigma, L2630)- **see Appendix 4** and also *E. coli* O26:B6 (Sigma L2762)- **see Appendix 5**. A final LPS concentration of 0.2 ng.L^{-1} (well below its critical micelle concentration (Santos et al., 2003) was used in all experiments bar as noted for some of the ITC measurements. It was made by vortexing a micellar suspension of 10 mg.L^{-1}, followed by multiple serial dilutions. The South African National Blood Service (SANBS) supplied human thrombin, which was at a stock concentration of 20 U/ml and was made up in a PBS containing 0.2% human serum albumin.

In experiments with added thrombin, 5µL of thrombin was added to 10 µL of PPP or fibrinogen. Human fibrinogen was purchased from Sigma (F3879-250MG). A working solution of 0.166 mg.mL^{-1} purified fibrinogen was prepared. This concentration was found to be the optimal concentration to form fibrin fibers in the presence of thrombin, similar to that of platelet rich plasma fibers from healthy individuals (Pretorius et al., 2013e).

Addition of LPS ± thrombin to whole blood, plasma and purified fibrinogen

LPS-incubated WB and purified fibrinogen was prepared for SEM without added thrombin. The LPS-incubated PPP and purified fibrinogen samples were prepared as above, but with added thrombin to create an extensive fibrin fiber network.

4.3.2. Isothermal Titration Calorimetry

E. coli O111:B4 lipopolysaccharide and human plasma fibrinogen were purchased from Sigma-Aldrich. Samples were reconstituted in warm phosphate buffered saline and incubated for 1 hr at 37 °C with shaking. Lipopolysaccharide was then sonicated for 1 hr at 60 °C. Fibrinogen solutions were passed through a 0.2 µm polyethersulfone syringe filter and concentrations were determined by UV absorbance ($E_{1\%}$ = 15.1 at 280 nm).

Samples were then diluted with buffer to the required concentration and degassed. ITC experiments were performed at 37 °C on a MicroCal Auto-iTC200 system (GE Healthcare) in high-gain mode at a reference power of 10 µcal.s^{-1}, with an initial 0.5 µL (1 s) injection followed by fifteen 2.5µL (5 s) injections with 300 s spacing. For longer titrations, the syringe was refilled and injections continued into the same cell sample. Control runs were performed in which cell samples were titrated with buffer and syringe samples were titrated into buffer, and data from these runs were subtracted from the experimental data as appropriate. Data analysis was performed in Origin, using the supplied software (MicroCal).

4.3.3. Thromboelastography®

TEG® was used to study the viscoelastic properties of the participants' blood, before and after addition of LPS. Whole blood TEG® was performed on the day of collection (after 10 min incubation time with LPS), PPP was stored in 500µL aliquots in a −70 °C freezer. Standard TEG procedures were followed with addition of $CaCl_2$ to activate the coagulation process as previously described (Nielsen, 2007, Nielsen, 2008, Nielsen and Pretorius, 2014b, Nielsen and Pretorius, 2014a). TEG® was also performed on 5 PPP samples 30s after adding O111:B4 LPS or O26:B6 LPS.

4.3.4. Confocal microscopy

Thioflavin T (ThT) was added at a final concentration of 5 M to 100 µL PPP. A second sample was also prepared by adding a final concentration of 0.2 ng.L^{-1} LPS before the addition of ThT. After an incubation time of 10 min, at room temperature and protected from light, 10 l of the LPS-incubated PPP (with and without LPS) was mixed with 5 µL thrombin (as above) and viewed under a Zeiss LSM 510 META confocal microscope with a Plan-Apochromat 63x/1.4 Oil DIC objective, excitation was at 488 nm, and emission measured at 505-550.

4.4. Results

Scanning electron microscopy of whole blood, plasma and purified fibrinogen

To investigate our hypothesis that LPS may cause hypercoagulation via an acute, and direct binding reaction (by interaction with plasma proteins directly involved in the clotting cascade), we investigated the effect of 2 LPS preparations from *E. coli* (viz. O111:B4 and O26:B6). These were added to whole blood (WB) of healthy individuals, to platelet (and cell) poor plasma (PPP), and to purified fibrinogen. **See Figure 4.2** shows the visual difference of the addition of LPS to healthy blood, that shows affected fibrin fibers and fibers not changed by LPS addition, found in the same sample.

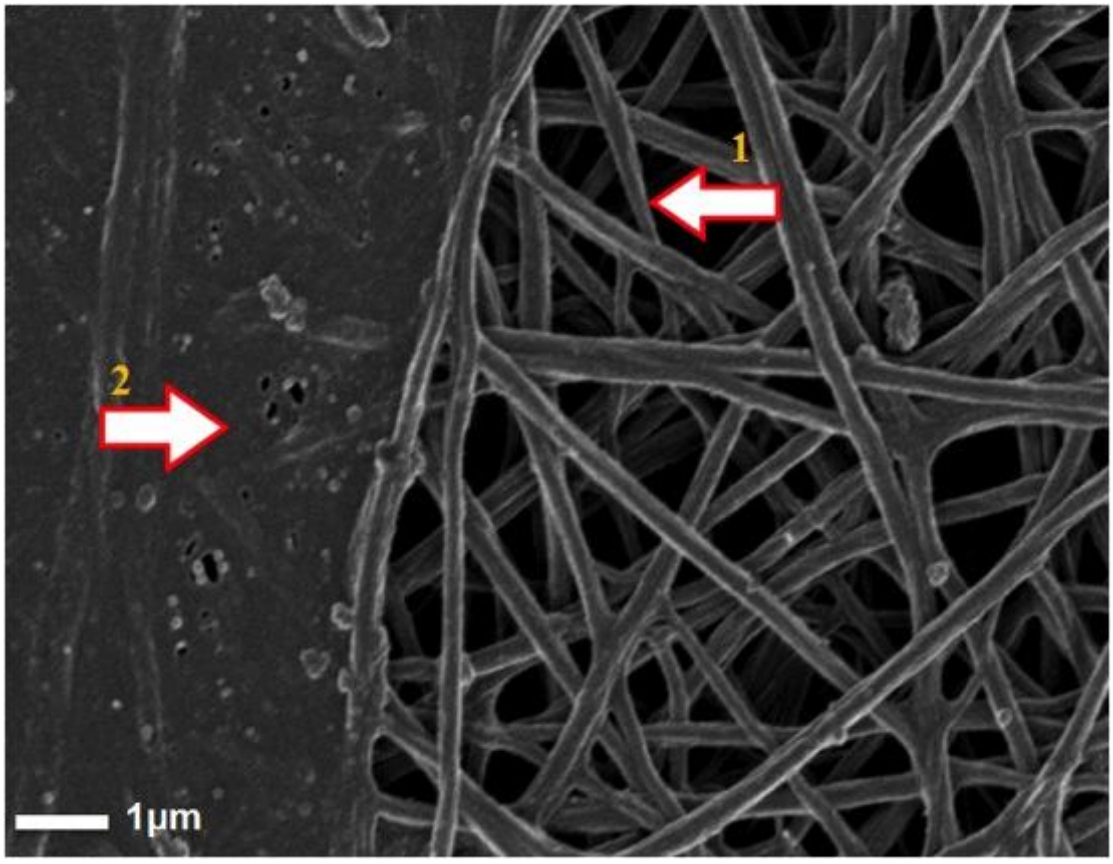


Figure 4.2: LPS treated blood smears taken from healthy individual. (**1**) is the site of normal fibrin fibers with visible healthy separated fibers and (**2**) represents the start of inflammatory response due to addition of small ($0.2ng.L^{-1}$) concentration of O111:B4 LPS for 10 minutes. Scale bar: 1 µm.

See Figure 4.3 shows PPP from 3 our controls with added thrombin, O111:B4 LPS was added for 30 seconds (short exposure time). Red arrows show fibrin fibers starting to agglutinate after 30 seconds of LPS exposure. Scale bar: 1 µm.

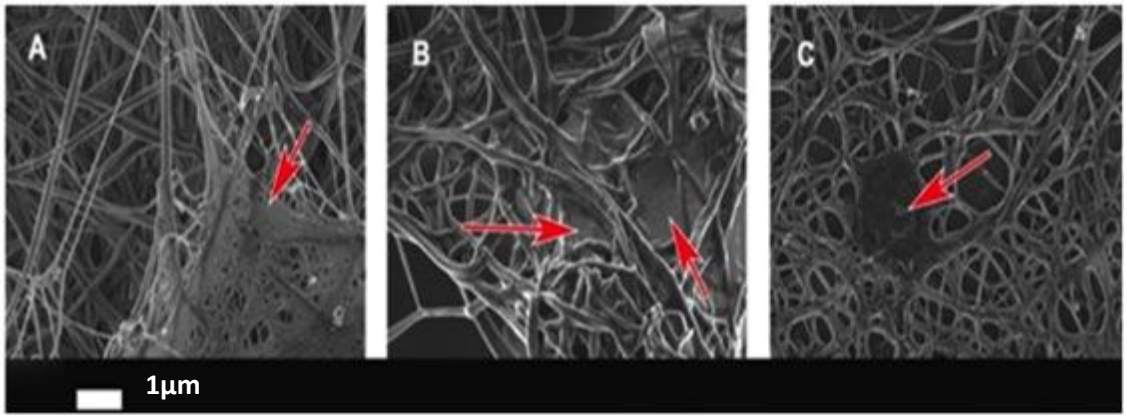


Figure 4.3: Induction of acute inflammation by LPS for 30 seconds in PPP of healthy individuals in micrograph (A-B) we see the start of mesh of fibrin fibers at different sites.

4.4.1. Purified fibrinogen

Because fibrinogen is present at significant concentrations in plasma, and its levels are increased during inflammation {refs above}, it is considered that it might be an important mediator of the LPS-induced hypercoagulation. Thus, we also added both LPS types to purified fibrinogen (30 seconds and 10 minutes exposure time) with and without added thrombin. **Figure 4.4 A to C** shows purified fibrinogen with and without O111:B4 LPS with added thrombin (30 seconds and 10 minutes exposure time). We see that even the 30 seconds exposure time changed the fibrin fibers to form dense matted deposits without any individual fibers visible, resembles inflammatory characteristics. This pattern was also witness with purified fibrinogen and added O26:B6 LPS (results not shown).

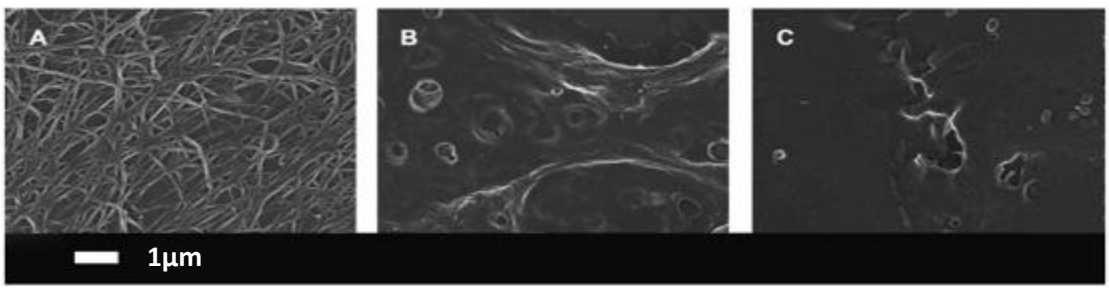


Figure 4.4: (**A)** Purified Fibrinogen with added thrombin; (**B)** purified fibrinogen with added O111:B4 LPS (30 seconds exposure) and (**C)** Fibrinogen and LPS 10 minutes exposure.

Although the physiological levels of LPS are said to be 10 - 15 ng.L^{-1}, and little or none of it is free (Kell and Pretorius, 2015c), in our hands the addition of LPS at these concentrations caused immediate coagulation when they were added to whole blood. **See Figure 4.5.** shows the effect of 0.2 ng.L^{-1} O111:B4 LPS when added to WB and incubated for 10 min. Dense matted deposits are spontaneously formed; these are not seen in healthy whole blood. **See Figure 4.6** fibrinogen with added O111:B4 or O26:B6 LPS with just 30s exposure (no thrombin added) also spontaneously formed matted deposits (results not shown).

4.4.2. Whole blood and LPS

The LPS from *E.coli* O111:B4 was incubated together with whole blood for a period of 10 minutes. See **Figure 4. 5** it shows the effects of LPS on whole blood (WB) without thrombin (activation of clotting system) for a much longer exposure time of 10 miuntes. Red arrows show abnormal thick matts deposits on the slide.

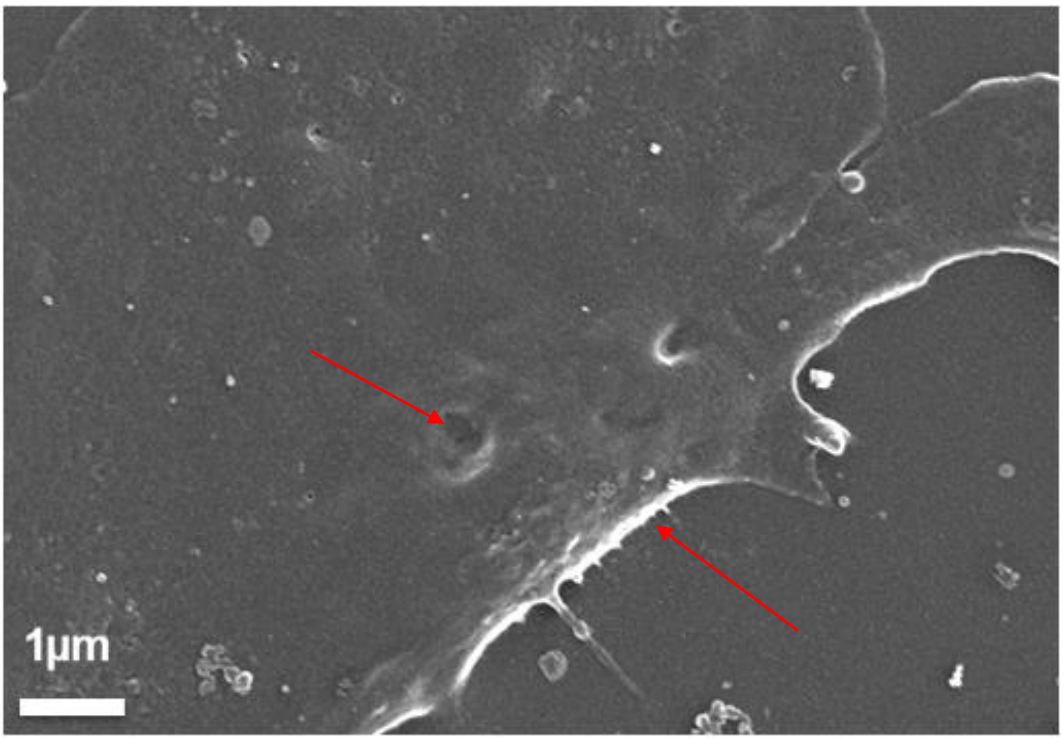


Figure 4.5: LPS and whole blood. Thick mass deposit seen smeared all over the canvas . Indicative of inflammation. This was without thrombin. Scale bar: 1 µm.

The following **Figure 4.6.** shows the effect of LPS on wb and thrombin on a larger scale with more of controls. A common trend was seen throughout the slide with SEM. Thick matts deposits showing clumping of small plasma particles highlighted in red boxes. Scale bar: 1 µm.

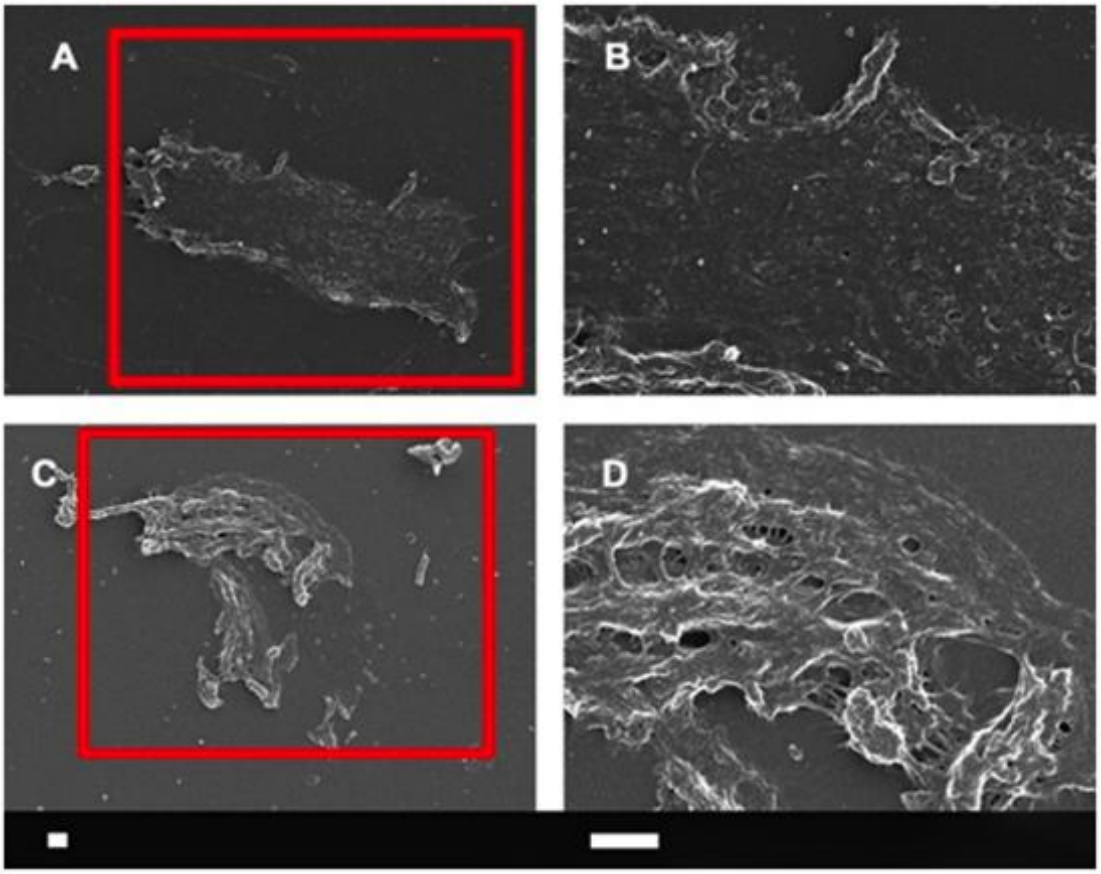


Figure 4.6: Effect of O111:B4 LPS (0.2 ng.L^{-1}) on whole blood (without thrombin), where dense matted deposits were spontaneously formed, not seen in control whole blood smears. Scale bar 1µm

4.4.3. Platelets poor plasma and LPS

The fiber thickness is much more heterogeneous after LPS is added; Clearly these tiny amounts of LPS are having enormous effects on the clotting process. These kinds of netted structures, that we have also termed 'dense matted deposits', were previously seen in inflammatory conditions such as diabetes (Lipinski and Pretorius, 2012b, Pretorius et al., 2013b, Pretorius et al., 2011a), iron overload and stroke (Lipinski and Pretorius, 2012b, Pretorius et al., 2013a, Pretorius and Kell, 2014, Pretorius et al., 2011b).

Typically healthy fibrin fiber networks form a net where individual fibrin fibers are seen in (**Figure 4.7**.**A**), but with added LPS a matted net develops as seen in **Figure 4.7** (**C**). There is a significant difference between fiber thickness before and after LPS treatment ($p < 0.0001$) in the presence of thrombin, this is revealed in **Figure 4.7** and **Figure 4.8**.

Note, that the distribution of the fibre thickness in the LPS-treated group varies from very thick to very thin. In some cases, continuous fibre plates are formed, where no individual fibres could be seen or measured. This explains the difference in n between the 'before' and 'after' treatment (1450 versus 1330 measured fibres). See **Figure 4.7** shows the effect of LPS on formation of thin fibres (**4.7.D**) these continue till they disappear (**4.7.B**) or rejoin other fibrin fibres to form much thicker mass of fibrin fibres seen in (**4.7.C**)

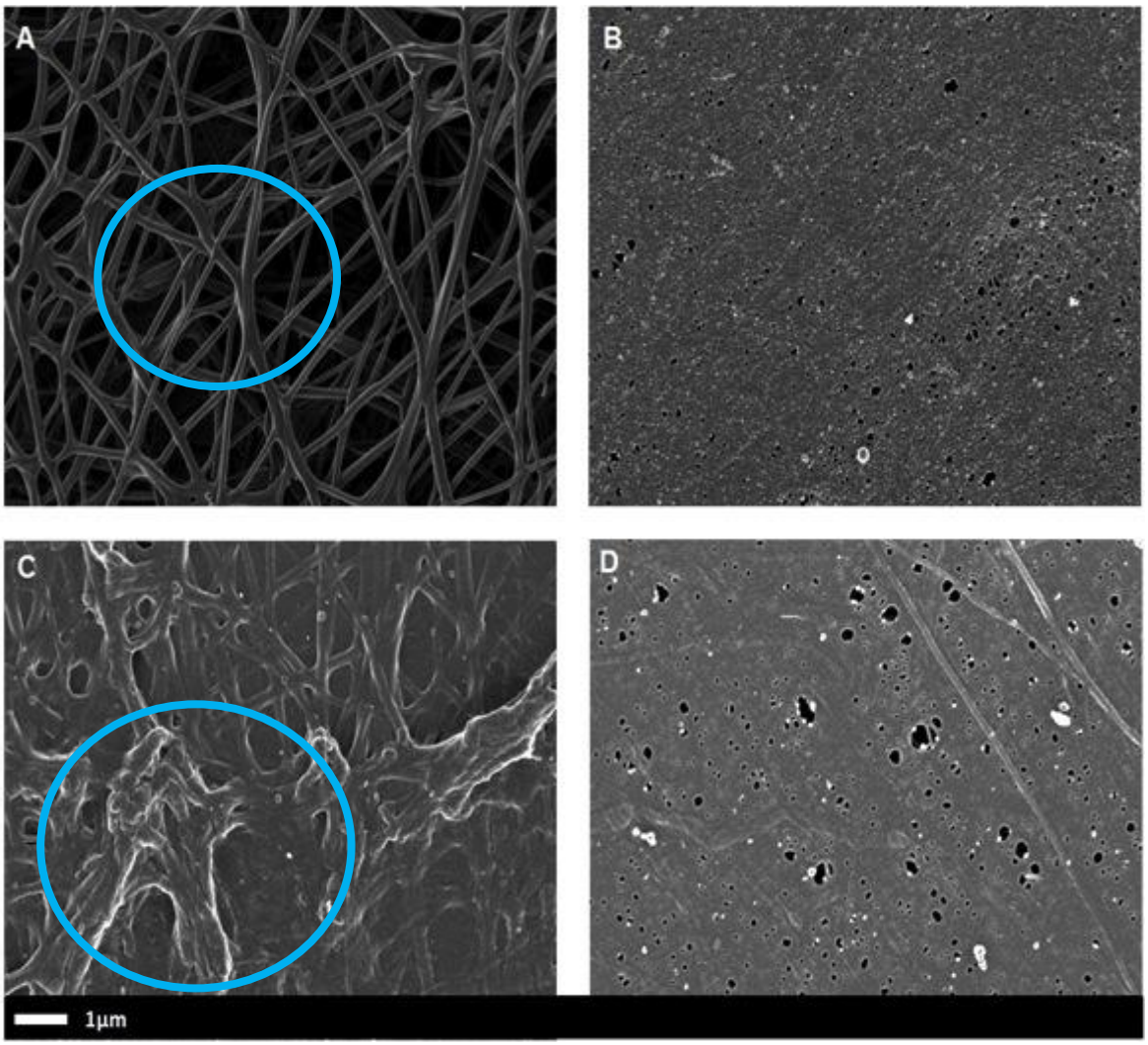


Figure 4.7: LPS and PPP. (A) Normal healthy fibrin fibres (B)Smooth networks no individual fibers seen (**C**) thick mass deposits of fibres clamping together encircled in blue (**D**) smooth thin fibrin fibres and perforated canvas signs of hypercoagulation Scale bar: 1 µm.

The experiment with the O111:B4 LPS was also repeated with a shorter 30s exposure time. Platelets poor plasma with LPS and added thrombin showed fiber agglutination starting to happen in only 30s of LPS exposure. These shorter experiments are to be contrasted with previously reported experiments that showed the much longer-term effect of LPS.

These involve cytokine production, including increased TF production via monocytes. By adding LPS to PPP (with thrombin) we bypass the possibility that LPS can stimulate TF production via the monocyte route suggested by (Landsem et al., 2015a, Landsem et al., 2013). In **Figure 4.8**. I show the formation of the contious thick matt deposits on fibrin fibres, the red arrows show site of thickened fibres compare to singular thin fibres seen in (**A and C**). To determine if another type of LPS would also cause the changes noted above, we also added *E. coli* O26:B6 LPS to PPP of 5 individuals (30s and 10 min exposure time), followed by addition of thrombin. SEM results showed the same trends as noted with the O111:B4 LPS (see Figure 4.4).

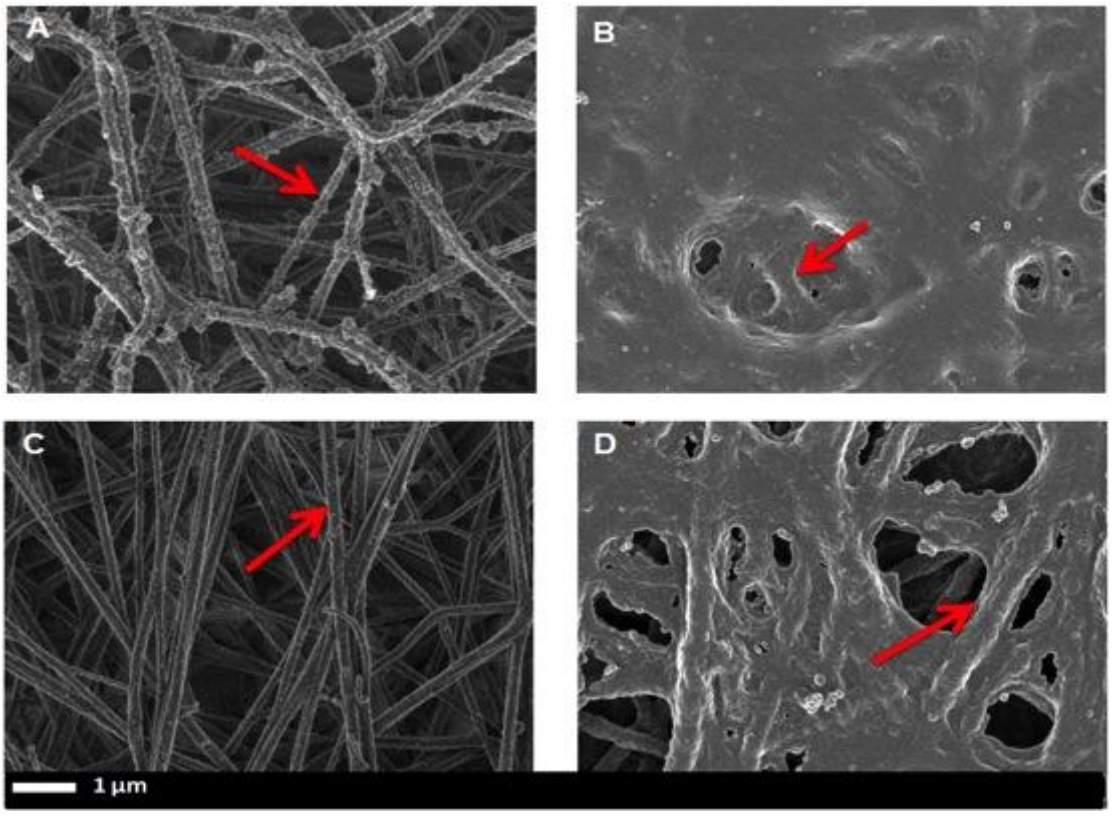


Figure 4.8: Thick matts deposits seen on the fibrin fibers when treated with LPS. In (A) represents the normal fibers from healthy individual before treatment with LPS. (B) Shows highly dense matted deposits with the red arrow showing one individual big fiber. (D) the red arrow points to the highly thick abnormal fibrin fibers. Scale bar: 1 µm.

In the following figure 4.9 I reveal the effects of thrombin on clot formation for healthy control (**4.9 A**) and when the PPP was pre-incubated for 10 min with 0.2 ng.L^{-1} O111:B4 LPS (two examples in (**4.9 B, C**). **Fig 4.9 D** shows the distribution of fiber thicknesses for the 30 individuals, with and without added LPS.

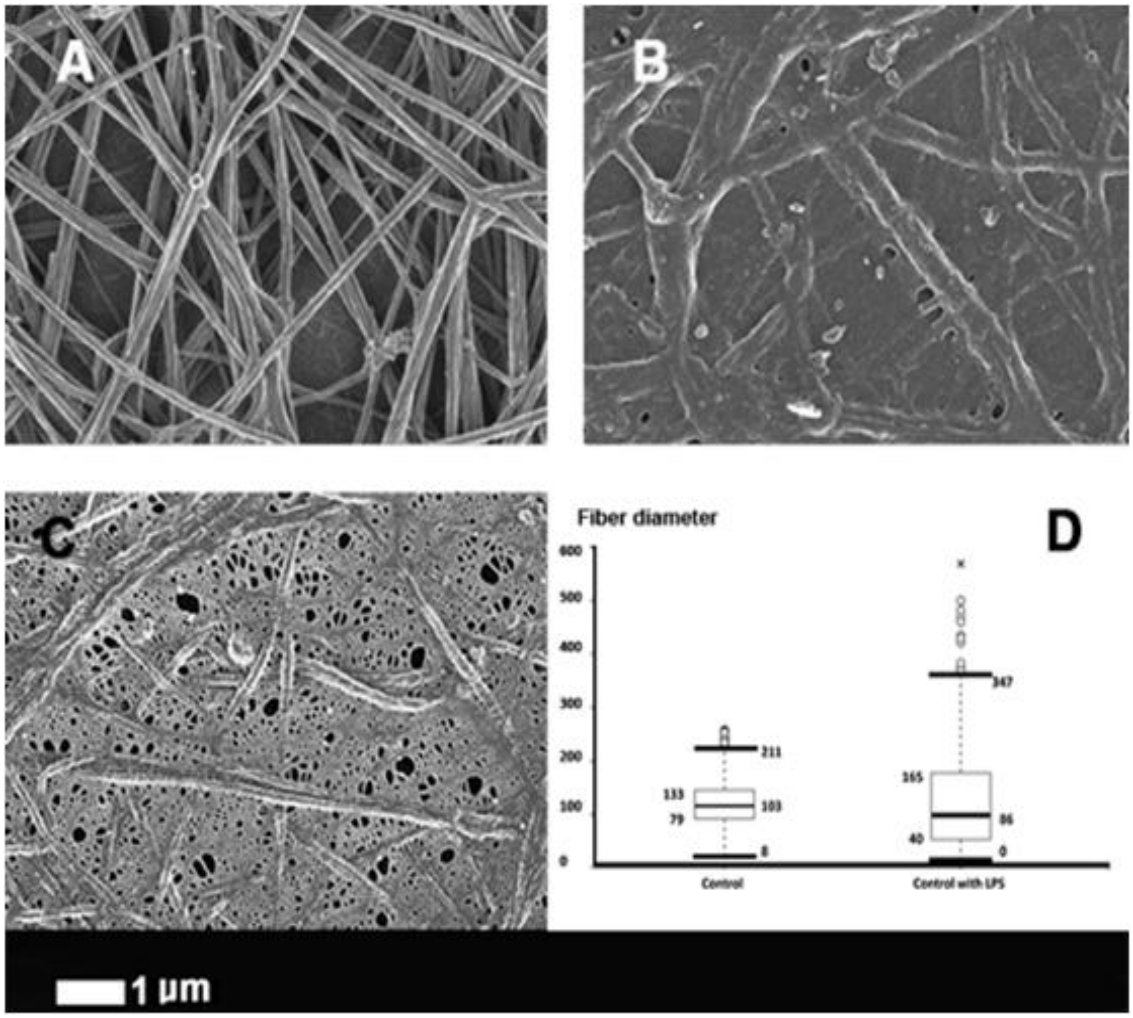


Figure 4.9: The effect of 0.2 ng.L^{-1} O111:B4 LPS on the morphology of fibrin fibers in the platelet poor plasma (PPP) of healthy individuals (with added thrombin). (A) Healthy fibers; **(B and C)** PPP with added LPS. **(D)** Fiber distribution of the control fibers and of controls with added LPS of 30 individuals. Note: in samples with added LPS there were areas of matted layers with no visible fibers to measure. Fibers were measured using ImageJ as described in Materials and Methods. Scale bar: 1 µm.

4.4.5. Confocal microscopy

The reaction, and presumed binding, of the hydrophobic LPS within fibrinogen fibers implies that they contain, or expose, significant hydrophobic elements.

Such elements, also common in amyloid-like fibrils (Liu et al., 2013), can be stained fluorescently using dyes such as thioflavin T (TFT) (Biancalana and Koide, 2010). We therefore studied the effect of 0.2 $ng.L^{-1}$ LPS on the ability of the fibrin fibers formed following thrombin treatment of PPP to bind TFT (**see Figure4.10**). In contrast to the LPS-free controls (**A**) there is a very substantial binding of TFT to the fibrin fibers formed in the presence of the LPS (**4.10 B**).

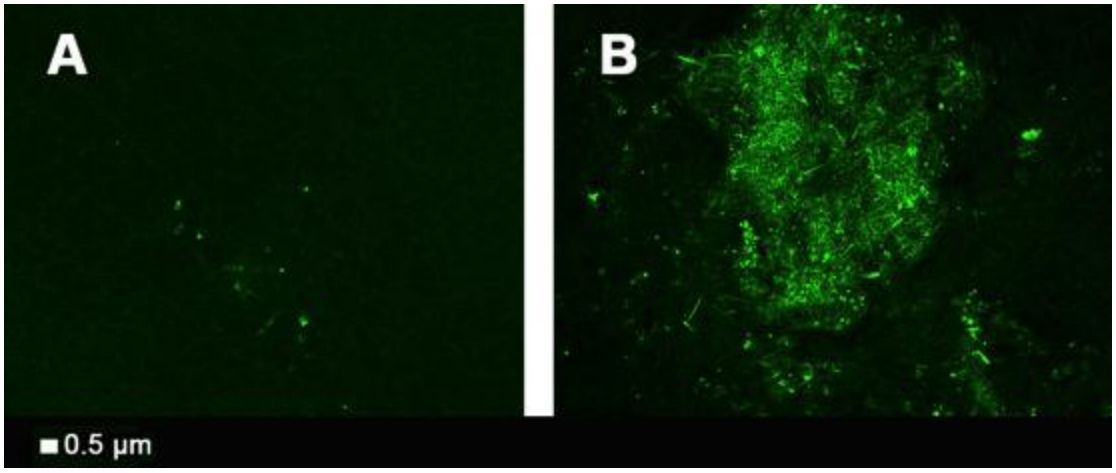


Figure 4.10: Confocal of LPS and PPP (A) Control PPP with TFT and thrombin and **(B)** as (A) but pre-incubated with 0.2 $ng.L^{-1}$ LPS. Scale bar 0.5µm

4.4.6. Purified fibrinogen

Because fibrinogen is present at significant concentrations in plasma (~3.5 $g.L^{-1}$), and its levels are increased during inflammation (see above), we considered that it might be an important mediator of the LPS-induced hypercoagulation. Thus, we also added both LPS types to purified fibrinogen (30s and 10 min exposure time) with added thrombin. Even the 30s exposure time changed the fibrin fibers to form dense matted deposits without any individual fibers visible (data not shown).

4.4.7. Isothermal Titration Calorimetry (University of Manchester)

Isothermal Titration Calorimetry (ITC) is a sensitive and convenient method for detecting biomolecular interactions by measuring the heat that is released or absorbed upon binding (Ward and Holdgate, 2001). Measurements are conducted directly in solution, without modification of immobilization of the interacting species. We used ITC to study potential interactions between human plasma fibrinogen and LPS from E. coli O111:B4.

Titration of fibrinogen into LPS resulted in strong endothermic injection heats with a clear sigmoidal saturation curve indicating a direct binding interaction (Fig 4.11 A). Assuming molecular weights of 340 kDa for fibrinogen and 20 kDa for monomeric LPS, we determined a binding stoichiometry (n) of ~0.135. This is consistent with each fibrinogen monomer binding to a micelle formed from ~75 LPS monomers. Reverse titrations were conducted, injecting LPS into plasma concentrations of fibrinogen (3 g.L-1 = 8.8 µM). Titration of 2.5 µM LPS into fibrinogen yielded endothermic injection heats greater than those observed for titration of 2.5 µM LPS into buffer alone (**Figure 4.11.B**), clearly indicating a direct binding of LPS to fibrinogen. Each injection added 125 ng of LPS into the instrument cell, increasing the LPS concentration by ~30 nM per injection. Although we expect LPS binds fibrinogen at sub-nanomolar concentrations, interactions at these concentrations are below the detection limits of the ITC instrument.

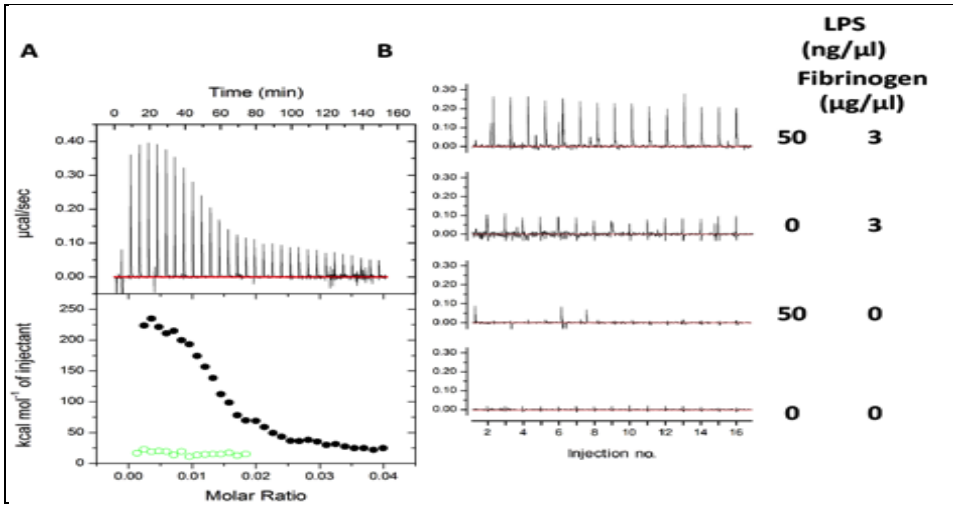
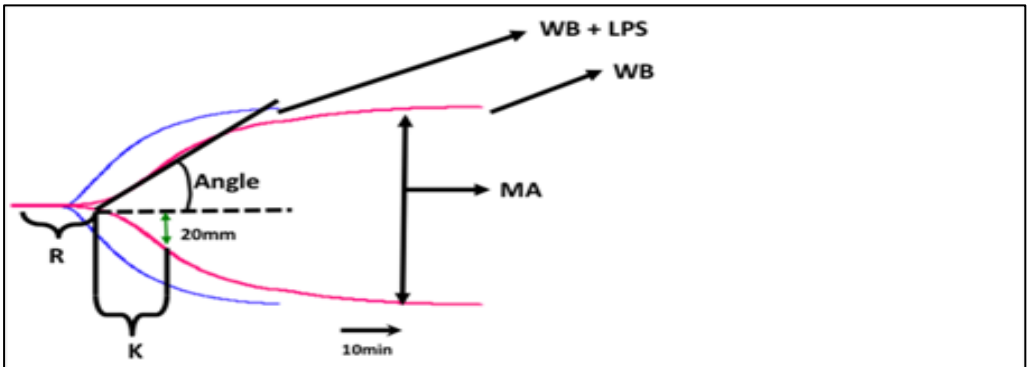


Figure 4.11: ITC analysis of the LPS−fibrinogen interaction. (**A**) Titration of 8.8 µM human plasma fibrinogen (*black circles*) or buffer (*green open circles*) into 100 µM of *E. coli* O111:B4 LPS. (**B**) Titration of 50 ng/µl LPS (2.5 µM) or buffer into 3 µg/µl fibrinogen (8.8 µM) or buffer as indicated. Experiments were conducted at 37 °C in phosphate buffered saline.

4.4.8. Thromboelastography of whole blood and PPP

Thromboelastography (Moreno-Navarrete et al.) is a viscoelastic technique for measuring the clotting properties of whole blood (Nielsen, 2007, Nielsen, 2008). TEG of WB and PPP was performed. TEG was not performed with purified fibrinogen because the coagulation activator in the TEG is $CaCl_2$ and fibrinogen is only activated by thrombin, not calcium. **Figure 4.12.** Represents a typical TEG trace from a control whole blood with and without added LPS, overlaid with lines that explain the parameters extracted by the instrument and the values for those traces. The statistics are given see **Table 4.1.**



	R (min)	K (min)	Angle (deg)	MA (mm)	MRTG (dynes.cm^{-2}.s^{-1})	TMRTG (s)	TGG (dynes/cm^2)
WB	11.3	7.9	40.2	51.9	2.2	21.2	566.7
WB + LPS	8.1	4.2	56.2	52.5	2.8	14.6	552.9

Figure 4.12: TEG overlay from a control whole blood sample with and without added LPS. R: Reaction time, first measurable clot formation; K: Achievement of clot firmness; Angle: Kinetics of clot development; MA: Maximum clot strength; MRTG: Maximum rate of thrombus generation; TMRTG: Time to maximum rate of thrombus generation; TGG: Final clot strength.

Whole blood TEG

TEG® analysis of whole blood (10 min incubation time with O111:B4 LPS) showed that the R, TMRTG and TGG are all significantly increased (**Table 4.1**). This suggests a hypercoagulable state as well as a denser clot, and is consistent with the SEM fibrin fiber thickness results.

We have previously shown that the same concentration of LPS as used in the current paper, when added to naïve uncitrated healthy blood but without added $CaCl_2$, also had an effect on coagulation after only 3 min incubation time (Bester et al., 2015a).
Both TMRTG and R of naïve whole blood were also significantly shorter, also showing hypercoagulation.

Platelet poor plasma TEG
We also performed similar TEG experiments with PPP (**see Table 4.1**). After 10 min exposure just the initial clotting time R was changed. The results were not specific for O111:B4 LPS as O26:B6 LPS added to PPP behaved similarly. The decreased R time is indicative of reaction time, and therefore the time to first measurable clot formation is also significantly decreased (as the initiation of the clot starts faster with than without LPS), confirming a hypercoagulable state with added LPS. After 30s exposure time, both the R and the TMRTG were shorter (in the 5 patients tested). This confirms our hypothesis that LPS causes (near) instant hypercoagulability. Here also, the results were not specific for O111:B4 LPS as O26:B6 LPS added to PPP also showed a decreased R-time.

4.5. Statistical Analysis

The non-parametric Mann–Whitney U test was performed using the STATSDIRECT software. **Table 4.1** shows a summary of the various viscoelastic properties (TEG experiments) after LPS has been added to WB and PPP. There are very substantial changes in a number of the clotting parameters. Following these 10-min exposure experiments, we shortened our experimental time to 30s and we repeated the experiments with 5 samples, using PPP, where significant changes were still observed. The results were not specific for O111:B4 LPS as O26:B6 LPS. We note that WB with added LPS showed a more pronounced change in relevant viscoelastic parameters than when LPS was added to PPP (s**ee Table 4.1**). In this table the medians, standard deviation and p-values (values lower than 0.05 are indicated in blue) obtained using the Mann-Whitney U test are shown for iron profiles, and TEG® of whole blood and plasma.

Table 4.1: Demographics of blood from healthy individuals with and without added LPS

Variables	Healthy individuals (n = 30)	Healthy individuals with added LPS (n = 30)	P-value
AGE (years)	29.5 (±13.81)		
GENDER			
Male	15 (50%)		
Female	15 (50%)		
IRON PROFILES			
Iron □M	16.9 (± 6.18)		
Transferrin $g.L^{-1}$	2.75 (± 0.46)		
% Saturation	25.0 (± 10.94)		
Serum Ferritin $ng.mL^{-1}$	42.5 (± 92.47)		
FIBRIN FIBER THICKNESS	**n = 1450**	**n = 1330**	
Fiber thickness (nm)	103 (± 40)	86 (± 82)	**< 0.0001**
THROMBOELASTOGRAPHY®			
THROMBOELASTOGRAPHY® OF WHOLE BLOOD - RECALCIFIED WITH $CaCl_2$ WITH ADDED O111:B4 LPS (10 MIN)			
MRTG ($dyn.cm^{-2}.s^{-1}$)	2.61(± 1.13)	2.89(± 0.90)	0.33
TMRTG (min)	13.9 (±3.53)	9.6 (± 3.01)	**<0.0001**
TTG ($dyn.cm^{-2}$)	615.0 (± 179.55)	527.9 (± 146.65)	**0.049**
R (min)	8.0 (± 1.64)	6.2 (± 1.77)	**<0.0001**
K (min)	4.9 (± 2.63)	4.2 (± 1.23)	0.07
Angle (in degrees)	49.8 (± 5.27)	56.2 (± 7.02)	**0.0066**
MA (mm)	55.0 (± 8.07)	51.3 (± 6.90)	0.092
THROMBOELASTOGRAPHY® OF PLATELET POOR PLASMA - RECALCIFIED WITH $CaCl_2$ WITH ADDED O111:B4 LPS (10 MIN)			
MRTG ($dyn.cm^{-2}.s^{-1}$)	3.6 (± 4.35)	4.2 (± 2.13)	0.36
TMRTG (min)	10.6 (± 3.22)	9.3 (± 3.68)	0.50
TTG ($dyn.cm^{-2}$)	203.9 (± 137.51)	211.6 (± 103.67)	0.70
R (min)	8.2 (± 2.64)	7.1 (± 2.70)	**0.026**
K (min)	4.4 (± 3.51)	3.8 (± 2.42)	0.18
Angle (in degrees)	63.2 (± 2.70)	54.4 (± 10.67)	0.23
MA (mm)	28.4 (± 8.34)	30.1 (± 9.27)	0.196
THROMBOELASTOGRAPHY® OF PLATELET POOR PLASMA - RECALCIFIED WITH $CaCl_2$ WITH ADDED O111:B4 LPS (30S)			
	n=5	**n=5**	
MRTG ($dn.cm^{-2}.s^{-1}$)	5.94 (± 1.8)	8.2 (± 2)	0.166
TMRTG (min)	11.58 (± 1.2)	9 (± 1.3)	**0.0159**
TTG ($dyn.cm^{-2}$)	244.4 (± 69.9)	290.2 (± 66.5)	> 0.99
R (min)	9.8 (± 1.2)	7 (± 1.5)	**0.031**
K (min)	2.8 (± 1.6)	2 (± 0.9)	0.119
Angle (in degrees)	63.6 (± 6.5	68.8 (± 8.2)	0.095
MA (mm)	32.8 (± 6.5)	36.7 (± 5.5)	> 0.99
THROMBOELASTOGRAPHY® OF PLATELET POOR PLASMA - RECALCIFIED WITH $CaCl_2$ WITH ADDED O26:B6 LPS (30S)			
	n=5	**n=5**	
MRTG ($Dynes.cm^{-2}.s^{-1}$)	6.3 (± 2.6)	6.2 (± 3.6)	0.70
TMRTG (min)	11.6 (± 2.1)	8.9(± 1.5)	0.15
TTG ($Dynes.cm^{-2}$)	276.7 (± 43.1)	230.8 (± 202.2)	0.69
R (min)	9.8 (± 1.5)	6.4 (± 1.4)	**0.05**
K (min)	2.1 (± 0.5)	2.1 (± 1.1)	0.88
Angle (in degrees)	64.4 (± 4.3)	70.8 (± 10.4)	> 0.99
MA (mm)	35.5 (± 3.3)	31.5 (± 13)	0.60

4.6. Discussion

In the introduction, we suggested that LPS might contribute to excessive blood clotting (or an activated coagulation state) via two possible routes: (i) via a direct and acute binding to plasma proteins (e.g. fibrinogen) or (ii) by an indirect or chronic (longer-term) process where it participates in an inflammatory activation via cytokine production. Here we demonstrated that the first process is indeed possible, and demonstrated it by both viscoelastic and ultrastructural methods. We also confirmed that LPS can change the viscoelastic properties of PPP within 30s of its addition. Furthermore, WB with added LPS, but without thrombin activation, showed spontaneously formed matted deposits. Purified fibrinogen experiments with O111:B4 LPS and O26:B6 LPS, with and without added thrombin showed a changed ultrastructure, suggesting that LPS indeed binds to the 340 kDa fibrinogen molecule and that the effects of this are visible ultrastructurally.

LPS, and especially its lipid A component, is highly lipophilic, and it therefore may be able to bind directly to plasma proteins, in an acute way. This might be one reason underlying the hypercoagulability (Kell and Pretorius, 2015c), as well as a denser clot structure (Calabrese et al., 2015, Chaby, 2004), as seen in various inflammatory diseases. Although we show here that exposure to even tiny amounts of LPS leads to an immediate (acute) change in the coagulability parameters, we recognize that this may happen simultaneously with the chronic (longer-term) reactions (**Figure 4.3**). Fibrinogen molecules are roughly 5x45nm, and their self-assembly is a remarkable process (some 5800 are involved in generating a fiber of 80-90nm diameter and 1µm length). This would explain why the highly substoichiometric binding of LPS can have such considerable effects, especially as observed in whole blood (**Figure 4.5 and 4.6**). At present, the exact mechanisms of action of these small amounts of LPS are not known, although we recognize that cascade effects may be possible.

Previously we coined the term “atopobiotic” microbes to describe microbes that appear in places other than where they should be, e.g. in the blood, forming a blood microbiome (Potgieter et al., 2015b). Here we suggest that the metabolic and cell membrane products of these atopobiotic microbes correlate with, and may contribute to, the dynamics of a variety of inflammatory diseases (Østerud et al., 2015a, Cunningham et al., 2005, Deng et al., 2014, Miklossy, 2008), and that LPS, in addition to (possibly low-grade) long-term inflammation via cytokine production, may lead an acute and direct hypercoagulatory effect by binding to plasma proteins, especially fibrinogen.

We showed here that, even with very low levels of LPS, a greatly changed fibrin fiber structure is observed. An urgent task now is to uncover the mechanism(s) of this acute and immediate effect.

It is well known that a variety of inflammatory diseases are accompanied by hypercoagulability, and a number of more-or-less longer-term signalling pathways have been shown to be involved. In recent work, we have suggested a direct and primary role for bacterial lipopolysaccharide in this hypercoagulability, but it seems never to have been tested directly. Here we show that the addition of tiny concentrations (0.2 $ng.L^{-1}$) of bacterial lipopolysaccharide (LPS) to both whole blood and platelet-poor plasma of normal, healthy donors leads to marked changes in the nature of the fibrin fibers so formed (**Figure 4.7 and 4.8**), as observed by ultrastructural and fluorescence microscopy. They resemble those seen in a number of inflammatory diseases, consistent with an involvement of LPS in their aetiology. These changes are mirrored by changes in their viscoelastic properties as measured by thromboelastography (**see Table 4.1**). Since the terminal stages of coagulation involve the polymerisation of fibrinogen into fibrin fibers, we tested whether LPS would bind to fibrinogen directly. We demonstrated this using isothermal calorimetry (**Figure 4.11**).

The observation of a direct effect of very low amounts of LPS on both fibrinogen and coagulation opens up this process to further mechanistic analysis and possible treatment. Short summary of the chapter **see Figure 4.13.**

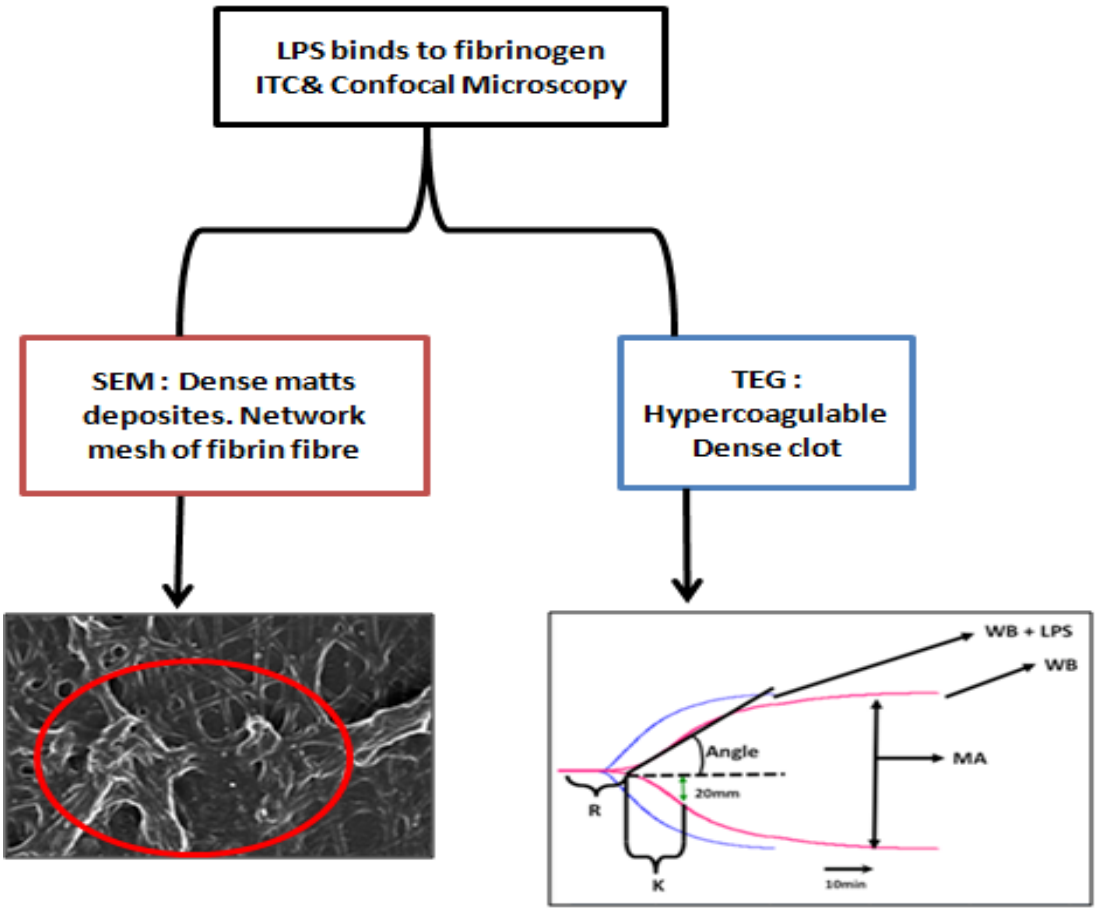


Figure 4.13: Summary of the chapter

4.6. Conclusion Remarks

We have suggested that one explanation is a dormant microbiome that can shed the highly inflammatory LPS. Such inflammatory diseases are also accompanied by a hypercoagulable phenotype. Our results here show directly that very low concentrations of LPS can affect the coagulation properties of blood and plasma significantly, and that these effects may be mediated via a direct binding of LPS to fibrinogen monomers proven by isothermal calorimetry.

CHAPTER 5: Studying RBCs ultrasturcure using SEM and morphology with LM

5.1. Chapter Objective

As plasma treated with LPS showed phenotypic characteristics of inflammation under SEM, here in this chapter I will pay special attention to structural changes in RBCs when using SEM after adding LPS to whole blood with added thrombin to activate normal clotting process that takes place in a normal functional body. Finally I will focus on the RBCs changes in shape and morphology using the light microscopy by comparing RBCs axial ratios of treated and untreated WB using the cell analyzer. **Figure 5.1.** Displays all possible factors that are known to affect the shape, and morphology of RBCs impairing the function of RBCs.

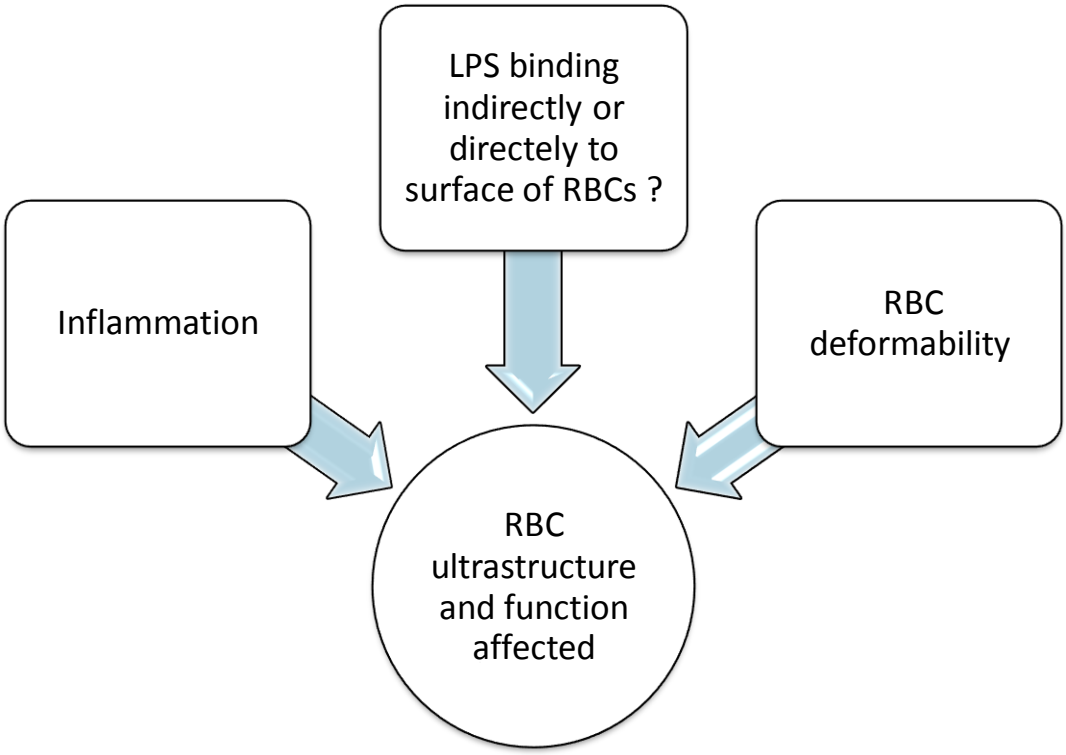


Figure 5.1: Schematic representative diagram of factors which are known to cause structural changes and decrease the function of RBC as discussed in this chapter.

5.2. Introduction

One of the hallmarks of inflammation is hypercoagulation, and it results from a cascade of inflammatory pathway events, linked to cytokine activation (Bern and McCarthy, 2013, Caprini, 2010, Chu, 2006b). Since LPS causes cytokine activation, others and have looked at the possible events leading to hypercoagulation in the presence of LPS. It was found that LPS cause hypercoagulation by acting on the coagulation pathway, via tissue factor (TF) upregulation (Monroe and Key, 2007, Koch et al., 2009). LPS from *E. coli* (100 $ng.mL^{-1}$) also activated the coagulation system when added to WB, via a complement- and CD14-dependent up-regulation of TF, leading to prothrombin activation and hypercoagulation (Landsem et al., 2015a). Recently, we also found that LPS might bind directly to circulating plasma proteins, particularly fibrinogen, and that this (rapid) binding might also cause pathological changes in the coagulation process (PNAS) (binding was shown in both 3 min and 10 minutes). We confirmed the binding potential of LPS to fibrinogen using isothermal calorimetry. Now the question that arose was if LPS binds to plasma proteins causing hypercoagulation, can it also bind to RBCs and if so, will this binding be visible as RBC shape and membrane changes. The usual LPS binding sites are either CD14 or TLR4. However, RBCs does not have these two receptors on their membranes, so binding must take place either at another receptor site, or as direct binding to any of the phospholipids or proteins on the RBC membrane. Currently, we simply do not know where LPS might bind on the RBC membrane, but in the discussion we discuss possible candidates, based on binding sites of malarial parasites (Satchwell, 2016). I will furthermore determine if LPS may cause, additionally to fibrin hypercoagulation, membrane changes to RBCs, and whether these changes can be seen with LM, SEM and AFM.

Red blood cells deformability improves blood flow in the microvessels and in large arteries at high shear rate. The major determinants of RBC deformability include cell geometry, cell shape and internal viscosity (i.e., mean cell haemoglobin (Hb) concentration and components of the RBCs' membrane). The deformability is measured by several techniques but filtration of RBCs through micro-pore membranes and ektacytometry are two sensitive techniques to detect changes in RBCs under varied experimental and diseased conditions (AlMomani et al., 2012). Diabetes mellitus (DM) is a metabolic disorder, characterized by varying or persistent hyperglycemia, which induces several changes in the RBCs membrane and its cytoplasm, leading to alteration in the deformability.

A decreasing trend of RBCs' deformability in these patients is observed (Pretorius et al., 2015). Fluidity of the membrane as measured in RBCs of these patients is decreased (AlMomani et al., 2012). With prolonged diabetic or any inflammatory conditions deformability of RBCs is further decreased, which may complicate the flow of RBCs in microvessels (Shin et al., 2007).

I have used the combination of Light Microscopy (LM) and Scanning Electrone Microscopy (SEM) to reveal the damaged caused by LPS on RBCs membrane. In the following paragraph I will discuss sample preparation and techniques I chose to use for this chapter.

5.3. Materials and methods

For sample size and selection refer to chapter 3, for bacterial selection refer to chapter 4.

5.3.1. Light microscopy

LM was used to study the axial ratios of RBCs using 100x magnification. 10ul of whole blood (with and without LPS) - where LPS was incubated for 10 minutes with whole blood was used to make a thin smear on a microscopic glass slide followed by usual LM staining with methylene blue (Pretorius et al., 2014c). Slides were viewed using a Nikon Optiphod transmitted light microscope. Axial ratios were determined from the LM micrographs, with the use of a program written in the C# programming language. The longest axis from each RBC was determined, referred to as the major axis, after which a perpendicular line was drawn in the centre of the major axis to establish the minor axis length. The axial ratio for each cell was obtained by dividing the major axis length by the minor axis length; a value of 1 represents a perfect circle (Pretorius et al., 2014c).

The steps discussed below were used to prepare the samples for LM:

Blood from our volunteers was collected in citrated tubes (5ml), blood was aliquoted into two separate Eppendorf tubes added with 490µl of 10ng/L diluted LPS concentration.

I then used 10µl of the blood, for each smear from untreated and treated samples and placed on glass slides and with another glass slide a thin smear of the blood will be made across the slide.

Two smears were made for each the naïve and treated sample. The samples were air dried for 24 hours followed by fixation of cells in methanol for 5 minutes and air dried again on a hot plate kept at constant 37˚C. This was conducted in a flow cabinet. After the samples have completely dried they were stained with Methylene blue and Eosin for one type of staining and GIEMSA second optional staining (stains white blood cells along with RBCs).

Eiosin staining technique: Slides were first stained in Methylene blue (dye) for a period of 5 minutes this was followed by the slides being rinsed with running tap water until the water is clear of the stain. The slides were air-dried and after left to dry for 3 hours, they were placed in the second stain, Eosin, for 30 seconds. After the 30 seconds the samples were rinsed with running tap water until the water washing out from the slide is clear of the stain. The samples were then air dried on a hot plate left overnight at 37°C. When the samples were completely dry a cover slip was mounted over the sample with Entellan. Micrographs of the smears were taken at 40 and 100x magnification, and the erythrocyte shape analyzed using the Cellanalyzer program.

Giemsa staining technique: After the slides have been fixed in methanol for 5 minutes, they were placed in the GIEMSA staining dye for a period of 4 hours. Followed by rinsing in tap water for 1 minute. The samples were then air dried on a hot plate left overnight at 37°C. When the samples were completely dry a cover slip was mounted over the sample with Entellan.

Scanning electron Microscope samples were prepared as indicated in chapter 4.

5.3.2. Scanning electron microscopy (SEM)

Whole blood smears with and without LPS was made on small glass coverslips fixed, dehydrated, dried, mounted and coated with carbon according to previously described methods (Buys et al., 2013). The final concentration of LPS in WB was 0.2 $ng.L^{-1}$. A Zeiss high resolution Crossbeam 540 was used to study the surface morphology of RBCs, and micrographs were taken at 1kV.

5.4. Results

Scanning electron microscopy of WB, looking specifically at RBCs membranes. We see increased agglutination of plasma proteins adhering to the surface of RBCs in LPS treated whole blood samples. There is visible distortion of the RBCs membrane seen in **Figure 5.1.**The shape of the RBCs looks irregular, not the typical biconcave phenotype seen in healthy individuals. Table 5.1 shows RBC axial ratios and Young's modulus with medians, standard deviation and p-values (obtained using the Mann-Whitney U test) for the current study. **Figure 5.2** shows the effect of 0.2 $ng.L^{-1}$ O111:B4 LPS when added to WB and incubated for 10 min, viewed with using light microscopy, scanning electron microscopy and atomic force microscopy.

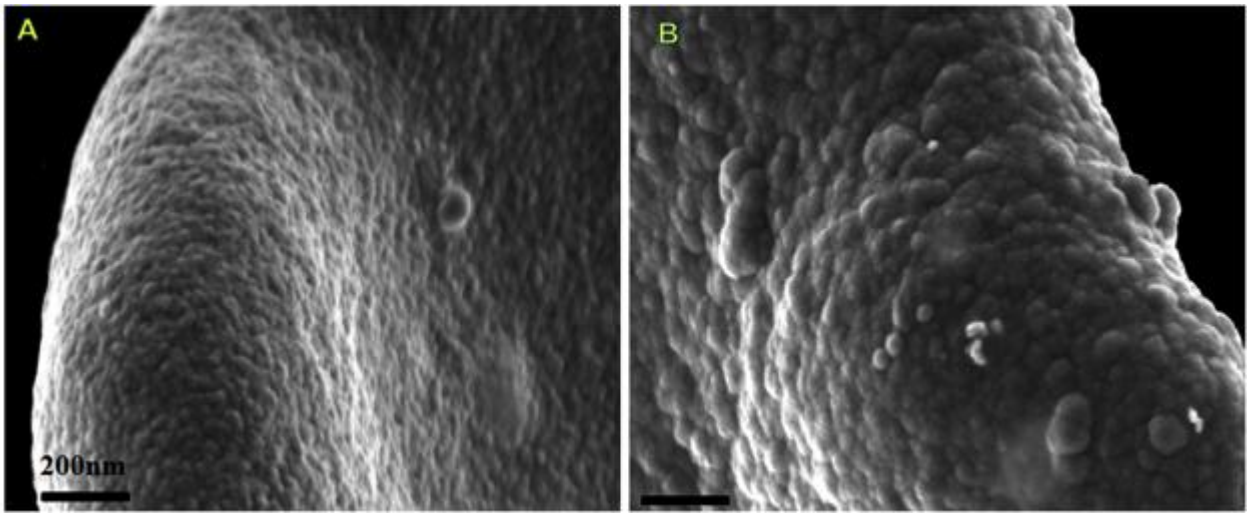


Figure 5.2: LPS and RBCs. Shows the surface of the RBC displaying the abnormal agglutination of plasma protein binding to the RBC both micorgraphs were taken on scale bar 200nm.

It is known that LPS causes agglutination of plasma protein from our recent publication Pretorius 2016 (PNAS). Here we see agglutination of plasma protein on the surface of RBCs see **Figure 5.3** were in micrograph (**C**) we see the tiny mass particles on the RBC's membrane and (**D**) reveals the closer-up of surface area indicated with a red box on micrograph. (**F**) In we see an enlarged surface area of RBC with small plasma proteins encircled within the red square. These mass particles are not present in normal healthy RBC see **Figure 5.3.E.**

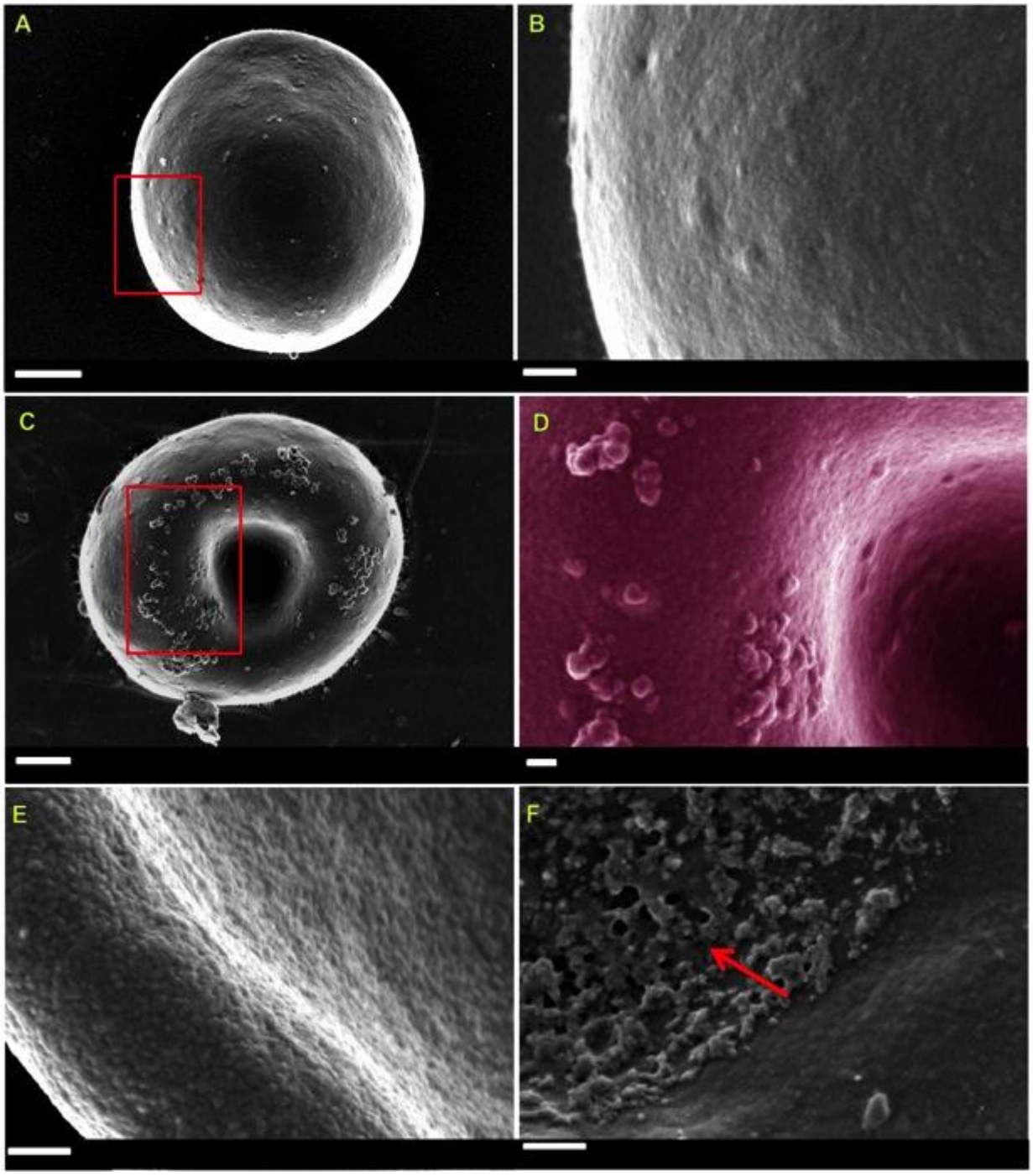


Figure 5.3: Attachment of plasma proteins on the surface of RBCs in LPS treated whole blood (**A**) is the normal back view of the RBC (**B**) shows the smooth surface with was taken from the same RBC in using scale of 200nm from SEM. (**C**) RBC that has the attached plasma proteins taken scale bar of 1µm and (**D**) a closer look at the surface area marked in red box was taken scale of 200nm **(E)** shows the inner curve from the untreated RBC**(F)** Shows the inside of the biconcave structure of RBC membrane from treated LPS whole blood with the little tiny particles highlighted in a red box.

For a much closer look at the membrane of RBCs affected by LPS adverse effects, we took a closer look at the membrane ultrastructure . **See Figure 5.4.**

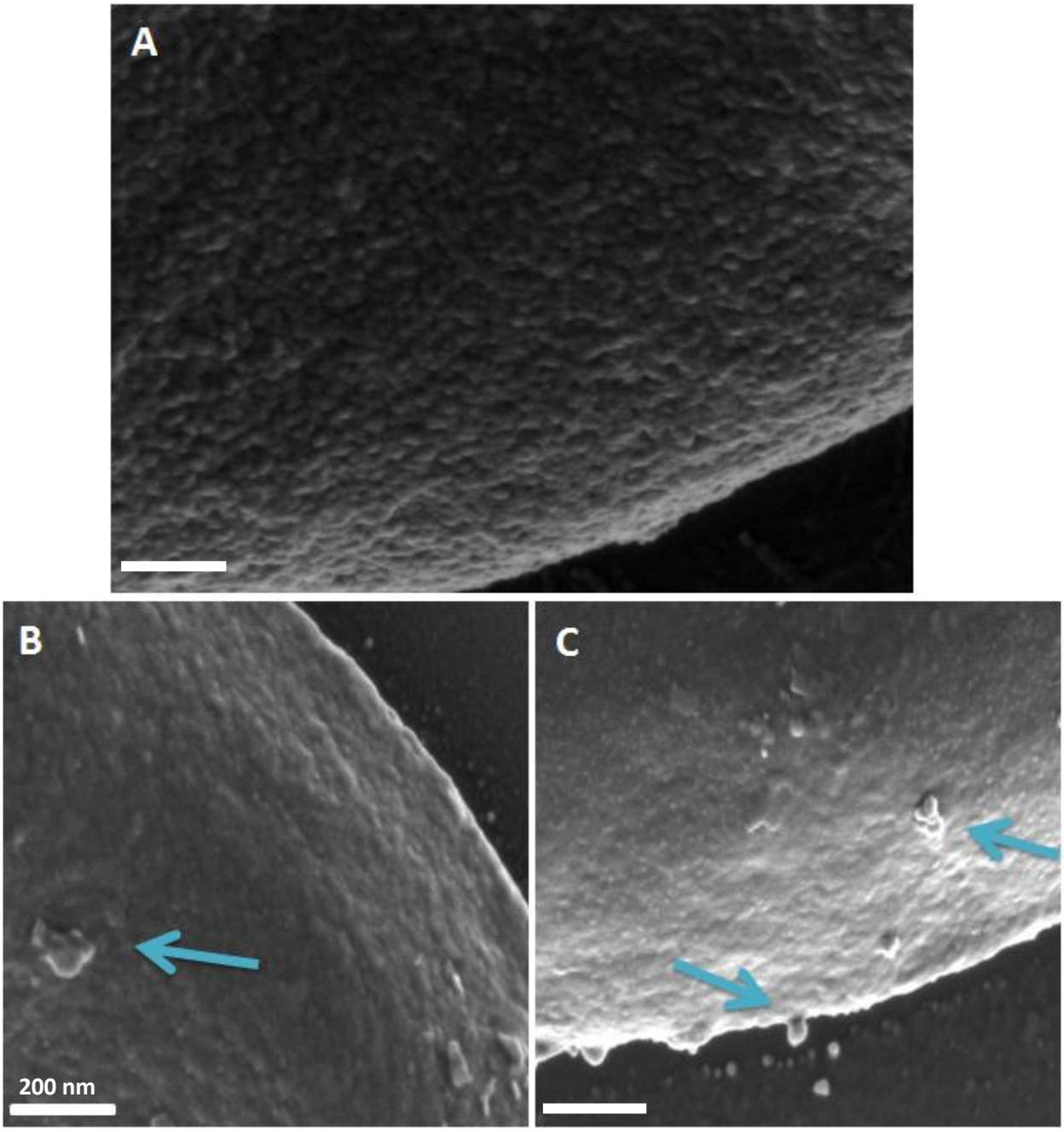


Figure 5.4: RBCs' ultrastructure. (**A**) is from untreated whole blood smooth membrane surface consistency unlike when we treated the same blood with 0.2ng.L-1 of LPS. (**B**) Bulging little plasma particles attached to the surface of the RBC. The attachment is consistant and prominent in other parts of the same RBC from the same LPS treated shown in (**C**). Little blue arrows indicate the abnormal attachments on the surface of the RBC scale of 200nm.

Lastly for SEM results, the study focused on the changes in the shapes changes of RBC as seen in **Figure 5.5.** Comparing the membranes of RBC before and after LPS addition to WB. Due to the agglutination of plasma protein on the surface of RBCs this leads to the change in the membrane and the RBC may lose it elasticity hence the shapes seen in **Figure 5.5 (B) and (C)**.

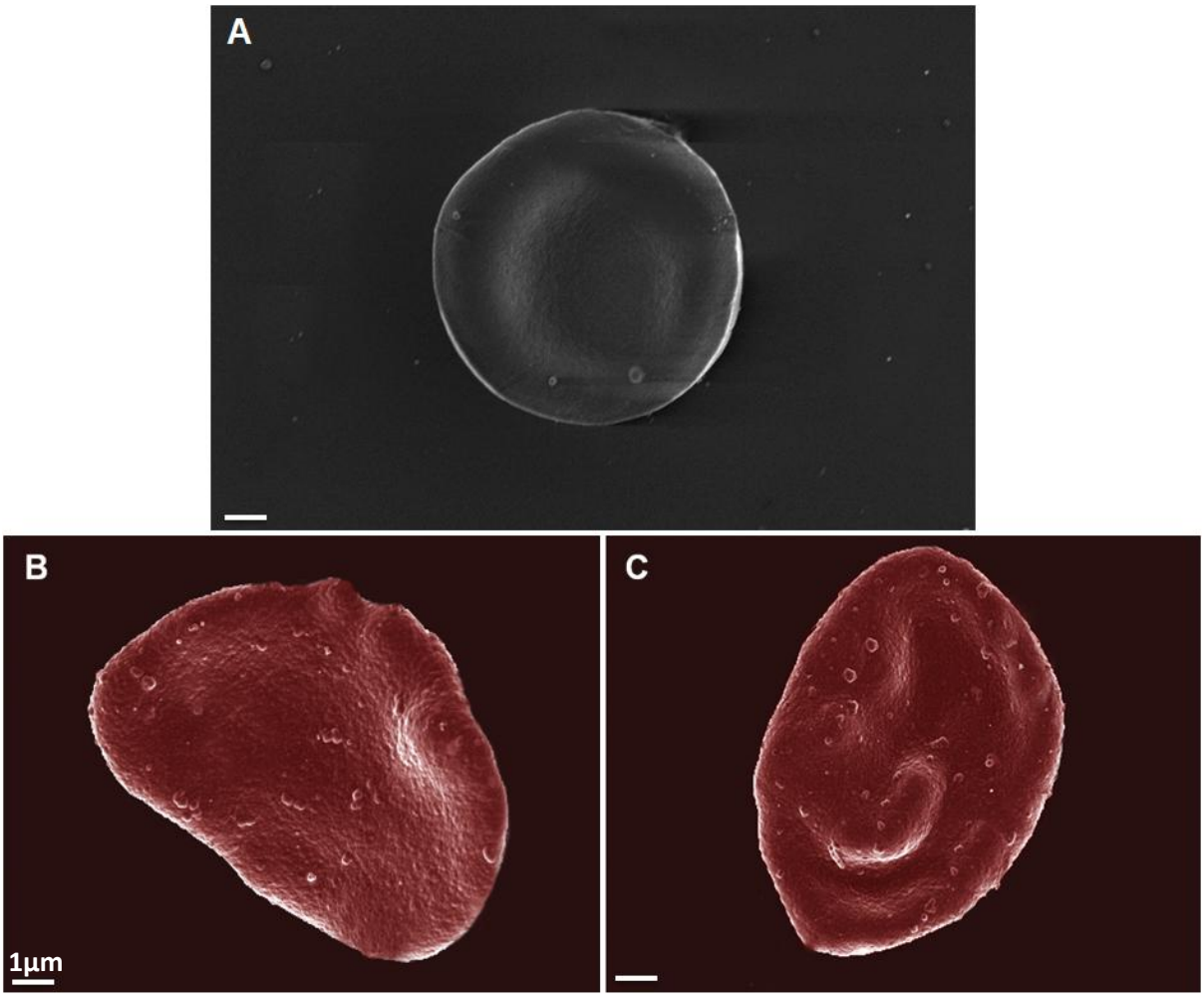


Figure 5.5: Displays the change in RBCs ultrastructure after incubation with LPS for 10 minutes, using a minuet concentration of 0.2ng.L^{-1} (**A**) shows the normal shape/structure of the RBC before incubation with LPS taken at a scale of 200 nm. (**B, C)** Shows the abnormal membrane of RBCs, after incubation with LPS scale bar 1µm.

In order to gain full knowledge and insight of the effect of this endotoxin (LPS), the study was directed to study the morphology of RBC using LM. **Figure 5.6** shows three different healthy individuals that LPS was added to invitro and studied the changes after incubation.

Since this is a direct comparative study, I placed every individual control blood directly next to their LPS treated blood sample. Micrograph (**A**) normal whole blood smear without LPS is the control of (**B**) which serves as our test, it represent the sample smear of whole that was incubated with LPS for 10 minutes.

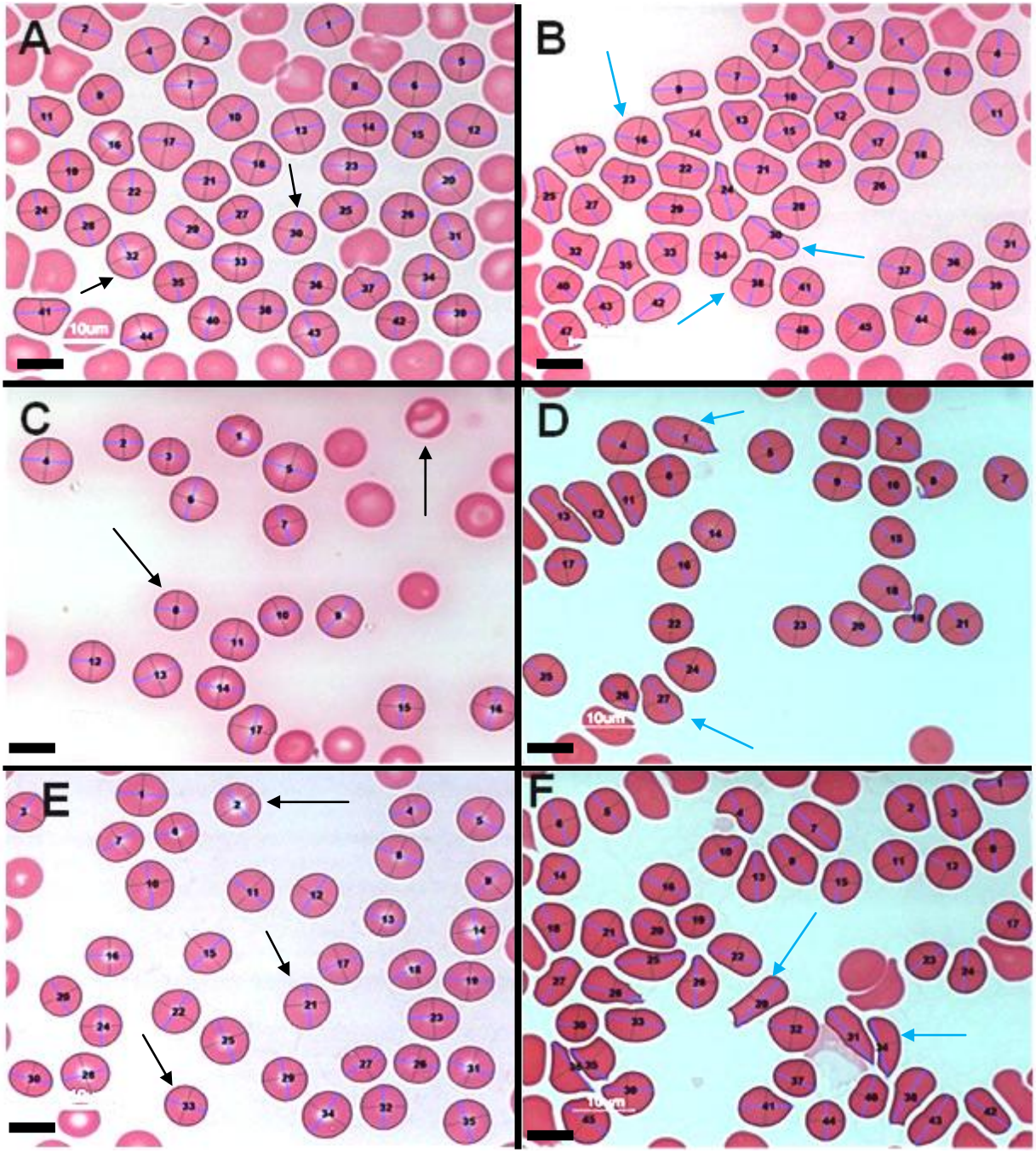


Figure 5.6: Light microscopy smears of RBCs from treated and un treated whole blood. Every individual served as their own control. One smear was made from whole blood that did not contain LPS (A,C,E) and after 10 minutes of incubating LPS balck arrows show normal size and shape of RBCs (final concentration of 0.2ng.L^{-1}) smears were made and visually seen in (B,D,F) were by blue arrows show abnormal elongated RBCs. Scale bar 10µm

All images were taken on Scale of 10µm at 100x magnification. The distorted, elongated RBCs seen in all whole blood treated with the same LPS. This is commonly seen in inflammatory conditions.

For the measurement of the axial ratios of RBCs, the cell analyser programme was used to measure every axle ratio of RBC in every slide. Axial ratios of RBCs from healthy individuals, and LPS treated samples, were captured using ImageJ (ImageJ is a public domain, Java-based image processing program developed at the National Institutes of Health: http://rsbweb.nih.gov/ij/). The differences we see on these micrographs were justified by statistical measurements by Mean Whiney see **Table 5.1** for all statistical measurements obtained from all measured axle ratios of RBCs. The Median obtained from untreated RBCs was (M 1.115). and from LPS treated blood the RBCs axle ration was (M 1.155). The difference between the two groups was statistically significant P<0.001. This is shown in a column graph Figure 5.7.the axle ratios from both groups were used to plot a graph using GRAPH PRISM version 6 2016. www.graphprism.com. The difference was slight not very visible when plotted in graph, but still statistically significant as seen using Mean Whiney.

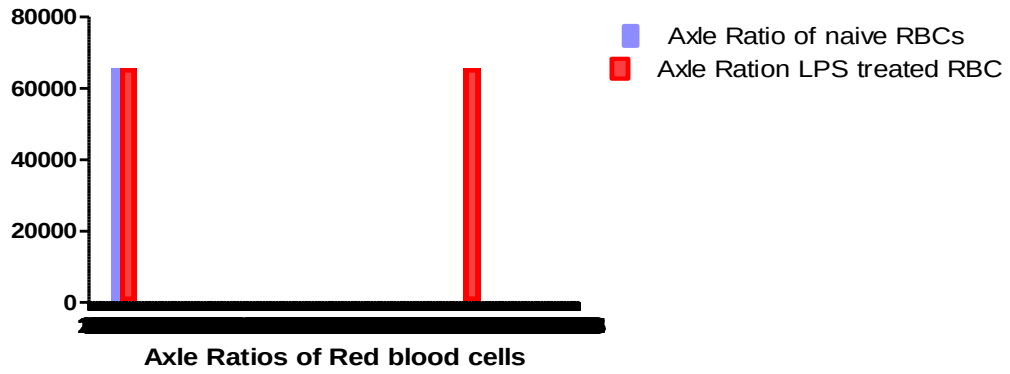


Figure 5.7: Bar graph showing axle ratios from the two groups

Statistical analysis of the results gave an indication of research outcomes, the next paragraph I will show how our research was proven to be successful by using statistical tools.

5.6. Statistical analysis

All axle ratios from obtained from the cell analyser program were statistically analysed by Mann-Whitney program see Table 5.5 for staitistical values obtained from both groups that were tested. The mean axe ratios of untreated vs treated RBCs was proven to be significantly smaller with the P-value <0.0001 showing the difference between the 2 groups RBCs axle ratios to be statistically significant.

Table 5.5 Statistical measurements Mann-Whitney U test was used from Cell Analyser

Statical values	Untreated whole blood	LPS treated whole blood
Number of RBCs (n)	1427	1759
Median	1.1	1.2
U'	1013946	1496147
Lower side P < 0.0001 Upper side P > 0.9999 Two sided P < 0.0001		

95% confidence interval for difference between medians or means:
K = 1204449 (approx) median difference = -0.035

To understand the dynamics and flow of blood in the circulation, it is essential that the physiological levels of RBCs and organs involved the shape and size of RBCs must be physiologically good condition. In the following paragraph I will further discuss the results obtained from both the LM and SEM techniques.

5.7. Discussion

Hypercoagulability is a hallmark of all inflammatory conditions and previously we have shown that the addition of the same tiny concentrations (0.2 $ng.L^{-1}$) of LPS and added thrombin, to both whole blood and platelet poor plasma of healthy donors, leads to marked changes in the nature of the fibrin fibers (PNAS). This was shown with both SEM and fluorescence microscopy in chapter 4. We could also mimic this hypercoagulability with thromboelastography (representing viscoelastic properties of a blood clot). Because RBCs plays a fundamental role in clot structure and in hypercoagulability, and we already have shown that LPS directly affects fibrin clot structure (Pretorius 2016 Science), we also now looked at RBC structure in the presence of LPS.

We have seen that LPS changes the RBC shape as well as membrane structure (**Figure 4.2**). Due to the short exposure time (10 minutes) LPS and the changes we saw, implies that LPS must bind to the RBC membrane and probably do not follow a cytokine activation of TF pathway or a non-canonical pathway that involves inflammasome LPS activation independent of TLR4 (Hagar et al., 2013, Kayagaki et al., 2013). Such a non-canonical pathway involves the activation and secretion of cytokines such as IL-1 and TNF-ϐ (Kell and Pretorius, 2015c).

However, direct LPS binding in cells (other than RBCs) typically happens via binding to the TLR4 [for an extensive review, see (Kell and Pretorius, 2015c), where LPS bound to its binding protein, LPS-binding proteins (LBP), are is carried to the TLR4 receptor via a CD14 co-receptor, resulting in the activation and production (this is the canonical pathway) (Suraweera et al., 2015). However, as mentioned previously TLR4 and CD14 are not present on the RBC membrane. However, because of the absence of the two most well-known LPS receptors, other receptors or membrane binding components should be involved.

Although Burton and Bruce gave an comprehensive review on the macromolecules embedded within the RBC membrane (Burton and Bruce, 2011), our knowledge regarding RBC membrane invasion receptors are mostly limited to malaria parasite invasion receptors for a recent informative paper, on malaria and RBC membrane receptors see (Satchwell, 2016). **Table 5.1** shows of the most important RBC receptors (usually associated with malarial parasite attachment) and their possible association with direct LPS receptor binding. A literature search suggests that mainly glycophorin A, complement receptor 1 and also CD55 may be possible direct RBC binding sites for LPS

LM results: We see there is a statistical significant difference in membrane shape and size between the two groups according to the statistical results seen above. According to the results it is evidence that LPS does indeed create shape changes to the membranes of RBCs, the P-value was significant from the two sides as seen in **Table 5.1**.With decreased in deformation of RBCs, the flow dynamics is interrupted this is mostly seen in chronic inflammatory diseases such as Alzheimer's and stroke patients. Where researchers have proven that up regulation of inflammatory cells increases inflammation therefore decreases RBCs deformability.

Light microscopic micrographs of RBCs that have been exposed to LPS are elongated, dis-shaped and closely in contact with each other. A slight agglutination of RBCs is seen in some instances. Changes on RBCs membrane and integrity is a characteristic of inflammation in the blood. In this investigation we agree with Pretorius and colleagues who previously have suggested that RBCs can be used one of the inflammatory markers. This phenotype matched those that are seen in patients with inflammatory diseases. This was justified visually by axle ratios plots from the two groups in **Figure 5.5.** The two columns are almost the same height but the is a slight difference in the height measured.

I will further discuss the results obtained of RBCs deformed shape changes seen using SEM.

SEM Results: When looking at the morphology of the red blood cell using SEM we see there are slight changes or damage to the ultrastructure of RBCs membrane. In adding LPS to whole blood a matted deposit of plasma protein are visible on the surface to RBCs this is seen in **Figure 5.3.** this is seen in chronic inflammatory patients. This is justified by the results we obtained from the LM there RBCs before treatment are oval and healthy people sparely arranged. However this is not the case when we added LPS to whole blood the RBCs change shape and size.There is limited research that suggest LPS binding to RBC membranes may take place at other areas, not associated with receptor/ligand binding and that this LPS binding cause increased membrane protein mobility, mainly in the spectrin-actin complex in membrane cytoskeleton (leading to loss of membrane integrity) (Gwoździński et al., 2003b, Gwoździński et al., 2003a). However, these effects were much more profound in isolated membranes than in intact RBCs. In an earlier study, RBCs in whole blood treated with LPS, showed marked conformational alteration in the cytoskeletal proteins, which according to the authors may, in part, explain the marked reduction in RBC deformability during septic shock (Bellary et al., 1995b). LPS can also bind directly to sphingomyelin, which forms part of the phospholipid bilayer, and this leads to activation of acid sphingomyelinase and production of ceramide (Cuschieri et al., 2007). Ceramide is important in eryptosis (programmed RBC death) (Föller et al., 2008, Lang et al., 2012, Lang et al., 2013, Qadri et al., 2011) and acid sphingomyelinase enhances RBC-derived microparticle (MP) generation (Awojoodu et al., 2014). Also sphingosine 1-phosphate (S1P), is stored at high concentrations (1 μM) in RBCs) and LPS and S1P cooperate to increase the expression of proinflammatory molecules such as IL-6, cyclooxygenase-2, and prostacyclin (Fernández-Pisonero et al., 2012).

Currently, we do know that LPS forms a fundamental part of the dynamics of a variety of inflammatory diseases (Østerud et al., 2015b, Cunningham et al., 2005, Deng et al., 2014, Miklossy, 2008), and that LPS, in addition to (possibly low-grade) long-term inflammation via cytokine production, is fundamentally associated with hypercoagulability. In all hypercoagulable states, aberrant RBC structure, including a changed elasticity, has been confirmed, as the RBC is intimately associated with pathophysiological clotting, and mostly irreversibly trapped inside the matted fibrin clots (Bester et al., 2013b, Pretorius, 2013a, Pretorius et al., 2014b, Pretorius and Kell, 2014, Pretorius et al., 2016a, Pretorius et al., 2014c).

So, although we do not completely understand where LPS binds to the RBC membrane, we do know that it causes a significant change in RBC shape as seen with LM and in SEM, and we also see that it causes a changed membrane elasticity (seen with AFM).

The current results, together with our previous results that showed that LPS may at least (in part) be responsible for hypercoagulability, in inflammatory conditions, confirm that LPS presence does (in part) cause systemic pathophysiological coagulation together with a changed RBC structure. An urgent task now is to uncover the mechanism(s) of this acute and immediate effect, particularly to determine the RBC membrane LPS binding sites.

5.8. Conclusion Remarks

From the above results it I conclude that RBCs membranes are sensitive to LPS and it was seen that LPS does indeed cause shape changes and membrane deformities in RBCs. This may lead to increase in RBCs deformability, making it difficult for flow of blood in microvessls. This is seen in inflammatory conditions such as stroke, which makes one thing that LPS does indeed have an effect seen in chronic inflammatory disorders.

As we have seen structural changes in RBCs with SEM, the following chapter will look at whether the elasticity of the RBCs are changed due to the addition of LPS.

CHAPTER 6: Analysis of red blood cells membrane elasticity using Atomic Force Microscope

6.1 Chapter Objectives

In the previous chapter I discussed the effect of LPS on RBCs ultra structure and morphology, in this chapter I will analyse the effects of LPS on the local mechanical properties of the RBC membrane, using Atomic Force Microscopy (AFM). The elastic modulus of the membrane will be measured to get an overview of the effect of LPS on the membrane and ultimately the function of the RBCs.

6.2 Introduction

Atomic force microscopy (AFM) is a unique technique which enables the quantitative determination of microelastic properties of living cells in an aqueous environment (Radmacher et al., 1996) as well as in fixed and dehydrated cells (Yang et al., 2010). Recent AFM techniques enables the solving of a number of problems in cell biomechanics due to the simultaneous evaluation of local mechanical properties and topography of living cells at a very high spatial resolution and force sensitivity (Kuznetsova et al., 2007). Imaging of elastic properties with the AFM is possible using force modulation, a magnetic force modulation method to measure the stiffness and viscosity of living cells using a modified AFM apparatus (Rebêlo et al., 2014)

It is generally well accepted that cellular function is essentially determined by structure (Kuznetsova et al., 2007, Rebêlo et al., 2014). At various hierarchy levels found, the structural organization present in cells is characterized by mechanical properties. It is expected that cellular structure should vary, both in a variety of physiological processes (such as cell growth, differentiation, adhesion) and under pathogenesis (thrombosis, oxidative stress, inflammation) (Launspach et al., 2014, Kuznetsova et al., 2007). Three types of filamentous proteins are found in the cytoskeleton of cells; intermediate filaments, actin filaments (microfilaments), microtubules, and a wide variety of associated proteins. Together all these proteins are responsible for the general shape of the cell, the movement of the cell, and notably the generation of force (Prabhune et al., 2012, Martens and Radmacher, 2008).

Atomic force microscope utilizes a cantilever with a sharp probe to scan a specimen surface (Rebêlo et al., 2014). The cantilever is attached at one end to a piezoelectric displacement actuator controlled by the AFM. At the other end of the cantilever is the probe tip that interacts with the surface (Grobelny et al., 2011). At close proximity to the surface, the probe experiences a force (attractive or repulsive) due to surface interactions, which imposes a bending moment on the cantilever. In response to this moment, the cantilever deflects, and this deflection is measured using a laser beam that is reflected from a mirrored surface on the back side of the cantilever onto a split photodiode (Grobelny et al., 2011, Grobelny et al., 2006). **See Figure 6.1** for a schematic diagram of the AFM.

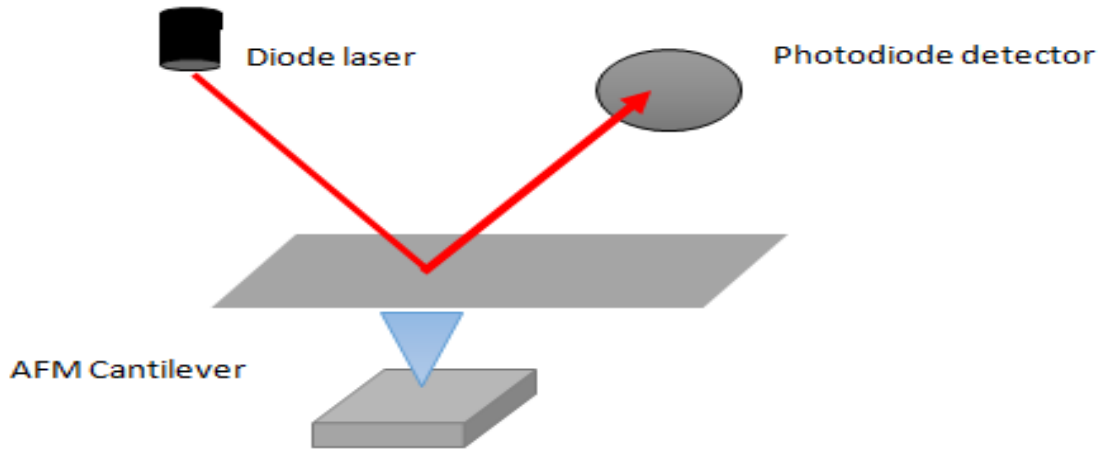


Figure 6.1: .Schematic diagram of the AFM. A laser beam is focused on the back of a cantilever that has a tip that interacts with a surface; the beam reflects into a four-quadrant photodetector. Normal forces between the tip and surface deflect the cantilever up or down. Lateral forces twist the cantilever left and right. These deflections are measured by monitoring the deflection of the reflected laser redrawn from (Grobelny et al., 2011)

Characterization of the cells was performed with a commercial AFM system (Dimension Icon with Scan Asyst, Bruker, USA) using the Peak Force™ QNM™ (Quantitative Nanomechanical Property Mapping) imaging mode. This method is similar to the standard tapping mode of scanning probe microscopy, where the probe and the sample are brought together occasionally, but in contrast to the more classical tapping mode (where the oscillation amplitude is kept constant), this mode operates by controlling the *maximum force* applied by the probe to the sample (Dufrêne, 2014).

At every pixel a rapid force-distance curve is performed and as the cantilever's deflection sensitivity and spring constant is calibrated before measurements, the curve can be analyzed quantitatively to obtain a series of specific property maps of the sample (Dufrêne, 2008, Kuznetsova et al., 2007).

The retract curve is used to calculate modulus and adhesion images (slope of the curve and the minimum of the curve, respectively). The variation between the zero and maximum force is used to calculate deformation. Energy dissipation can be measured as tip sample interactions as hysteresis is caused between the approach and retract curves, and by measuring the area between these curves the loss of mechanical energy can be determined (Berquand et al., 2005). See Figure 6.2 for a schematic diagram illustrating how the AFM measure the peak force of RBCs membrane elasticity from both the treated and untreated RBCs.

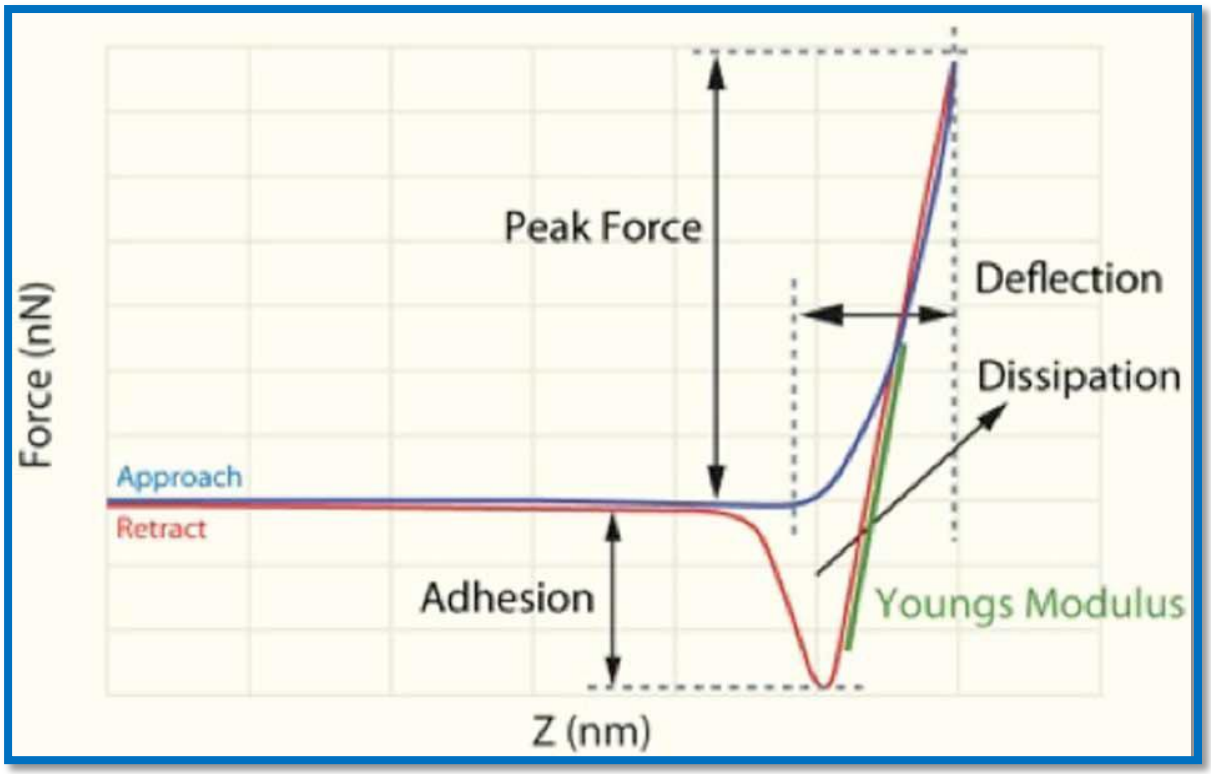


Figure 6.2: Schematic representation of force/separation plot illustrating the type of the information that can be obtained adapted from (Bester et al., 2013a)

Atomic force microscopy provides a novel technique for differentiating the mechanical properties of various cell types. Cell elasticity is abundantly used to represent the structural strength of cells in different conditions.

All of these cues show a significant influence on the cell elasticity assessment. Sharp AFM tips create a two-fold increase in the value of the effective Young's modulus (E_{eff}) relative to that of the blunt tips. Higher indenting force at the same loading rate generates higher estimated cell elasticity.

Increasing the operation temperature of the AFM leads to decreases in the cell stiffness because the structure of actin filaments becomes disorganized. The physiological cues include the presence of fetal bovine serum or extracellular matrix-coated surfaces, the culture passage number, and the culture density. Both fetal bovine serum and the extracellular matrix are critical for cells to maintain the integrity of actin filaments and consequently exhibit higher elasticity.

For researchers who use AFM to assess cell elasticity, results provide basic and significant information about the suitable selection of physical and physiological cues (Chiou et al., 2013). Scientists used this quantitative method to measure mechanics of biological cells (Sokolov et al., 2013). They demonstrated how the elastic modulus of the cell body should be measured when the cellular brush is taken into account. The brush is an essential inelastic part of the cell, which surrounds all eukaryotic (the brush is mostly microvilli and glycocalyx) and gram-negative prokaryotic cells (the brush is polysaccharides). The other main feature of the described method is the use of a relatively dull AFM probe to stay in the linear stress-strain regime. In particular they showed that the elastic modulus (aka the Young's modulus) of cells is independent of the indentation depth up to 10-20% deformation for the eukaryotic cells studied (Sokolov et al., 2013). Besides the elastic modulus, the method allowed obtaining the parameters of cellular brush, such as the effective length and grafting density of the brush. Although the method is demonstrated on eukaryotic cells, it is directly applicable for all types of cells, and even non-biological soft materials surrounded by either a brush or any field of long-range forces (Sokolov et al., 2013).

For this study we used the Young’s modulus to measure the elasticity of RBCs from LPS treated and untreated blood. With an intension to prove that LPS does indeed cause shape changes seen from **Chapter 5** (SEM).

6.3 Materials and methods

6.3.1. Samples preparations

We incubated blood from our control subjects with LPS as mentioned in the previous chapter to create our test, and for the control we used the same individual sample but without added LPS.

The following techniques were used to prepare sample for Atomic Force Microscopy:

Whole blood was centrifuged at 1000 rpm for 30 seconds. The supernatant (plasma, platelets and white blood cells) was discarded to obtain only the RBCs that will be investigated. The remaining pellet (RBCs) was suspended in 4% formaldehyde for 30 minutes, followed by rinsing three times with 0.075 M Phosphate Buffer, 10 minutes each (centrifuge the mixture, discard supernatant and re-suspend pellet). For the post-fix step the samples were be immersed in 1% OsO_4 for 30 minutes (to ensure the preservation of membrane phospholipids).

After this post-fixation step the samples were once again rinsed three times with 0.075 M Phosphate Buffer, 10 minutes each (centrifuge the mixture, discard supernatant and re-suspend pellet). This rinsing step was followed by dehydration of the RBCs in a series of ethanol, 10 minutes each 30%, 50%, 70%, 90%, 3x100% (to avoid osmotic shock), (centrifuge the mixture, discard supernatant and re-suspend pellet) for all 5 concentrations of ethanol. After the introduction of 100% (the third exposure) the pellet was then suspended in HMDS, for 30 minutes. This was followed by centrifuging the sample and discarding supernatant, then suspending the pellet in HMDS. A drop of the suspended material was dropped onto a glass cover-slip, ensuring an even distribution of cells and will be left to dry overnight before viewing on the AFM.

6.3.2 Imaging and probes used on the AFM

Quantitative determination of the local elastic properties of RBCs was carried out using the Dimension Icon AFM system manufactured by Bruker. Peak Force™ QNM™ tapping mode was used, which operates by applying a controllable, constant force at each data point and then using the resulting force-distant curve for image formation and further calculations.

Young's modulus describes tensile elasticity of an object (in our case) it is the tendency of an object to deform along an axis when opposing force is applied along that axis. To calculate the Young's modulus of each data point, the slope of the unloading or retract curve is fit to the using Derjaguin–Muller–Toporov (DMT) model, which is based on the well-known Hertz models geometry, but this model additionally considers attractive interactions. Silicon Nitride probes (TAP525 MPP 13120-10, Bruker, USA) with a force constant of 200 N/m (newton meter), a resonant frequency between 430 and 516 kHz (kilohertz) and a nominal tip radius of 15nm (nanometer) was employed in all AFM measurements.

A total number of 140 cells from each group was scanned by selecting a 1µm by 1µm scan area and carrying out force curves at 128 by 128 data points using a peak force of 6 µN. The outside of the cell was chosen for analysis as to avoid any variability that might be caused by the concavity of the cell center. Fifty force curves was chosen at random and was processed and fit to above mentioned DMT model by offline software (NanoScope Analysis version R3, Bruker, USA). Only force curves with a goodness of fit of 0.85 and above was used for elasticity measurements. To obtain images of the whole cell selected few samples was imaged using tapping mode using TESPA probes (Bruker, USA) with a spring constant of 20 – 80 N/m, a resonant frequency between 382 – 405 kHz and a nominal tip radius of 8 nm).

6.4. Results

It was found that RBCs membrane elasticity decreased comparing with the same individual's untreated whole blood. Looking at the **Figures 6.3.** AFM topography micrographs of RBCs from the same individuals captured when treated with LPS and untreated RBCs. Micrographs obtained from AFM in **Figure 6.3** (**A;B**) Shows RBCs from untreated whole blood these served as controls for the LPS test, after LPS has been added whole blood acute inflammation was seen proven see **Figure 6.3** (**B;D;E**) show the shape changes in RBC membrane. Where by micrograph **(E)** are shows the RBCs membrane roughness from treated whole blood samples. As it is known that elasticity of the RBC membrane is responsible for the integrity, function and shape of the biconcave RBCs, when affected the membrane will be deformed affected the movement and dynamic flow of blood.

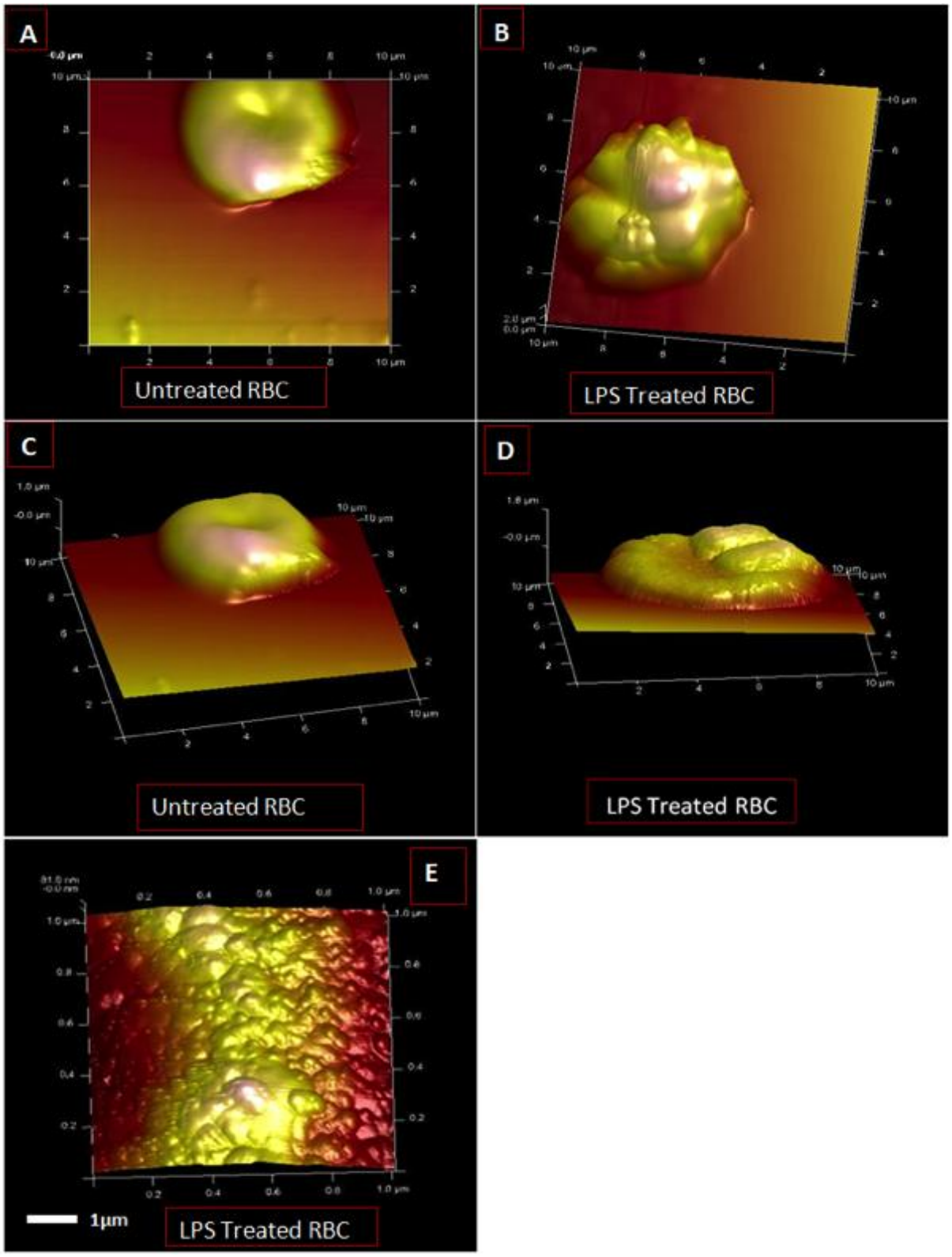


Figure 6.3: AFM topographical micrographs of RBCs (A) smooth membrane surface of RBC from untreated whole blood. (**B**) rough membrane of RBC from the same individual who's whole blood was treated with LPS.(C) Shows the top view of the membrane and (**D**) reveals the RBC membrane at an angle showing the consistent roughness of the membrane. (**E**) Rough membrane surface from LPS WB. Scale bar 1µm

A peak force QNM technology (Bruker) in the form of quantitative Force-distance curves and subsequent modulus value calculation were used to determine the elasticity of the RBCs (Bester et al., 2013a). For elastic modulus calculations see **Table 6.1**. it showed that the Young's modulus of RBCs taken from **LPS treated WB to be Mean of (51812.29MPa)** was statistically significant higher than the one obtained from RBCs taken sampled from **untreated WB to be Mean of (44784.18MPa)**. This increase in the Young's modulus explains the decrease in elasticity and this can be seen in the force-distance curves (Bester et al., 2013a) in **Figure 6.4**. where a steeper slope and decreased probe displacement of the high LPS treated RBCs curve is prominent. As seen with SEM micrographs the surfaces of the cell appear to have altered or increased in roughness. Our results show that with the same amount of force applied to the RBCs, the RBCs that have been introduced to LPS take longer to bounce back to their original shapes.

The force distance curves - (force) as a function of the distance travelled by the cantilever of tip on top of RBCs using the AFM

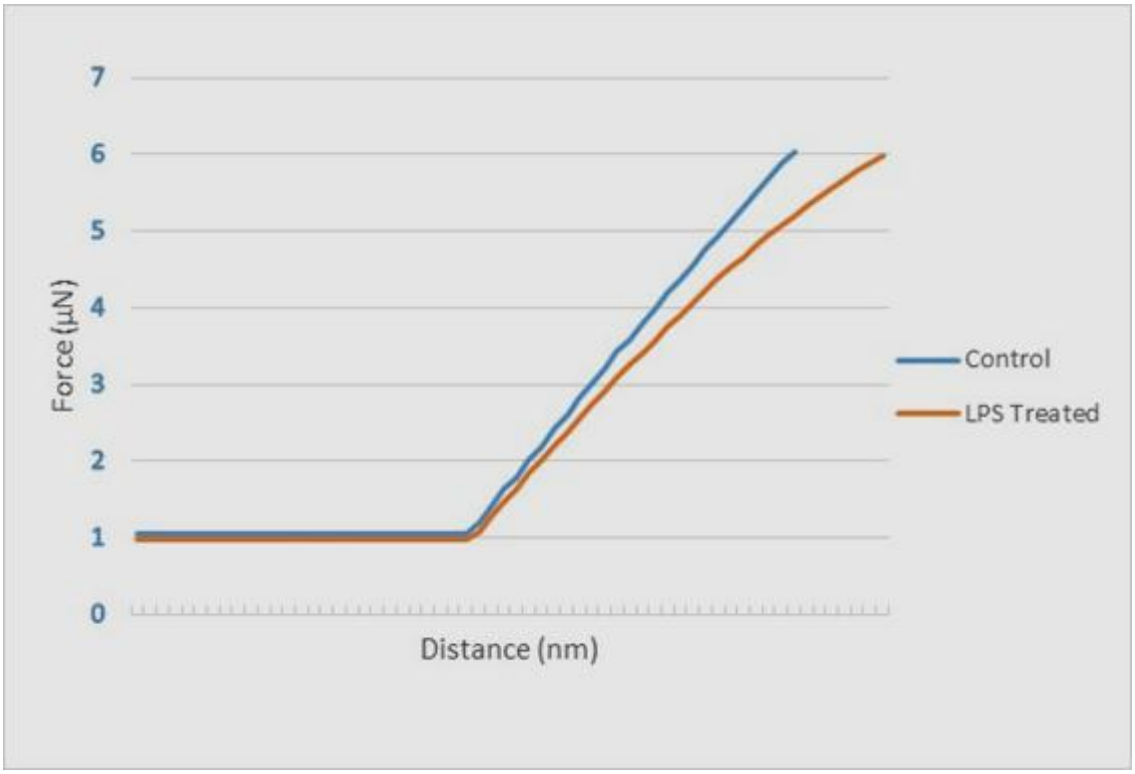


Figure 6.4: Force- distance curve shows the AFM cantilever deflection ranges on the surface of RBCs. The red line represents displacement curve of LPS treated RBCs and the blue line is the displacement curve of RBCs from normal healthy individuals 'controls'.

6.5. Statistical analysis

The Mann-Whitney U test was used to determine statistical values, the differences in elasticity between the two groups was statistically significant with a ($P < 0.0001$) see **Table 6.1** for elastic modulus statistical calculations**,** it is conclusive detailed comparative list of statistical values obtained from control versus LPS treated whole blood.

Table 6.1: Statistical analyses using Mann-Whitney U test for elasticity of membrane from LPS treated versus untreated blood

Description	Control statistic values	LPS treated statistic values
Total no. Of analyzed cells	5951	5571
Median	12597	18224
Mean	44784.2	51812.3
Mode	48372	51812
Standard Deviation	196047	465882.5
Kurtosis	1237.7	1720.7
Skewness	28.6	39.8
Range	10004974	22764135
Lower side $P < 0.0001$ (Results show there is a statistical difference between the two groups)		
Upper side $P > 0.9999$		
Two sided $P < 0.0001$		

95% confidence interval for difference between medians or means

6.6. Discussion

Red blood cells are highly deformable and elastic, these physical properties contributes significantly to assist in blood flow when RBCs circulates through the smallest blood vessels (Baskurt et al., 2004, Gallagher, 2013). A decrease in RBCs' deformation can lead to serious pathophysiology (Pretorius, 2013b, Bester et al., 2013a). The extent of deformability may be affected by many factors such as, alterations of the properties and associations of membrane skeletal proteins(Koshino et al., 2012).

In healthy individuals where the RBCs have optimal functioning, they have the ability to deform which improves blood flow in the micro-vessels at high shear rates (Shi et al., 2014).

Moreover the membrane structural design also plays an important role in the mechanical stability of the cells in the presence of shear forces (Nans et al., 2011). Based on the results that were obtained from AFM, it is shown that LPS treated RBCs are deformed and irregular shaped see **Figure 6.3** LPS treated RBCs are lumpy and do not have the biconcave structure of normal RBCs. A change in structre and membrane of the treated RBCs was confirmed with statistical tools see **Table 6.1.** an increase in the Young's modulus values. According to researchers that have use the same technique (AFM) they established that a decrease in elasticity results in a decrease in RBC deformation (Bester et al., 2013a). In **Figure 6.4** the force distance curve it is evident that LPS treated RBCs are irregular in shape seen by an increase in the distance travelled by the tip when force is applied onto the RBCs' membrane. These results coincides with previous research that was conducted by Dulinska and colleague, they found that an increase in Young's modulus values resulted in pathological RBCs (Dulińska-Molak et al., 2014).

In a study conducted by (Pöschl et al., 2003) they found that RBCs deformation is decreased in septic patients, after *in vitro* incubation of washed red blood cells with lipid A and of whole blood with LPS. They found that LPS did not influence red blood cell deformation after incubation with washed red blood cells. For their research the decrease of RBCs deformation was related to the amount of hydroxymyristic acid measured in red blood cell membranes, suggesting that endotoxin binding directly affects mechanical properties of red blood cells. We on the other hand found that LPS does indeed have an effect directly on the RBCs membrane and causes decrease the elasticity. However the differences between the two findings is highly due to the fact that in the research conducted by (Pöschl et al., 2003) and his team they used washed RBCs, we believe that it is the washing of cells that acts as a detergents on RBCs decreasing the binding of LPS to RBCs, Kell and Colleagues also agree (Kell and Pretorius, 2015b).

Our results show a significant difference between the two groups i.e. untreated and treated RBCs ($P < 0.0001$) this P-value is significant, and also there is a strong 95% confidence interval for difference seen between medians or means (**see Table 6.1**). Our results prove that LPS when binds to RBCs causes a decrease in deformation and elasticity of membrane.

Researchers have suggested that the elasticity of RBCs depends on the dynamic rearrangement of spectrin dimmers otherwise known as tetramers under the shearing forces experienced in circulation (Baines, 2009); and that stable intact helical linker regions are needed to maintain the soft elasticity of spectrin (Martens and Radmacher, 2008).

As mentioned previously in chapter 2, **LPS DOES NOT** bind to RBCs (there is no evidence that LPS can bind directly to RBCs). Through research, scientists found that LBP in blood facilitates the binding of LPS to CD14, a glycoprotein which is expressed only on monocytes and neutrophils **BUT NOT ON RBCs**. Scientists however found that the LPS-binding protein found in blood facilitates the binding of LPS to RBCs (Ding et al., 2013, Opal et al., 1999, Tsutsumi-Ishii et al., 2008).

Never the less some researchers have found that the binding of LPS to RBCs membrane therefore correlates with the presence of hydrophobic nature of both the LPS molecule as well as the RBC membrane (Kell and Pretorius, 2015b, Peters et al., 2009). Hydrophic LPS can potentially interact with a great many lipoproteins, and LPS also has the ability to convert prions to their more toxic form (Kell and Pretorius, 2015b), causing more stress to cells, as seen by the decrease deformability and increasing the stiffness of RBCs membrane with AFM (Bester et al., 2013a, Kuznetsova et al., 2007). A decreased deformability is also seen in inflammatory conditions such as diabetes (Shin et al., 2007).

Therefore it seems as if a decreased deformability and increase in stiffness is common in inflammatory disease, and can be ascribed to a changed elasticity as well as stability of RBCs' membrane. Changes in elasticity impact on the membrane fluidity and deformability of the cell as it moves through the circulation. This may result in atypical tissue perfusion, leading to functional deteriorations, ultimately resulting in disturbed vascular properties (Baskurt and Meiselman, 2012, Baskurt and Meiselman, 2013). **See Figure 6.8**. for a brief summary of the RBCs chapters as discussed in this chapter.

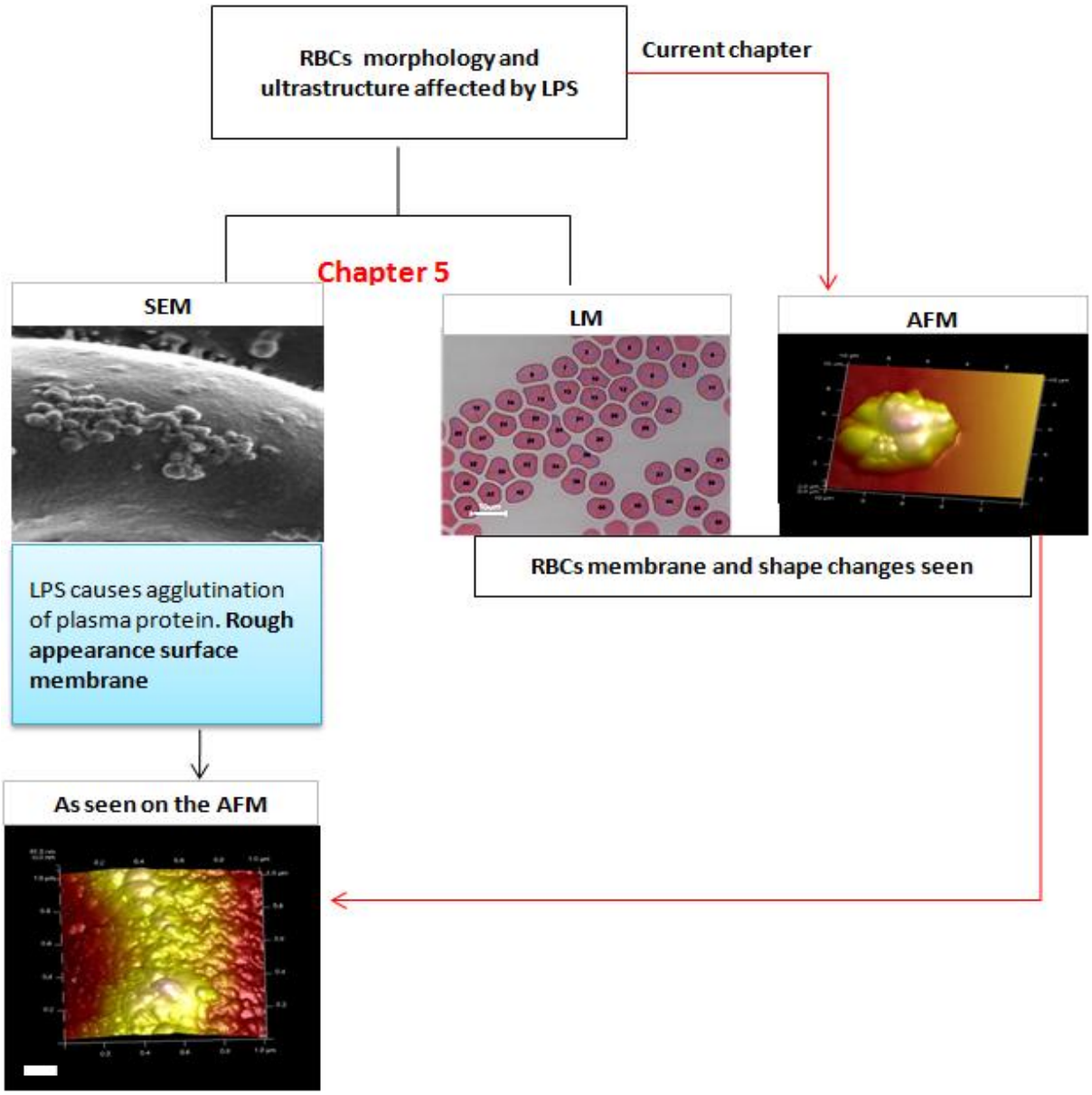


Figure 6.5: Summary of the RBCs chapters

6.7. Conclusion remarks

Based on these results it is evidence that LPS can elucidate inflammation causing shape changes typically affecting the elasticity of the membranes, seen as seen with the AFM. The decrease in elasticity of RBCs membrane decreases the RBCs deformability (affecting the blood flow) which may results in pathophysiological effects as seen in chronic inflammation disorders.

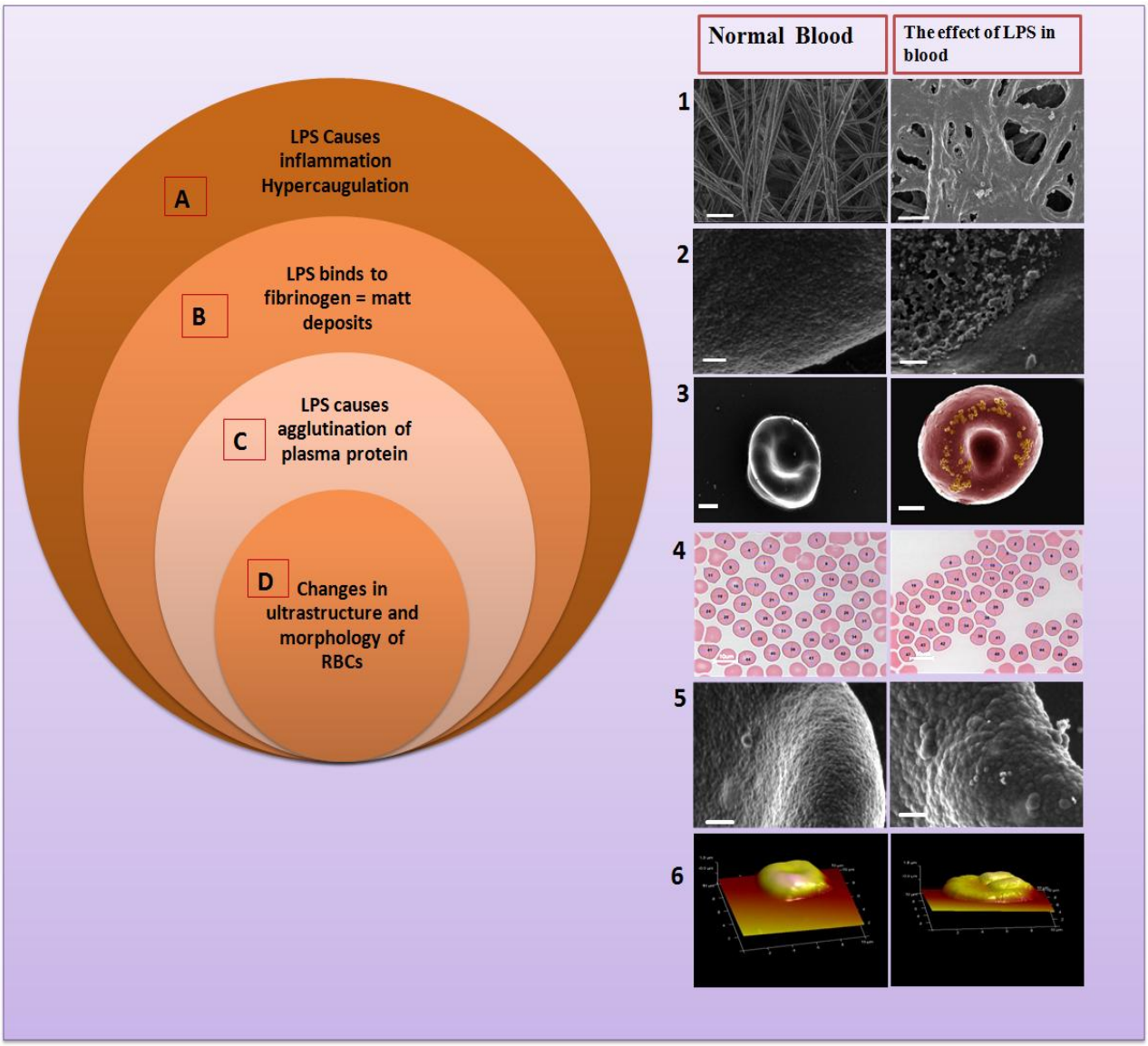
CHAPTER 7: Conclusion

7.1. Chapter objective: In this chapter my main focus is to sum up all the our research chapters (4; 5 and 6) in this thesis **see Figure 7.1**.for a conclusive diagram

Based on these results it is evidence that this endotoxin (LPS) molecule can elicit inflammation causing shape and elastic changes seen on the RBCs membrane when viewing with the AFM, SEM and LM. The change in RBC membrane structure was indicated by a decrease in membrane elasticity in samples that were treated with LPS (when tested with AFM). A decrease in elasticity of RBCs membrane decreases the RBCs deformability which results in pathophysiological effects as seen in chronic inflammation disorders.

It was shown directly that very low concentrations of LPS can affect the coagulation properties of blood and plasma significantly using SEM and TEG. These techniques showed a rise in clotting time and strength of these clots formed were stronger indicating that LPS causes Hypercaugulation using TEG. These dense clotting trends were seen when viewing with SEM- matted mass deposits were seen on fibrin fibers. These masses are common trends seen in chronic inflammatory diseases. All these inflammatory reactions were mediated by the direct binding of LPS to fibrinogen monomers as assessed with ITC. It seems that this endotoxin is highly inflammatory and poses very devastating effects in our systems even at low physiological concentrations of 0.2ng.L^{-1}. **Results shown here prove without doubt that LPS does indeed play a role in chronic systemic inflammatory diseases, when there is a bacterial component present.**

See Figure 7.1 for a full description of thesis research outcomes.



Matches of figures and descriptions	
Description	**Figures**
A	1
B	1,2
C	2
D	3;4;5;6

Figure 7.1: Summary of all results outcomes from on this thesis

7.2. Future research and comments from the author

Judging from the results that were obtained from the study it is clear now that LPS does indeed change the surface roughness of RBCs. Therefore the actual measurements of membrane roughness could be implemented in feature research using AFM. It was found that LPS binds to fibrinogen using TIC and SEM, but the question now lies in where on the RBCs does the LPS molecule bind to? It would be advantageous to identify the precise LPS binding site on the RBC for future treatment. The mass spectrometer was used to find the mass of individual masses of both fibrinogen and LPS and mass when they have bonded, we used this technique to confirm our SEM results. The results we got were adequate but future referencing of better methods to calculate mass of bonded products would be beneficial.

Appendix 1: Patients Consent Forms

INFORMATION LEAFLET AND INFORMED CONSENT FORM:

Ethics number:

TITLE OF STUDY:

The effect of the neurotoxin, lipopolysaccharide on the coagulability and red blood cell ultrastructure of blood from healthy individuals

You act as a healthy control blood donor for this study

Principal investigator: Sthembile Mbotwe; Department of Physiology

INTRODUCTION

You are invited to participate in a research study where we are looking at blood cells, conducted by the Department of Physiology (School of Health Sciences) from the University of Pretoria. This information leaflet is to help you to decide if you would like to participate. Before you agree to take part in this study you should fully understand what is involved. The medical practitioner will explain the reason for the drawing of blood. One tube will be given to the research team at the University of Pretoria for research purposes, and the second blood tube will be sent to a Pathology laboratory to determine your iron levels.

PURPOSE OF STUDY

The researcher is investigating how your blood, consisting of red blood cells, platelets and fibrin, will be influenced by adding LPS (neurotoxins) to your blood. We will view your blood under a specialized microscope that can magnify up to 100 000x (called an electron microscope). The iron levels will be used to show high or low iron levels, this will give us an indication of any inflammatory diseases that could be present. That could influence the shape of the red blood cells, platelets and fibrin.

Who will draw the blood?

Dr Prashilla Soma will draw 3 blood tubes from you.

PROCEDURES

Three tubes of blood will be drawn. **TUBE 1 and 2:** Blood will be drawn in a citrate tube and EDTA tube. This blood will be given to Sthembile Mbotwe, who will use it to study your blood cells and fibrin, using specialised microscopes. **TUBE 3:** A third blood tube will be drawn to determine your iron levels.

HAS THE TRIAL RECEIVED ETHICAL APPROVAL?

The Faculty of Health Sciences Research Ethics Committee, University of Pretoria, approved this protocol and the committee has granted written approval. 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

The current laboratory study is not intended to benefit you, but will add to our knowledge about your blood clotting patterns, red blood cell ultrastructure influenced by neurotoxins introduced to your blood.

MAY THE PROCEDURES RESULT IN DISCOMFORT OR INCONVENIENCE OR SIDE EFFECTS?

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

INSURANCE AND FINANCIAL ARRANGEMENTS

Neither you nor your medical scheme will be expected to pay for your participation in the study. No study-related injury is expected, as a Clinician takes the blood.

CONFIDENTIALITY

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

ADDITIONAL CONSIDERATIONS

Our research group has various on-going studies, including a study where we use control samples (ethics approval number 506/2014: E Pretorius).

All excess blood (plasma and whole blood) not used in the experiments discussed in this protocol will be stored in formaldehyde or at -70°C and will used, under ethics approval 506/2014, in order not to recollect or waste healthy control samples.

INFORMED CONSENT

I hereby confirm that I have been informed by a 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.

Healthy donor's name (Please print)

……

Patient's signature ……..**Date**……..

Patient ID number:……

Principal investigator's signature (Sthembile Mbotwe)

……

INFORMATION FILLED IN BY Dr SOMA/Phlebotomist

Age	
Sex	
Inflammatory condition?	
Does the donor smoke?	
Is the donor taking birth controls if female	
Any other chronic condition?	
Pills that the donor uses?	

Appendix 2: Letter from Biostatistician

Date: 24 / 3 /2015

LETTER OF CLEARANCE FROM THE BIOSTATISTICIAN

This letter is to confirm that the student(s),

with the Name(s) Ms Sthembile Mbatsi

Studying at the University of Pretoria

discussed the Project with the title The effect of the neurotoxin, Lipopolysaccharide on the coagubility and red blood cell ultrastructure of blood from healthy individuals

with me.

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

The analytical tool that will be used will be Pilot study to estimate parameters required to estimate sample size in main study. Data analysis for main study will employ descriptive statistics, Hotelling's paired T^2 test, Students paired t-test. Please also refer to achieve the objective(s) of the study. to section from protocol.

Name PJ Becker Date 24/3/15

Signature Tel: 012-319-2203

Department or Unit Research Office, Fac Health Sci, UP

BIOSTATISTICS
Faculty of Health Sciences
Research Office
2015 -03- 24
UNIVERSITY OF PRETORIA

Official Stamp of Biostatistician

Appendix 3: Ethics approval letter

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002667, Approved dd 22 May 2002 and Expires 23 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

20/08/2015

Approval Certificate
New Application

Ethics Reference No.: 213/2015

Title: The effect of the neurotoxin, lipopolysaccharide on the coagulability and red blood cell ultrastructure of blood from healthy individuals

Dear Ms Sthembile Mbatwa

The **New Application** as supported by documents specified in your cover letter dated 6/07/2015 for your research received on the 11/08/2015, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 12/08/2015.

Please note the following about your ethics approval:
- Ethics Approval is valid for 2 years
- Please remember to use your protocol number (**213/2015**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

012 354 1677 0866518047 deepeka.behari@up.ac.za http://www.healthethics-up.co.za
Private Bag X323, Arcadia, 0007 - 31 Bophelo Road, HW Snyman South Building, Level 2, Room 2.33, Gezina, Pretoria

Appendix 4: LPS (1) O111:B4 data sheet

SIGMA®

3050 Spruce Street
Saint Louis, Missouri 63103 USA
Telephone 800-325-5832 · (314) 771-5765
Fax (314) 286-7828
email: techserv@sial.com
sigma-aldrich.com

ProductInformation

Lipopolysaccharides
from *Escherichia coli* O111:B4

Product Number **L 2630**
Storage Temperature 2-8 °C

Product Description

Synonym: LPS

This product is phenol extracted from *E. coli* serotype O111:B4. The source strain is from a private collection. This LPS serotype has been used to stimulate B-cells[22] and induce NOS in human hepatocytes.[23]

Lipopolysaccharides (LPS) are characteristic components of the cell wall of Gram negative bacteria; they are not found in Gram positive bacteria. They are localized in the outer layer of the membrane and are, in noncapsulated strains, exposed on the cell surface. They contribute to the integrity of the outer membrane, and protect the cell against the action of bile salts and lipophilic antibiotics.[1]

Lipopolysaccharides are made up of a hydrophobic lipid (lipid A, which is responsible for the toxic properties of the molecule), a hydrophilic core polysaccharide chain, and a hydrophilic O-antigenic polysaccharide side chain. In most cases, O-specific chains are built of repeating units of oligosaccharides which exhibit a strain-specific structural diversity. The sugar constituents, their sequence, and their mode of linkage determine the serological O specificity of respective strains. They are the main determinants of the classifications of the serotypes of *Salmonella* species. The diversity of O chains in *Enterobacteriaceae* may have developed during evolution to allow enteric bacterial to escape the host's immune system by developing new specificities on their cell surface (specific to the bacterial serotype).[1]

Since lipopolysaccharides confer antigenic properties on the cell, they have been termed O antigens. As the main antigen, lipopolysaccharides are involved in various host-parasite interactions. They seem to protect Gram negative bacteria from phagocytosis and lysis.[1] Bacteria with common serotypes have surface antigens (group O, group H, or LPS) which generate the same antibody response. Examples of serotypes are O55:B5 and O26:B6 for the *E. coli* bacterium. The designations are immunological classifications, which specify which antibody recognized which strains. Different strains may have some common antigenic determinants.

If a wild strain of bacterium is irradiated with UV light or exposed to mutagenic compounds, it will mutate. The few mutations that are not lethal result in viable mutants (rough strains) which are generally not found in nature, and which possess some unique characteristics. The genes that encode lipopolysaccharide formation may also be altered in the mutants, and LPS with shorter polysaccharide chains may be formed. Ra, Rb, Rc, Rd, Re, etc. (where a, b, c, etc... designate 1st, 2nd, 3rd, etc... degree, respectively) designate the polysaccharide length of a given LPS. Ra and Re designate the mutants with the longest and shortest chain lengths, respectively.[2] The most extreme mutants are the Re mutants which produce an LPS which is made up of Lipid A and 3-deoxy-D-manno-octulosonic acid (2-Keto-3-deoxyoctonate, KDO) as the sole constituent of the core.[2] Lipid A and lipopolysaccharides from rough strains are tested for KDO content.[3]

Purified endotoxin is generally referred to as lipopolysaccharide or LPS, to distinguish it from the more natural complexed cell membrane associated form. The core portion of the polysaccharide chain is common to LPS from wild and mutant bacterial strains.

Removal by hydrolysis of the polysaccharide chain from LPS produces lipid A, either as the naturally occurring, cytotoxic diphosphoryl form[4] or the less toxic, monophosphoryl form.[5,6] The longer the polysaccharide chain is, the longer and more difficult the hydrolysis. LPS with a long polysaccharide chain has a relatively low lipid A content, which must be purified from a large amount of hydrolysis byproducts (oligosaccharides and saccharide monomers). Thus, the yield of lipid A is low and recovery is poor. LPS with a short polysaccharide chain (LPS from mutant bacteria) is therefore used to produce lipid A products. Removal of the fatty acid portions of lipid A results in a detoxified LPS[7] with an endotoxin level about 10,000 times lower than that of the parent LPS.

The molecular structure of LPS has been studied.[8,9] Since LPS is heterogeneous and tends to form aggregates of varying sizes, the molecular weight is not very meaningful. However, there is a reported range of 1-4 million or greater. When the LPS is treated with SDS and heat, the molecular weight is in the range of 50 to 100 kDa. In their purest form, in the presence of strong surface active agents, and in the absence of divalent cations, bacterial endotoxins consist of 10-20 kDa macromolecules. In the absence of surface active agents and in the presence of divalent cation sequestering agents such as EDTA, LPS is believed to arrange itself into a micellar structure with a molecular weight of approximately 1,000 kDa. This is the smallest form of bacterial LPS that is likely to exist in aqueous liquids. In the presence of divalent cations such as Ca^{2+} and Mg^{2+}, a bilayer structure appears to exist that passes through a 0.2 µm membrane, but does not pass through a 0.025 µm membrane. LPS vesicles up to 0.1 µm in diameter may also be formed in water in the presence of divalent cations. The self aggregation of LPS is generally a function of the lipid A component of the molecule, which also confers the ability to bind to hydrophobic surfaces.

LPS can be prepared by TCA,[10] phenol,[11] or phenol-chloroform-petroleum ether (for rough strains)[12] extraction. The TCA extracted lipopolysaccharides are structurally similar to the phenol extracted ones. Their electrophoretic pattern and endotoxicity are similar. The main differences are in the amounts of nucleic acid and protein contaminations. The TCA extract contains approximately 2% RNA and approximately 10% denatured proteins. The phenol extract contains up to 60% RNA and less than 1% protein. Purification by gel filtration chromatography removes much of protein present in the phenol-extracted LPS, but leaves a product that still contains 10-20% nucleic acids. Further purification using ion exchange chromatography, will yield an LPS product which contains <1% protein and <1% RNA. Sigma offers LPS with various levels of protein and/or RNA.

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LPS preparations are used extensively for research in the elucidation of LPS structure,[13] metabolism,[14] immunology,[15] physiology,[16] toxicity,[17] and biosynthesis.[18] They have also been used to induce synthesis and secretion of growth promoting factors such as interleukins.[19]

FITC (fluorescein isothiocyanate), TRITC (tetramethyrhodamine isothiocyanate), and TNP (trinitrophenyl) conjugates have been prepared by reacting LPS with either FITC, TRITC or 2,4,6-trinitrobenzenesulfonic acid, respectively.[20] They are used in research on the T-independent B cell immune response to bacterial LPS.[20]

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For cell culture use, LPS should be reconstituted by adding 1 ml of sterile balanced salt solution or cell culture medium to a vial (1 mg) and swirling gently until the powder dissolves. Solutions can be further diluted to the desired working concentration with additional sterile balanced salt solutions or cell culture media.

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Appendix 5: LPS (2) O26:B6 data sheet

SIGMA

3050 Spruce Street
Saint Louis, Missouri 63103 USA
Telephone 800-325-3010 • (314) 771-5765
Fax (800) 325-5052
email: techserv@sial.com
sigma-aldrich.com

ProductInformation

Lipopolysaccharides
from *Escherichia coli* O26:B6

Product Number **L 2762**
Storage Temperature 2-8 °C

Product Description
Synonym: LPS

This product is extracted from *E. coli* serotype O26:B6 and purified by gel filtration. The source strain is ATCC 12795.

LPS from serotype 026:B6 is the only LPS from *E. coli* which exhibits short chain-length behavior on SDS-PAGE. The short chain-length of this LPS is closer to that of the mutant rough strain LPS.

This LPS serotype has been used at 1 µg/ml to stimulate neutrophils.[22]

Lipopolysaccharides (LPS) are characteristic components of the cell wall of Gram negative bacteria; they are not found in Gram positive bacteria. They are localized in the outer layer of the membrane and are, in noncapsulated strains, exposed on the cell surface. They contribute to the integrity of the outer membrane, and protect the cell against the action of bile salts and lipophilic antibiotics.[1]

Lipopolysaccharides are made up of a hydrophobic lipid (lipid A, which is responsible for the toxic properties of the molecule), a hydrophilic core polysaccharide chain, and a hydrophilic O-antigenic polysaccharide side chain. In most cases, O-specific chains are built of repeating units of oligosaccharides which exhibit a strain-specific structural diversity. The sugar constituents, their sequence, and their mode of linkage determine the serological O specificity of respective strains. They are the main determinants of the classifications of the serotypes of *Salmonella* species. The diversity of O chains in *Enterobacteriaceae* may have developed during evolution to allow enteric bacterial to escape the host's immune system by developing new specificities on their cell surface (specific to the bacterial serotype).[1]

Since lipopolysaccharides confer antigenic properties on the cell, they have been termed O antigens. As the main antigen, lipopolysaccharides are involved in various host-parasite interactions. They seem to protect Gram negative bacteria from phagocytosis and lysis.[1] Bacteria with common serotypes have surface antigens (group O, group H, or LPS) which generate the same antibody response. Examples of serotypes are O55:B5 and O26:B6 for the *E. coli* bacterium. The designations are immunological classifications, which specify which antibody recognized which strains. Different strains may have some common antigenic determinants.

If a wild strain of bacterium is irradiated with UV light or exposed to mutagenic compounds, it will mutate. The few mutations that are not lethal result in viable mutants (rough strains) which are generally not found in nature, and which possess some unique characteristics. The genes that encode lipopolysaccharide formation may also be altered in the mutants, and LPS with shorter polysaccharide chains may be formed. Ra, Rb, Rc, Rd, Re, etc. (where a, b, c, etc... designate 1st, 2nd, 3rd, etc... degree, respectively) designate the polysaccharide length of a given LPS. Ra and Re designate the mutants with the longest and shortest chain lengths, respectively.[2] The most extreme mutants are the Re mutants which produce an LPS which is made up of Lipid A and 3-deoxy-D-manno-octulosonic acid (2-Keto-3-deoxyoctonate, KDO) as the sole constituent of the core.[2] Lipid A and lipopolysaccharides from rough strains are tested for KDO content.[3]

Purified endotoxin is generally referred to as lipopolysaccharide or LPS, to distinguish it from the more natural complexed cell membrane associated form. The core portion of the polysaccharide chain is common to LPS from wild and mutant bacterial strains.

Removal by hydrolysis of the polysaccharide chain from LPS produces lipid A, either as the naturally occurring, cytotoxic diphosphoryl form[4] or the less toxic, monophosphoryl form.[5,6] The longer the polysaccharide chain is, the longer and more difficult the hydrolysis. LPS with a long polysaccharide chain has a relatively low lipid A content, which must be purified from a large amount of hydrolysis byproducts (oligosaccharides and saccharide monomers). Thus, the yield of lipid A is low and recovery is poor. LPS with a short polysaccharide chain (LPS from mutant bacteria) is therefore used to produce lipid A products. Removal of the fatty acid portions of lipid A results in a detoxified LPS[7] with an endotoxin level about 10,000 times lower than that of the parent LPS.

The molecular structure of LPS has been studied.[8,9] Since LPS is heterogeneous and tends to form aggregates of varying sizes, the molecular weight is not very meaningful. However, there is a reported range of 1-4 million or greater. When the LPS is treated with SDS and heat, the molecular weight is in the range of 50 to 100 kDa. In their purest form, in the presence of strong surface active agents, and in the absence of divalent cations, bacterial endotoxins consist of 10-20 kDa macromolecules. In the absence of surface active agents and in the presence of divalent cation sequestering agents such as EDTA, LPS is believed to arrange itself into a micellar structure with a molecular weight of approximately 1,000 kDa. This is the smallest form of bacterial LPS that is likely to exist in aqueous liquids. In the presence of divalent cations such as Ca^{2+} and Mg^{2+}, a bilayer structure appears to exist that passes through a 0.2 µm membrane, but does not pass through a 0.025 µm membrane. LPS vesicles up to 0.1 µm in diameter may also be formed in water in the presence of divalent cations. The self aggregation of LPS is generally a function of the lipid A component of the molecule, which also confers the ability to bind to hydrophobic surfaces.

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LPS Table

Source organism	Extraction method	Gel Filtration	Gel Filtration γ-irr.	Ion-exchange	Detoxified	Gel Filtration FITC label	Gel Filtration TNP label
O26:B6 *E. coli*	Phenol - L8274 TCA – L3755	L2762	L2654			F7037*	T7143*
O55:B5 *E. coli*	Phenol - L2880 TCA – L4005	L2637	L6529	L4524	L9023	F8666	T6682
O111:B4 *E. coli*	Phenol – L2630 TCA – L4130	L3012	L4391	L3024	L3023	F3665	T3382
O127:B8 *E. coli*	Phenol – L3129 TCA – L3880	L3137	L4516	L5024	L8654	F3540*	
O128:B12 *E. coli*	Phenol – L2755 TCA – L4255	L2887					T6769
E. coli EH-100 (Ra mutant)	Ph/Chl/Pet - L9641						
E. coli F-583 (Rd mutant)	Ph/Chl/Pet – L6893						
E. coli J5 (Rc mutant)	Ph/Chl/Pet – L5014		L7520*				
E. coli K-235	Phenol – L2143 TCA – L2268	L2018					
Klebsiella pneumoniae	Phenol – L4268 TCA – L1519	L1770*					
Pseudomonas aeroginosa 10	Phenol – L9143 TCA – L7018	L8643					
Salmonella abortus equi	Phenol - L5886 TCA – L6636	L1887					
Salmonella enteritidis	Phenol - L6011 TCA – L6761	L2012	L7770	L4774	L3773		
Salmonella minnesota	Phenol - L6261 TCA – L7011	L2137	L4641		L1523*	F4665*	T3520*
Salmonella minnesota strain R5	Ph/Chl/Pet - L8893						
Salmonella minnesota strain R7 (Rd mutant)	Ph/Chl/Pet - L9391						
Salmonella minnesota strain Re595 (Re mutant)	Ph/Chl/Pet - L9764		L7645*				
Salmonella typhimurium	Phenol – L6511 TCA - L7261	L2262	L6143		L2525	F4790*	T4145*
Salmonella typhimurium strain SL684 (Rc mutant)	Ph/Chl/Pet - L5891						
Salmonella typhimurium strain SL1181 (Re mutant)	Ph/Chl/Pet - L9516						
Salmonella typhimurium strain TV119 (Ra mutant)	Ph/Chl/Pet - L6016						
Salmonella typhosa	Phenol - L6386 TCA - L7136	L2387	L7895			F4292*	
Serratia marcescens	Phenol - L6136	L2512*	L4766*				
Shigella Flexneri A1	Phenol – L4393 TCA – L7143	L9018					
Shigella flexneri (Re mutant)	Ph/Chl/Pet - L6643						
Vibrio cholerae serotype Inaba 569B	Phenol - L0385					F5009*	T1271*

* = discontinued product number
Ph/Chl/Pet = phenol:chloroform:petroleum ether

Appendix 6: Publications and conference attendance

Article

[1,*]Etheresia Pretorius, [1]Sthembile Mbotwe, [1]Janette Bester, [2,4,5]Christopher Robinson & [3,4,5,*]Douglas B. Kell. Acute induction of anomalous blood clotting by highly substoichiometric leves of bacterial lipopolysaccharide **(***Submitted to Royal society***)**

Conference

MMSA presentation Microscopy Society of South Africa (MSSA) 2015

Oral

The effect of the neurotoxin lipopolysaccharide on the coagulability of plasma from healthy individuals

S Mbotwe, E Pretorius

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