The effects of bacterial inflammagens from *Porphyromonas gingivalis* on blood clotting

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

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Declaration of Originality

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Abstract

Introduction: Abnormal blood coagulation, systemic inflammation and microbial dysbiosis are shared pathological characteristics of cardiovascular and neurodegenerative diseases. In addition, defective clotting processes and vascular complications are proposed as prominent links between these two disorders as they present simultaneously in respective patients. Another seemingly unrelated pathology that is seen associated with cardiovascular and neurodegenerative disease is periodontitis – a chronic inflammatory condition characterized by oral tissue degradation which is predominantly caused and driven by dysbiotic microbe populations. Out of several species of bacteria that have been identified to contribute to the pathogenesis of periodontal disease, *Porphyromonas gingivalis* – a gram negative anaerobe – has been deemed as a keystone pathogen capable of causing periodontitis in solitude. This bacterium has been implicated in both cardiovascular disease (CVD) and neurodegeneration. In this current study, the aims are to identify an integral virulent product, specifically a protease called gingipain R1 (RgpA), from *P. gingivalis* in the blood of individuals suffering from neurodegeneration and determine the effects that this protease and lipopolysaccharide (LPS) from the same species impose on normostatic blood coagulation. Research on the effect of these bacterial inflammagens on clot kinetics, rheology and fibrin network formation is and thus emphasizes the novelty of this study. The primary neurodegenerative disease of choice is Parkinson's disease (PD) as it is also significantly associated with periodontal pathologies and hence likely involves *P. gingivalis* infection exterior to the oral cavity. We also probed for its protease in the haematological system of patients with Alzheimer's disease (AD), another neurodegenerative closely correlated to periodontitis. Identifying membrane components of this oral pathogen in the blood of PD patients, and other neurodegenerative diseases such as AD, is of particular importance and may provide further insight into the early pathogenesis and cardiovascular involvement in neurodegenerative disease as a whole.

Aims and objectives: This thesis is divided into two main objectives. The first being the identification of a RgpA produced by *P. gingivalis* in blood samples of PD and AD patients; the second objective is to study the effects of this bacterium's protease and LPS on coagulation using a healthy blood model and a purified fibrinogen model. To achieve the first objective, the aim is to identify, using polyclonal antibodies against the protease in question, RgpA, in the blood of individuals suffering from PD and AD. To achieve the second objective of this study we characterized the effects of RgpA and LPS on blood coagulation kinetics and terminal fibrin network structure by using a healthy blood model as well as a purified fibrinogen model. The techniques utilized include thromboelastography, rheometry, confocal microscopy and scanning electron microscopy (SEM).

Results: Using a polyclonal antibody against RgpA, it is shown that RgpA is present in the haematological system of both a PD population and AD population; this finding was quantitatively significant ($p < 0.0001$) in both groups as measured *via* fluorescent intensity. Additionally, the impact of RgpA on the viscoelastic parameters of blood clotting is extensive. RgpA exerts an inhibitory effect on clot kinetics, increases the stiffness of fibrin networks and decreases the total clot load *via* fibrin(ogen)olytic mechanisms in platelet-poor plasma (PPP) and whole-blood (WB). In purified fibrinogen models, however, this effect was exaggerated as minimal or no fibrin formed after RgpA incubation. This effect was abrogated in the presence of LPS from *P. gingivalis* whereby a decrease in fibrin load was absent; however, LPS still induced anomalous clot formation characterized by dense matter deposits – a pathological form of fibrin networks.

Conclusion: There exists a significant correlation between periodontitis and neurodegeneration, yet findings of *P. gingivalis*, the chief periodontopathic bacterium, in the blood of PD and AD patients are inexistent. Here, for the first time, it is demonstrated that RgpA is evidently present within the haematological system of individuals suffering from PD and AD. These findings pave way for a new view of neurodegenerative pathology and offers insight into prospective preventative and therapeutic interventions. Furthermore, RgpA may provide useful as a biomarker in these debilitating disorders and therefore requires such attention. In the context of the coagulation system, RgpA is highly active. In contrast to the general prothrombotic state observed in most chronic inflammatory conditions, RgpA seems to shift the normostatic state of coagulation to hypocoagulation. Furthermore, the proteolytic activity of this particular gingipain inhibits the formation of fibrin formed thereby decreasing the clot load, the total amount of fibrin formed. The magnitude of this effect differs in plasma and purified fibrinogen and most likely exits due to the presence of inhibitory and target molecules in plasma such as albumin and other proteins. Another important finding is that the proteolytic capability of RgpA is dampened in the presence of LPS from the same bacterial species which offers insight into realistic physiological functioning whereby LPS and gingipains are co-secreted by *P. gingivalis*. These data emphasises the degree of influence that bacterial species and inflammagens have on the coagulation system, and highlights their presence in PD and AD.

Table of Contents

List of Abbreviations:

AD – Alzheimer's disease

CAD – Coronary artery disease

CNS – Central Nervous System

CVD – Cardiovascular disease

FITC – Fluorescein isothiocyanate

Kgp – Gingipain K

LPS – lipopolysaccharide

LPS PG – Lipopolysaccharide from *P. gingivalis*

NCD – Non-communicable disease

LTA – Lipoteichoic acid

PAMP – Pathogen-associated molecular patterns

PBS – Phosphate buffered saline

PD – Parkinson's disease

PDP – Platelet depleted plasma

PPP – Platelet poor plasma

RANKL - Receptor activator of nuclear factor kappa-B ligand

RBC – Red blood cell

RgpA – Gingipain R1

RgpB – Gingipain R2

SEM – Scanning electron microscopy

T2DM – Type II diabetes mellitus

TEG – Thromboelastography

TLR – Toll-like receptor

UPDRS – Unified Parkinson's Disease Rating Scale

WB – Whole-blood

WHO –World Health Organisation

List of Figures:

List of Tables:

CHAPTER 1: INTRODUCTION

In the context of this thesis, it is assumed that the two neuro-inflammatory diseases, namely Parkinson's and Alzheimer's diseases are also characterized by the presence of systemic inflammation, and are thus referred to as inflammatory conditions.

Inflammatory conditions such as Parkinson's disease (PD) PD, Alzheimer's disease (AD) and type II diabetes mellitus (T2DM) continue to burden the global healthcare as the leading causes of death (Pahwa and Jialal, 2019). These disorders are also significantly related in a comorbid manner (Ursum et al., 2013, Garcia-Olmos et al., 2012, Holmstrup et al., 2017), whereby cardiovascular (CVD) and its complications – according to the World Health Organisation (WHO) – is the leading cause of death in these inflammatory conditions. It is, in fact, well-known that most inflammatory diseases are also associated with a prothrombotic state prompted by aberrant coagulation (Herzberg, 2001, Bester and Pretorius, 2016, Randeria et al., 2019, Kell and Pretorius, 2015). In such inflammatory conditions, clotting defects contribute significantly to an occurrence of a cardiovascular event (Zhao and Schooling, 2018) - a common mode of mortality among these patients (Vogelgsang et al., 2018, Holmstrup et al., 2017, Ursum et al., 2013, Potashkin et al., 2019). Common to inflammatory diseases is also an impairment and/or over activity of immune function; this presents with the dysregulation of inflammatory cytokines including interleukin (IL) -1β, IL-6, IL-8, tumour necrosis factor (TNF)-α and CRP (Chen et al., 2018b, Bester and Pretorius, 2016). This chronic, proinflammatory state is therefore a prominent driver of systemic (and neuro-) inflammation and further disease pathogenesis. Interestingly, systemic inflammation is a major link between the aforementioned chronic inflammatory conditions and poses as a principal target of clinical therapy (Bui et al., 2019, Straub and Schradin, 2016, Randeria et al., 2019). However, even though the contribution of immune dysfunction in chronic inflammatory conditions is well recognised, the complexity of pathophysiology, inefficacious treatment and overlapping mechanisms between comorbidities allows the prevalence of these disorders to grow.

A closer look at anomalous clotting in inflammatory conditions

A major hallmark of systemic inflammation – a commonality among and driver of most systemic diseases (Biava and Norbiato, 2015, Hunter, 2012) – is a dysregulation of clotting processes which manifest as a hypercoagulable and hypofibrinolytic state (Foley and Conway, 2016, Kell and Pretorius, 2015). Hypercoagulability entails the tendency of blood to undergo rapid, excessive clotting and acts as a major risk factor for thrombosis (Bridge et al., 2014). Hypofibrinolysis is characterised by a decreased degradation of fibrin clots (Kell and Pretorius, 2015). These two phenomena contribute to an increased prothrombotic state and cardiovascular risk (Maino et al., 2015). This prothrombotic state poses as a therapeutic target that can significantly decrease cardiovascular complications and provide prognostic benefit (Smalberg et al., 2011, Kearney et al., 2017). It has been demonstrated that certain inflammatory cytokines, specifically IL-1β, IL-6 and IL-8, activates platelets and promote more rapid clot kinetics characteristic of a hypercoagulable state (Bester et al., 2018, Bester and Pretorius, 2016); these cytokines also increase the time taken for fibrin degradation thereby increasing the propensity of hypofibrinolysis (Bester et al., 2018).

Both PD and AD have been denoted to exhibit abnormal coagulation (Rosenbaum et al., 2013, Pretorius et al., 2014, Suidan et al., 2018). Recently, analysis of clotting kinetics in PD and AD populations revealed a hypercoagulable state (Bester et al., 2015); furthermore, platelets exhibited phenotypes characteristic of hyperactivation (Adams et al., 2019). A hypercoaguable state in AD has also been elucidated. It was previously also shown that, in AD, a pathology hallmarked by amyloid β plaques, fibrinogen and amyloid β fragments amalgamate and subsequently increase amyloid β load, impaired cerebral blood flow and further neuroinflammation (Cortes-Canteli et al., 2012). This hypercoaguable state and fibrinogen dyshomeostasis, along with systemic inflammation, is responsible for the observed prothrombotic state observed in neurodegenerative disease (Gupta et al., 2005). In consideration of the impact that clotting defects exert on neurodegenerative disease, drugs targeting hypercoagulability, particularly in AD, has been developed (Maiese, 2015).

Additionally, it was also shown that in PD and AD (as well as T2DM) the biochemical nature of fibrin(ogen) clots are atypical and exhibit the presence of amyloid fibrils (de Waal et al., 2018, Pretorius et al., 2017b). In these diseases, lipopolysaccharide (LPS)-binding protein can be used as an intervention to ameliorate abnormal clotting seen in PD, AD and T2DM blood samples (Pretorius et al., 2018c, Pretorius et al., 2018a, Pretorius et al., 2017b). This opened a question, as to whether bacterial inflammagens like bacterial LPS might be one of the many novel circulating biomarkers that drive systemic inflammation, and whether LPS and other

bacterial membrane components (and thus microbial activity) may contribute to the clotting processes of PD and AD.

Pathological clotting processes thus seem to be notably implicated in neurodegenerative disease and therefore possibly linked to neuropathology and cardiovascular mortality. Circulating bacterial inflammagens also seem to be important novel biomarkers for AD, PD and other inflammatory conditions. Therefore, further investigation into etiological factors and the possible role of bacterial inflammagens are thus required to reduce the burden of such pathophysiological mechanisms. The following section will discuss the latest research with regards to bacteria and/or their membrane components as drivers of pathology in systemic inflammatory conditions such as PD and AD.

The implication of microbes in non-communicable disease

Recently, microbes have been more widely implicated in non-communicable diseases (NCD) such as CVD (de Waal et al., 2018), AD (Poole et al., 2013), PD (Ranjan et al., 2018), T2DM (Sharma and Tripathi, 2019), rheumatoid arthritis (du Teil Espina et al., 2019) and respiratory disease (Bansal et al., 2013). It is known that many patients suffering from these chronic inflammatory conditions also suffer from gut dysbiosis, leaky gut (Obrenovich, 2018, Kell and Pretorius, 2018) and oral dysbiosis (Carding et al., 2015, Griffiths and Mazmanian, 2018) which manifests as periodontal disease (Winning and Linden, 2017, Bui et al., 2019).

Periodontal lesions and ‘leaky’ gut epithelia may assist the entry of periodontopathic bacteria into the haematological system whereby their virulent and immunogenic constituents can act as potent inflammagens. This bacterial translocation phenomenon may account, in part, for the relation between bacterial inflammagens in circulation and the presence, in part, of systemic inflammation seen in the aforementioned inflammatory diseases. With the implication of microbes in chronic inflammatory conditions whereby defective coagulation associated, the question that this thesis aims to establish if bacterial membrane inflammagens are indeed present in inflammatory conditions such as PD and AD; and if these membrane inflammagens have a pathological effect on clotting processes.

The question that now arises is if there is any evidence that specifically oral pathogen inflammagens might play a more central role in inflammatory conditions, particularly in PD and AD.

Shifting focus to the oral microbiome

While much focus has been aimed at the gut microbiome in the context of NCD, the oral microbiome is beginning to receive the attention it deserves due to its impact on systemic

(patho)physiology (Sudhakara et al., 2018). In oral pathology, the commensal community of organisms become dysregulated and shift their normostatic microbial profile to a dysbiotic one (Deng et al., 2017). This pathological community, dominated by bacteria, is what induces oral tissue degradation and overt periodontal disease. According to the WHO, the Global Burden of Disease Study 2016 estimated that oral disorders such as periodontitis and gingivitis affect half of the world's population (close to 4 billion individuals) – a staggering statistic that calls for action, particularly against periodontopathic bacteria. *Porphyromonas gingivalis* – a gram negative, anaerobic bacterium that possesses an infamous inventory of virulent factors – is well recognised as a keystone pathogen capable of causing oral dysbiosis and ensuing chronic periodontitis (Darveau et al., 2012, Meuric et al., 2017). Interestingly, periodontal disease exhibits a tight relation with neurodegeneration in the form of PD and AD (Imamura et al., 2001a, Chistiakov et al., 2016, Cowan et al., 2019), and cardiovascular disease (Ilievski et al., 2018, Leishman et al., 2010). Periodontitis also presents with abnormalities in clotting processes and a subsequent prothrombotic state (Imamura et al., 2001a, Dikshit, 2015, Senini et al., 2019, Bizzarro et al., 2010). Periodontal pathogens – predominantly *P. gingivalis* – and their effect on 'isolated' clotting processes have been studied with detail by Imamura and colleagues (Imamura et al., 2001b, Imamura et al., 1997, Imamura et al., 2001a, Imamura et al., 2000, Imamura et al., 1995). However, a more comprehensive analysis using modern techniques such as thromboelastography, rheology, and microscopy would offer a more insight. Hence, detailing the role of periodontopathic bacteria on systemic coagulation is one of the objectives of this study.

Is there a link between the presence of oral pathogens and impaired clotting?

P. gingivalis infection has indeed been associated with impaired clotting and an increased thrombotic risk (Papapanagiotou et al., 2009, Zhan et al., 2016). Relevantly, *P. gingivalis* has been noted to enter circulation *via* dental procedures and more importantly periodontal lesions (Horliana et al., 2014) – a phenomenon which may account for the relation between oral and cardiovascular pathology. The bacterium has been found in atherosclerotic tissue (Kozarov et al., 2005, Velsko et al., 2014), vascular cells (Deshpande et al., 1998, Dorn et al., 2000, Olsen and Progulske-Fox, 2015) and cerebral clots of acute ischemic stroke patients (Patrakka et al., 2019). Also, periodontitis is significantly associated with PD and AD (Chen et al., 2017a, Laugisch et al., 2018, Kaur et al., 2016) Interestingly, neither this bacterium nor its molecular signatures have yet been identified in the haematological system of individuals suffering from neurological disorders of which a strong correlation with periodontal disease stands (Cicciu, 2016). However, numerous mice studies have found *P. gingivalis* disseminated in physiological systems exterior to the oral cavity (Velsko et al., 2014, Poole et al., 2015) which suggests its infection exterior to the oral cavity is likely.

Causative factors of this haematological disturbance includes dysregulated inflammatory markers (Adams et al., 2019, Gupta et al., 2005, Bester and Pretorius, 2016) and more relevant to this study, the presence and influence of microbial products such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA) (Pretorius et al., 2016, Kastrup et al., 2008, Borenstein, 2008). A recent study detected LPS from *Escherichia coli* associated with fibrin molecules in Parkinson's and Alzheimer's disease, as well as type II diabetes (de Waal et al., 2018). Apart from simply being present in the blood of these individuals, LPS has been shown to impact the kinetics of the clotting cascade resulting in hypercoagulability and cause aberrant fibrin network ultrastructure (Pretorius et al., 2016, Pretorius et al., 2018b). As a gram-negative bacterium *P. gingivalis* produces LPS. This species' endotoxin also alters coagulation and has been shown to do so by activating platelets, causing their spreading and inducing hypercoagulability (Senini et al., 2019). Furthermore, this species' LPS upregulates neuroinflammation and causes cognitive deficits in mice (Zhang et al., 2018). Hence, the influence of LPS and therefore microbial activity in neurodegeneration seems convincing.

Room for a novel inflammagen?

While the pathological activity of LPS is well recognised, there are other virulent factors – specific proteases called gingipains – produced by *P. gingivalis* that are deemed the pivotal factor to its pathogenicity (Guo et al., 2010). Gingipains are a group of cysteine proteolytic enzymes produced by various strains of *P. gingivalis* which are essential for this bacterium's survival, nutrient acquisition, proliferation and success in terms of virulence (Sheets et al., 2008, Jia et al., 2019). There exist three major forms of the enzyme: gingipain R1 (RgpA) and gingipain R2 (RgpB) which are both arginine-specific, as well as gingipain K (Kgp) which is lysine-specific (Li and Collyer, 2011). In oral health, these proteases degrade gingival tissue and give rise to periodontal lesions (Imamura, 2003). It should be noted that this chronic degradation of oral tissue acts as a route for microbes into the blood stream. With relevance to coagulation, gingipains have been shown to exert fibrin(ogen)olytic activity (Imamura et al., 1995) – a phenomenon which epitomizes the essence of this study. Another function of these proteases is too interfere with the immune system and degrade (nullify) cytokines (Guo et al., 2010, Slocum et al., 2016, Hajishengallis, 2011, Mezyk-Kopec et al., 2005) thereby acting as a link between periodontal disease, systemic inflammation and associated comorbidities such as cardiovascular disease and neurodegeneration. More recently, gingipains have been discovered in the brains of Alzheimer's patients (Dominy et al., 2019). These findings alone are strongly suggestive of a possible causative mechanism of neurodisease whereby microbial influence interior and exterior to the oral cavity may initiate, drive and/or contribute to neurodegeneration. Furthermore, if this bacterium is so intimately involved in neurodegenerative diseases (especially in physiological systems exterior to the oral cavity),

the use of its inflammagens as biomarkers may provide clinical utility in terms of diagnostic and prognostics.

The question as to whether this microbe or its exclusive proteases can be found in other forms of neurodegeneration such as PD is of particular importance, especially to unveil the role of *P. gingivalis* in neurodegeneration and there is any potential for biomarker utility. Furthermore, with the belief that this bacterium is persistent in systemic disease, the effects of gingipains on clotting cascades and overall fibrin formation require investigation; especially since defects in coagulation contribute to the burden of cardiovascular disease and other chronic inflammatory conditions (Lowe and Rumley, 2014). There is minimal research regarding the effects of these bacterial inflammagens – RgpA and LPS from *P. gingivalis* – on blood coagulation parameters; hence, this study is one of the first that explores such phenomena in detail.

Therefore, the following research questions direct this thesis:

- Can bacterial membrane inflammagens from *P. gingivalis* be detected in blood samples of the two most common neuro-inflammatory conditions, namely PD and AD?
- How does inflammagens from *P. gingivalis* affect blood clotting?

Models used

Our objectives are to use a clinical model (PD and AD), as well as two laboratory models, namely a healthy blood model and a purified fibrinogen model, to detect and study the effects of various membrane inflammagens from *P. gingivalis*.

Methodology

Thromboelastography and rheometry are used to investigate changes in clot kinetics, viscoelasticity and deformable capabilities. In addition, confocal and scanning electron microscopy are utilized to study terminal clot structure, network and load.

Our hypothesis

We hypothesize that RgpA will be detected, for the first time, in PD blood samples using immunohistochemistry. Furthermore, the same technique will be applied to AD samples thereby identifying RgpA in the blood (not brain tissue) of these patients.

Furthermore, due to its fibrinogenolytic activity, fibrinogen incubated with RgpA will result in a much lower load of fibrin formed when exposed to thrombin. Plasma clot kinetics and formation will shift towards a hypocoaguable state when exposed to RgpA while the rheology of clots will be less pliable and stiffer. These effects of RgpA will be altered when co-incubated with LPS from the same species of bacteria, *P. gingivalis.*

Considering these hypotheses, the following **aims** will frame the working order of this thesis:

Aim 1:

To identify *P. gingivalis'*¸ integral virulent protease, RgpA, in PD and AD blood samples.

Aim 2:

To determine the effects of RgpA and LPS from *P. gingivalis* on clot kinetics and rheology using a healthy blood model.

Aim 3

To determine the effects of RgpA and LPS from *P. gingivalis* on terminal fibrin formation and network structure using a purified fibrinogen model.

CHAPTER 2: LITERATURE REVIEW

In this chapter, we review literature that shows a close link between a microbial presence and systemic inflammatory conditions. The literature review aims to bring together various points of view and research data, suggesting that patients with systemic inflammatory conditions (with the focus directed at neuroinflammatory conditions) often have leaky gut (dysbiosis) and conditions such as periodontitis, that allow bacteria to enter into the blood circulation, therefore directly influencing cells and proteins of the haematological system. These bacteria may then shed their membrane inflammagens, and that these inflammagens might indeed play an important role in driving systemic inflammation, and in particular abnormal blood clotting – an important hallmark of systemic inflammation seen in conditions such as Parkinson's and Alzheimer's diseases. This chapter is divided into the following sections:

Microbes and their role in non-communicable diseases: Here we discuss, in the light of how commensal microorganisms become dysregulated and transform into a dysbiotic community capable of causing host dyshomeostasis.

The oral microbiome: The implication of oral microbial populations in periodontal disease, and its burden is discussed here.

Periodontal disease: An introduction into the characterisation and pathology of periodontal disease.

Defects in coagulation in periodontal disease: This section highlights abnormalities observed within periodontal disease and

Overlapping defects of coagulation in neurodegenerative disease: Here we discuss how PD and AD exhibit abnormalities in clotting and the general profile is similar to that of periodontal clotting.

The influence of bacterial inflammagens on clotting processes: The role of bacterial membrane products on certain parameters of blood clotting is mentioned here.

Inflammation and associated systemic dyshomeostasis coupled to periodontal disease: This section highlights maladaptations in the immune system which prompt systemic inflammation and dyshomeostasis in periodontitis.

Periodontitis and its links with cardiovascular disease: Associations of periodontitis with cardiovascular disease is emphasised here, along with experimental evidence aimed at elucidating overlapping mechanisms.

Periodontitis and its links with neurodegenerative disease: Associations of periodontitis with neurodegenerative disease is emphasised here, along with experimental evidence aimed at elucidating overlapping mechanisms.

Chief periodontal pathogen discovered in the brains of Alzheimer's disease patients: Here we discuss recent findings from early 2019 whereby *P. gingivalis'* DNA and certain virulent factors were found in the brains of Alzheimer's patients – the source of inspiration for this present thesis.

Porphyromonas gingivalis: An introduction into the chief periodontopathic bacteria implicated in periodontitis and its necessity for oral detriment.

Gingipains – major virulent factors of *P. gingivalis:* Here we discuss the role and necessity of gingipains in pathology.

Inflammation as a disturbance of coagulation in periodontitis – the possible role of *P. gingivalis:* How periodontal inflammation, induced by *P. gingivalis*, may contribute to the abnormal coagulation observed in periodontal disease.

The impact of gingipains on the coagulation system: Here we highlight papers that studied the effects of gingipains on parameters on clotting, and how these may relate to systemic disease.

Lipopolysaccharide from *P. gingivalis*: The influence of LPS from *P. gingivalis* on inflammation and coagulation is mentioned here.

Concluding remarks: Overall, the impact that dysbiotic oral microbes and gingipains produced by *P. gingivalis* have on inflammation and clotting constituents and processes is summarised here.

2. Microbes and their role in non-communicable disease

The presence of microbes as well as their activity is being implicated in non-communicable disease (Ogoina and Onyemelukwe, 2009, Bui et al., 2019, Deleon-Pennell et al., 2013). Conventionally, microbes – such as those found within the gut and oral microbiomes – have thought to play little or no role in the pathogenesis of chronic, inflammatory, systemic disorders including cardiovascular and neurodegenerative disease. However, research conducted in recent decades has implicated the existence and functionality of microbial populations in these diseased states (Tang et al., 2017, Leishman et al., 2010, Ilievski et al., 2018, Kaur et al., 2016, Byndloss and Baumler, 2018) such as CVD, AD, PD, type II diabetes mellitus, rheumatoid arthritis and anxiety disorders. Considering that there exist roughly the same amount of microbial cells as host cells in the human body (Sender et al., 2016), albeit commensal organisms, it seems plausible that a dysregulation of such symbiotic relationships can contribute to diseased states. Whilst commensal microbes play an integral role in maintaining physiological homeostasis in terms of human physiology, risk factors such as genetics, physical inactivity, poor dietary choices and exposure to harmful substances alter the normostatic profile of human microbiomes and allow opportunistic microbes – that are often present at low, non-pathogenic concentrations – to proliferate to levels that prompt microbial dysbiosis and subsequent host dyshomeostasis (DeGruttola et al., 2016). This is known to occur in the gut microbiome whereby an increased risk of developing or driving T2DM (Sharma and Tripathi, 2019), CVD (Jin et al., 2019) and neurology-related conditions (Spielman et al., 2018, Ambrosini et al., 2019), ranging from anxiety disorders to Alzheimer's pathology, is observed. Furthermore, the gut dysbiosis is often accompanied by leaky gut characterised by increased permeability of the intestinal epithelia, especially in the context of neurodegenerative disorders (Maguire and Maguire, 2019). It has been proposed that bacteria are able to traverse the gut wall and enter circulation whereby bacterial inflammagens can elicit an immune response and that chronic persistence of this mechanism drives systemic inflammation (Kell and Pretorius, 2018).

2.1. The oral microbiome

While much focus has been directed at the gut microbiome in the context of NCD, the oral microbiome is beginning to receive the attention it deserves. In the past, it was routine to view the oral microbiome's functionality with value only to dentists and orthodontologists. However, it soon became apparent that periodontal disorders inflict detriment on not just the oral cavity but also systemic physiology (Kim and Amar, 2006). Periodontal disease – characterised by soft and dense tissue loss within the oral cavity – is a NCD driven solely by dysbiotic (dysregulated) bacterial activity, particularly as a primary aetiological factor (Leishman et al., 2010). Interestingly, this disorder is strongly associated with CVD (Slocum et al., 2016), AD (Singhrao and Olsen, 2019) PD (Chen et al., 2018a). Poor dental hygiene and hence periodontal disease exhibits a significant correlation with both of these neurodegenerative disorders (Chen et al., 2018a, Schwarz et al., 2006, Singhrao and Olsen, 2018, Chen et al., 2017a) and cardiovascular disease (Slocum et al., 2016, Sudhakara et al., 2018). These significant correlations are suggestive of overlapping microbial activity whereby chief pathogens in periodontitis contribute to the pathogenesis of cardiovascular and neurodegenerative disease.

A predominant mechanism of comorbid manifestation includes immune dysregulation caused by periodontopathic bacteria (Holmstrup et al., 2017, Velsko et al., 2014, Ilievski et al., 2018, Hajishengallis et al., 2015); however, while inflammatory sequela governed from the oral cavity is a convincing tie between periodontal disease and CVD, AD and PD, oral pathogens most likely operate in a more atopobiotic manner. Atopobiosis, the infection of microbes in atypical locations such periodontopathic bacteria in the haematological or central nervous system, allows organisms to exert a more direct effect on specific tissues, i.e. pathological influence on neurons in the hippocampal region of the brain, thereby contributing more directly to AD than simply orchestrating inflammation from the oral cavity. Relevantly, this atopobiotic phenomenon in periodontal disease has been supported by the finding that oral pathogens enter the haematological system *via* periodontal lesions (Olsen and Progulske-Fox, 2015, Ambrosio et al., 2019, Tomas et al., 2012). Since oral pathogens are noted to enter blood, their effects on haematological and cardiovascular constituents are of particular importance.

As reported by the WHO, in consideration of epidemiology, NCDs are responsible for the majority of global mortalities; this emphasizes the need to comprehensively denote possible aetiological factors – such as the role of microbes – that contribute to NCD with the aim of optimizing preventative and treatment options. Out of the list of NCDs, CVD is responsible for an estimated 18 million annual deaths and is therefore regarded as the leading cause of global

mortality. An interesting trend observed with cardiovascular disease is its propensity to exist as both a primary disease and a comorbidity to other NCDs including PD (Potashkin et al., 2019), AD (Tublin et al., 2019) and periodontal disease (Leishman et al., 2010, Herzberg, 2001). This, along with the persistence of CVD as the leading cause of mortality, makes CVD the primary mode of death among these chronic inflammatory disorders and beckons for investigation into overlapping mechanisms – especially those that pose relevance to cardiovascular health such as the functionality of periodontopathic bacteria in coagulation.

2.2. Periodontal disease

Periodontal disease – a chronic inflammatory disorder driven by microbial dysbiosis – encompasses the infection, inflammation and destruction of oral structures such as the gingiva, periodontal ligament and supporting bone structures. According to the WHO, the Global Burden of Disease Study 2016 estimated that nearly half of the world's population is affected by periodontal disorders and therefore is regarded as one of the most common non-communicable diseases alongside cardiovascular pathologies. On the lower end of the severity spectrum is gingivitis, the infection of the gums. While this is reversible, if left untreated it is likely that gingivitis will progress into periodontitis (Donley, 2019, Page et al., 1978). The degradation of connective tissue as a result of periodontal disease is the most common cause of tooth loss in adults (Kinane et al., 2017). Periodontitis is routinely diagnosed by measuring the sulcus between teeth and gums as well as assessing the propensity for bleeding upon probing, the two major hallmarks of this disorder; other relevant parameters involved in diagnostics are plaque levels, furcation (loss of bone in root trunk of teeth), gum recession and tooth mobility (Preshaw, 2015). Figure 1 shows the prevalence of periodontitis among certain age groups in several countries. It is true that the burden of periodontitis increases with age. In Figure 1A, the rating of severity of periodontal pathology – called the community periodontal index (CPI) – is depicted. Bleeding upon probing as a solitary symptom receives a rating of one at the lower end of the scale. Bleeding along with calculus, a form of hardened dental plaque is rated 2. At the more severe end of the scale, periodontal pockets of 4-5mm and more than 6mm are indicative of a CPI rating of 3 and 4, respectively. These stages are considered overt periodontitis.

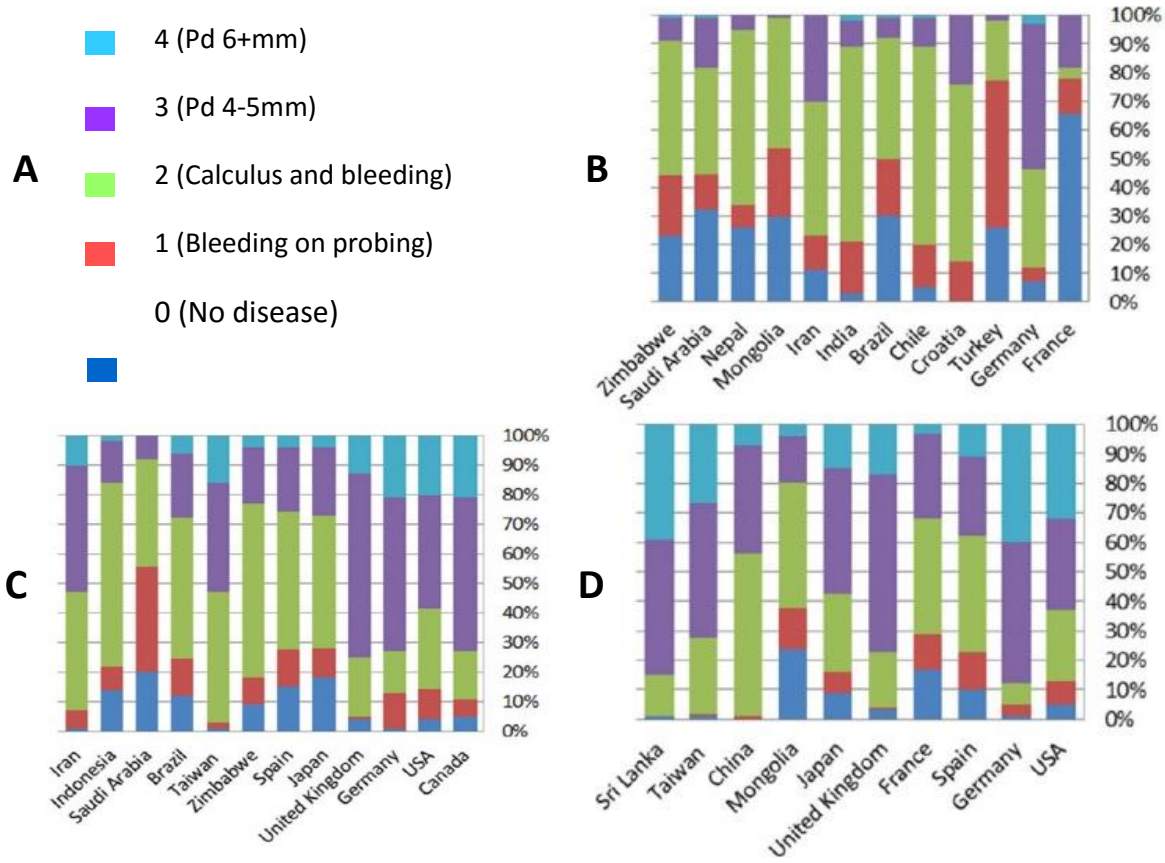


Figure 1: The prevalence of periodontal disease and severity among different age groups and countries using the community periodontal index (CPI). A) Degree of periodontal severity. B) Prevalence results among a 15-19 year-old population. C) Prevalence results among a 25-44 year-old population. D) Prevalence results among a 65-74 year-old population. (Adopted from (Nazir, 2017)).

Periodontal disorders are more common in adults where it seems that, over decades, certain genes (da Silva et al., 2017, Michalowicz, 1994) and lifestyle factors such as poor dental hygiene, diet, minimal exercise and immune dysfunction culminate into oral dyshomeostasis which manifests as chronic periodontitis (Tatakis and Kumar, 2005). Interestingly, bacterial dysbiosis – which arises as a result of the aforementioned factors – underlies the pathogenesis of periodontitis (Sudhakara et al., 2018, Meuric et al., 2017). There exist over 700 known species of bacteria in the oral cavity all of which grow in a polymicrobial community of commensal biofilms (Aas et al., 2005). Symbiosis between these bacterial communities and human cells and tissue is integral for host homeostasis; however, it is when these microbial cliques become dysregulated or imbalanced (dysbiosis) that oral dyshomeostasis ensues. Dysbiosis allows for pathogenic species and harmful population levels to develop which exerts

an implicative effect on the immune system (Levy et al., 2017). Oral dysbiosis in particular dysregulates inflammatory markers (Zhang et al., 2019, Abe et al., 2018), causes pathological immunomodulation (du Teil Espina et al., 2019, Devine et al., 2015) and degrades host gingival and periodontal tissue *via* direct (proteolytic) and indirect (inflammatory) mechanisms (Fouillen et al., 2019, Slocum et al., 2016). Accordingly, periodontal phenotypes present with systemic maladaptations of the immune system (Cekici et al., 2014, Leira et al., 2018, Hajishengallis, 2015). It is *via* these mechanisms – the disruption of host immune function – that periodontitis is, in part, believed to contribute to the pathogenesis of comorbidities such as neurodegeneration and cardiovascular disease.

2.2. Defective coagulation in periodontal disease

Coagulation is an extremely crucial function in both healthy and diseased states. In healthy states, coagulation functions to repair cardiovascular tissue injury in the form of hemostasis. While hemostasis still plays an integral role in disease, coagulation processes often exhibit maladaptations and defects in kinetics and the efficacy of clot initiation, formation, and breakdown (Marshall, 2001). Among CVD deaths, complications in the form of myocardial infarctions and strokes remain the predominant implication; more so, coagulation faults are present during these events (Undas et al., 2009, Sfredel et al., 2018). Defects in coagulation, such hypercoagulability and hypofibrinolysis, stand as a major contributing factor to CVD and the manifestation of a cardiovascular event (Lowe and Rumley, 2014, Zhao and Schooling, 2018). Efficacy of the coagulation system's activity is therefore an important factor in the burden of global mortality.

Inflammation has a major effect of the clotting system (Bester and Pretorius, 2016, Randeria et al., 2019). Relevantly, common to cardiovascular, neurodegenerative and periodontal pathology is systemic inflammation (Glurich et al., 2002, Velsko et al., 2014, Tublin et al., 2019, Fowler et al., 2001) – this overlap between pathophysiological biomarkers in the chronic inflammatory diseases in question makes mechanisms of comorbid causation difficult to discern. Regardless, dysregulated and zestful inflammatory processes are prominent aetiological and driving factors for the pathogenesis of most systemic diseases. In relation to the clotting kinetics, an inflammatory state of the haematological phenotype is often associated with defective coagulation characterised by hypercoagulation, excessive, rapid clotting, and hypofibrinolysis, a reduced clot degradation (Kell and Pretorius, 2015, Bester et al., 2018).

Relatively, periodontal disease presents with abnormal coagulation that is often characteristic of increased clot propensity (Sato et al., 2003, De Luca et al., 2017, Dikshit, 2015). These defects in clots have been associated with an increased risk for thrombosis in periodontal disease afflicted patients (Dikshit, 2015, Senini et al., 2019). Implementation of non-surgical therapy reduced platelet counts in periodontal patients (Banthia et al., 2013); another study demonstrated that similar treatment had favourable effects on markers of the clotting system. It has been put forth that periodontal-induced inflammation (as a result of bacterial dysbiosis) gives rise to this prothrombotic state (Weickert et al., 2017). Whether the dyshomeostasis in the clotting system observed in periodontal disease is directly (inflammagens acting directly on clotting machinery) or indirectly (inflammation) caused by periodontopathic pathogens is yet to be fully explained. Nonetheless, this prothrombotic state, inspired from dysregulated inflammatory cues (Bester and Pretorius, 2016, Randeria et al., 2019, Kell and Pretorius, 2015), is thus a major risk factor for the occurrence of a cardiovascular event and hence poses as an important link between periodontal disorders and (cardiovascular) mortality.

2.3. Overlapping defects of coagulation in neurodegenerative disease

Alzheimer's and Parkinson's disease both exhibit abnormalities in clotting processes which contributes to an increase in the overall prothrombotic state of these individuals (Maiese, 2015, Kalita et al., 2013) – similar to that of periodontal pathology. Hyperactivation of the intrinsic clotting pathway is present in neurodegenerative disease (Suidan et al., 2018). It has been demonstrated that clotting kinetics and strength in AD patients is impaired due to increased clot formation, hyperactivated platelets, increased levels of fibrinogen and decreased fibrinolysis (Suidan et al., 2018, Cortes-Canteli et al., 2012, Klohs et al., 2012); furthermore, this was also found in AD mice models and these researchers correlated levels of amyloid beta – the hallmark molecular defect of this disease – and cognitive defects with biomarkers of coagulation. Fibrinogen – the zymogen to the clot constituent fibrin – has been found entangled with amyloid beta plaques which leads to aggregates of fibrinolytic-resistant clots (Cortes-Canteli et al., 2012), not to mention increased loads of amyloid β deposits. Decreasing the levels of fibrinogen in diseased mice relays ameliorative effects on neuropathology including lessened blood brain barrier (BBB) permeability, decreased amyloid beta load, improved cognitive function and reduced inflammation (Cortes-Canteli et al., 2012). This suggests that abnormalities within the coagulation system may root the manifestation of certain phenotypes observed within AD, especially in relation to amyloid beta and resultant degradation-resistant clots.

Abnormal clotting kinetics and cases of thrombosis has also been observed in PD populations (Cortes-Canteli et al., 2010). Amyloid formation of fibrin(ogen) in PD plasma has been discovered (Pretorius et al., 2018c). Hypercoagubility and hyperactivated platelets contribute to the prothrombotic state observed in PD (Adams et al., 2019) – the same phenotype observed in periodontal disease. This may contribute to altered hemostasis and fibrin formation observed between these disorders. Erythrocytes – once thought to be bystanders in terms of coagulation – participate in determining terminal clot architecture and blood flow. Eryptotic erythrocytes pose as viable diagnostic and therapeutic biomarkers in PD (Pretorius et al., 2014) and likely contribute to impaired blood flow through thrombi. Additionally, Parkinsonism drugs impact coagulation and decreases the fibrinolytic capabilities of the clotting system (Sato et al., 2003).

Certain microbes exert a more direct effect on the clotting system. Microbial cells of non-oral origin and their molecular products have been found within T2DM, PD and AD clots (Armstrong et al., 2013, de Waal et al., 2018). Other bacteria have been known to degrade fibrin clots *via* enzymatic means (Loof et al., 2014). These data indicate that microbes, or at least their shed membrane inflammagens, are present and bioactive in the blood system of individuals suffering from chronic inflammatory conditions that were once viewed as sterile. These findings substantiate a relationship between aberrant coagulation in systemic disease and the presence of microbes. It is thus of interest to identify oral pathogens – which show a strong relation to inflammatory conditions such as AD, PD and CVD – in the haematological system of individuals suffering from systemic disease and assess their impact on coagulation, especially as their atopobiotic presence is eminent in overt periodontal disease. Furthermore, targeting these periodontopathic bacteria may provide clinical benefit in those disorders that exhibit a strong correlation with periodontal disease.

Ultimately, PD, AD and periodontal disease share similarities in their phenotypic clotting profile. Since the potential of microbial contribution to chronic inflammatory disease, particularly neurodegeneration and CVD (Figure 2), has been highlighted, and that coagulation efficacy is compromised in these disorders, identifying microbes in such disorders and understanding their influence on the clotting system is warranted. Relevantly, the oral microbiome is under the spotlight because of its correlation with PD, AD and CVD.

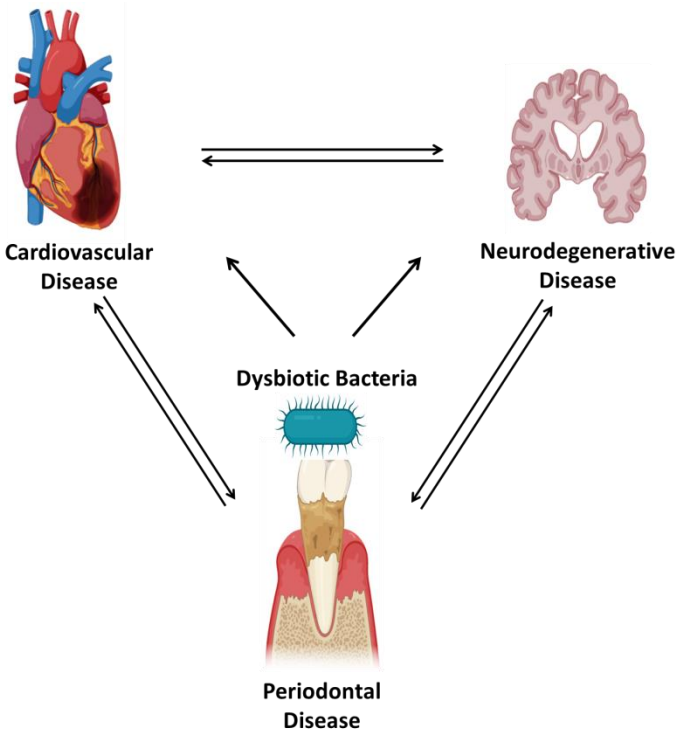


Figure 2: Intimate association between CVD, neurodegenerative disease and periodontal disease which is predominantly driven by dysbiotic bacteria in the oral cavity.

2.4. The influence of bacterial inflammagens on clotting processes

Bacterial products have been implicated in the efficacy of clotting processes (Loof et al., 2014, Pretorius et al., 2018b). Fibrinogen – the zymogen of the clot constituent – is a 340kDa glycoprotein present within circulation at a concentration of 2-4mg/ml. It is composed of two sets of three polypeptide chains, namely Aα, Bβ, and γ. What should be noted is that particular variations in the cleavage of these polypeptides during enzymatic conversion to fibrin result in a number fibrin variations that constitute clots with some that pose a degree of cardiovascular risk (Wolberg, 2016); proteolytic bacteria capable of cleaving fibrinogen may favour the production of these particular clots. Two well studied bacterial inflammagens, LPS as well as LTA, from gram-negative and gram-positive bacteria respectively, exert an implicative effect on clot kinetics and terminal structure. A study conducted by Pretorius *et al* (2018) demonstrated that LPS from *Escherichia coli* and LTA from various gram-positive bacteria altered the biochemical nature of fibrin(ogen) as well as clot kinetics. These bacterial endotoxins increased amyloidogenesis – albeit in different manners – of fibrin(ogen) which resulted in aberrant fibrin networks. Furthermore, the kinetics of coagulation was altered favouring a more hypercoaguable state when samples were exposed to these inflammagens. These data emphasize the eminent effect that low-levels (0.4-1 $ng.L^{-1}$) of bacterial products

have on the coagulation system. While it is true that inflammatory responses mediated by LPS alters the clotting cascade *via* platelets and endothelial cells (Dayang et al., 2019), purified fibrinogen models exposed to LPS has also shown aberrations in fibrin structure (Pretorius et al., 2016) – this implies that LPS interferes with cleavage and processing of fibrinogen and its polypeptides into fibrin which is independent of inflammatory cues as platelets and white blood cells are absent in a purified fibrinogen model. LPS-binding protein has also been demonstrated to reverse or ameliorate defective clotting observed in plasma from Parkinson's individuals (Pretorius et al., 2018c). This indicates that LPS is likely present in the haematological system in PD and act as a potential therapeutic target, especially in the context of coagulation. Also the alteration of the biochemical nature of fibrin(ogen) in the presence of certain inflammagens and in diseased states has been discovered. Amyloid formation of this clotting protein pair is induced by inflammation, bacterial products and disease thereby resulting in a terminal clot with undesired properties such as resistance to fibrinolysis and increased resistance blood flow (Pretorius et al., 2016, Pretorius et al., 2018b, Pretorius et al., 2018d).

2.5 Inflammation and associated systemic dyshomeostasis coupled to periodontal disease

Inflammation is important in the context of coagulation as it exerts profound effects on the state of clotting – it can induce a hypercoagulability in the proinflammatory state (Bester and Pretorius, 2016). Hence, it is important to detail the inflammatory component of periodontitis in order to tie relations to abnormal coagulation observed in oral pathologies as well as associated comorbid diseases such as PD and AD, both of which show overlapping characteristics in clotting defects. As mentioned, the detriment induced by periodontal disorders is not limited to the oral cavity. Periodontitis has an intimate correlation to a cohort of systemic diseases including CVD (Levy et al., 2017, Stewart and West, 2016), rheumatoid arthritis (Bingham and Moni, 2013, Rodriguez-Lozano et al., 2019), T2DM (Chee et al., 2013), respiratory infections (Saini et al., 2010, Bansal et al., 2013), AD (Teixeira et al., 2017, Singhrao and Olsen, 2018, Chen et al., 2017a) and PD (Chen et al., 2018a, Kaur et al., 2016, Chen et al., 2017b). These associations thereby infer a possible link between the microbes that orchestrate chronic periodontitis and systemic dyshomeostasis. Chronic inflammation is a major commonality among systemic diseases (Straub and Schradin, 2016, Kaur et al., 2016) and stands as a strong target of therapy to ameliorate a primary condition as well as prevent the manifestation of comorbidities (Gonzalez-Gay et al., 2014, Newcombe et al., 2018, Ursum et al., 2013). As a chronic inflammatory disease, periodontitis is likely associated with CVD,

AD and PD due to reasons related to the immune system and inflammation (Holmstrup et al., 2017, Chistiakov et al., 2016, Torrungruang et al., 2018, Kaur et al., 2016, Hajishengallis, 2015).

Periodontal pathogens are inflammophilic (Hajishengallis, 2014); a chronic inflammatory state is thus ideal for the infection of these microbes. The phenotype of periodontitis presents with upregulated inflammatory markers such as interleukin (IL) 1β, IL-6, IL-12, IL-17, IL-23, interferon-γ, vascular endothelial growth factor, C-reactive protein (CRP), tumour-necrosis factor (TNF) α and serum amyloid A (Leira et al., 2018, Zekeridou et al., 2019, Kalburgi et al., 2014, Singh et al., 2014, Hirai et al., 2019). These markers are common to other chronic inflammatory disorders such as CVD, AD and PD (Ramos et al., 2009, Watanabe et al., 2016, Luan and Yao, 2018). These overlapping inflammatory cues are suggestive of the possibility that immune-related matters are in part responsible for the link between periodontitis and the aforementioned diseases.

Both the innate and adaptive immune system are implicated during the pathogenesis of periodontitis (Cekici et al., 2014). In the initial stages of periodontal disease, predominantly gingivitis, the immune response to relevant pathogens in the oral cavity is a defensive one. In these early stages, subgingival plaque can be resolved and homeostasis can be returned – this immune response is not necessarily pathological. However, it is when these microbial communities and periodontal lesions persist that defects in immune regulation occur. While the first line of defense, components of the innate immune system, is the first to become dysfunctional in periodontitis, it is a combination of maladaptations in both the innate and acquired immune system that drives pathology.

Acute inflammation observed in the early stages of periodontal disease is governed by the innate immune system, whose components – typically the complement system and toll-like receptors (TLRs) present on phagocytes and other cells (Hajishengallis et al., 2015) (Daniel and Van Dyke, 1996) – are impaired. There are also physical defense barriers in the oral cavity such as tight intercellular junctions that prevent bacteria and their molecular by-products from entering tissue. However, during periodontal pathology, these epithelial cells become inflicted by pathogens and this mucosal barrier becomes ‘leaky’ thereby allowing bacteraemia to ensue (Chen et al., 2019). Systemic bacteraemia induced by periodontal pathogens leads to persistent activation of inflammatory processes (Hirschfeld and Kawai, 2015) – this could be a possible link between periodontal disease and cardiovascular and neurodegenerative disease.

The complement system is a group of humoral and cell-associated proteins apart of the innate immune system responsible for maintain microbial homeostasis. Bacterial evasion of the complement system is eminent in periodontitis and leads to chronic and maladaptive functioning of the immune system (Hajishengallis, 2015). Components of this system are degraded by bacterial proteases which lead in ineffective clearance its targets (Popadiak et al., 2007). In the complement system, there are generally three modes of activation – the classical pathway, the lectin pathway and the alternative pathway – all of which result in bacterial cell destruction in a similar manner. The classical pathway is stimulated by immunoglobulins which ultimately lyse bacterial cells *via* the membrane attack complex composed of the terminal complement proteins, C6 and C9. The lectin pathway converges with the classical pathway at the activation of C3, an integral convertase of the system; prior to this commonality, mannose-binding lectin targets carbohydrates present on the surface of microbial cells which subsequently interacts with complement proteins required to activate C3. The alternative pathway – which is significantly activated during periodontal disease (Cekici et al., 2014) – is stimulated by bacterial inflammagens such as lipopolysaccharide (LPS). While antibodies, utilized in the classical pathway, against periodontal pathogens are widespread in periodontal disease, the alternative pathway still predominates complement activity in this disorder; this may be due to the fact that oral bacterial products have the ability to cleave immunoglobulins thereby nullifying pathway activation (Vincents et al., 2011).

The involvement of the complement system in periodontal inflammation seems to be significant. In a rat model of periodontitis, a C5a receptor antagonist was used which resulted in a significant decrease in periodontal inflammation and bone loss (Hajishengallis et al., 2015), thereby demonstrating that complement functioning is an important factor in disease progression. In comparison to C5a and its receptor, C3 is common to all three complement pathways. The activity of C3 is therefore is independent of the mode of complement activation; this makes C3 a favoured therapeutic target (in the context of the complement system). In experiments using non-human primates, a C3 inhibitor significantly prevented inflammation and tissue destruction (Maekawa et al., 2014a). To quantitatively express the reduction in inflammation brought about by the C3 inhibitor, researchers measured levels of TNF, IL-1β, and IL-17 – all inflammatory biomarkers were lowered in the presence of the inhibitor. This study offers valuable insight into the complement system's role in periodontal disease. In a disease as immunologically complex as periodontitis, it is therapeutically useful to identify such impactful biomarkers such as C3. Furthermore, C3 activity is known to modulate toll-like receptor (TLR) signalling, typically in a positive manner (Hajishengallis and Lambris, 2010). Thus, the inhibition of C3 may also help to reduce periodontal and thus systemic inflammation mediated by TLR signalling. Ultimately, components of the complement system – specifically

C3 and C5a receptor – exhibits crosstalk with TLRs which leads to leukocyte mobilisation and further inflammation, all of which contributes to gingival and bone degradation (Hajishengallis et al., 2019)

TLRs are a group of receptors apart of the immune system that acts to recognize pathogen-related markers, formally termed pathogen-associated molecular patterns (PAMPs). These receptors recognize bacterial LPS, and other pathogen markers such as LTA, which results in a cascade of immune-related reactions that orchestrate inflammation in the context of both innate and adaptive immunity. Periodontitis is governed by uncontrolled, pathogenic bacterial populations; hence, the levels of PAMPs are high which increases TLR signalling thereby leading to further inflammation. Interestingly, TLR2 and TLR4, the two forms of the receptor most notably compromised in periodontal disease, have been proposed as reliable diagnostic markers for periodontal disease (Ilango et al., 2016).Dendritic cells, lymphocytes, macrophages and epithelial cells all possess these receptors, and are implicated in periodontal pathology (Ilango et al., 2016). When this receptor is activated by respective agonists, a stream of intracellular processes occur which ultimately results in cytokine, chemokine and antimicrobial peptide synthesis and secretion (Schaefer et al., 2004). These inflammatory markers become dysregulated and contribute to systemic inflammation – another link between oral dysbiosis and systemic disease. Osteoclast precursors also express TLRs; modulation of this particular receptor by periodontal pathogens lead to increased osteoclast activity and thus increased bone resorption (Hienz et al., 2015) – a characteristic pathophysiological factor of periodontitis. Ultimately, activated TLRs in a periodontal context is largely responsible for excessive production of inflammatory molecules (Parthiban and Mahendra, 2015), those which drive periodontal pathology. TLRs prompt both innate and acquired immune system processes and therefore pose as a reasonable target for the reducing systemic inflammation.

Neutrophils also play an important role in the progression of periodontal disease. These white blood cells are actually the most common type found within periodontal lesions (Hajishengallis and Hajishengallis, 2014) thereby emphasizing their importance in combating microbial activity in the oral cavity. Individuals with congenital defects in neutrophil function and recruitment exhibit an increased risk of IL-17-driven bone loss (Moutsopoulos et al., 2014). However, with that being said, it has also been shown that levels of neutrophils in periodontal regions also correlate with the severity of periodontitis (Landzberg et al., 2015). This is indicative of the possibility that neutrophil functioning becomes dysregulated. Relevantly, neutrophils have been shown to contribute to local, harmful tissue inflammation and degradation in periodontal lesions (Ryder, 2010). It has been suggested that pathogens are able to evade their defence mechanisms (Hajishengallis, 2015). With relation to the

complement system and TLRs, interference from oral pathogens lowers the phagocytic capabilities of neutrophils (Maekawa et al., 2014b); this bacterial evasion of neutrophils requires direct crosstalk between complement factors, specifically C5a receptor, and TLRs which are present on relevant cell types. Although their ability to digest bacteria is hindered, neutrophils are still functional in the sense that they contribute to inflammation via cytokine and chemokine production – this leads to constant leukocyte recruitment to infected areas. Additionally, to aid the transmigration of neutrophils through tissues, connective tissue is degraded (Maekawa et al., 2014b). In the context of periodontal lesions, these neutrophil implications aggravate tissue loss. The dysregulation of this immune consortium – consisting of complement system, TLRs, phagocytes – certainly contributes to chronic, systemic inflammation and thus may be largely responsible for correlations made between periodontitis, cardiovascular and neurodegenerative pathologies.

If the primary innate response is insufficient to remove the microbial threat, pathogenic antigens will be presented to cellular components of adaptive immunity, T and B lymphocytes. Dendritic cells, macrophages and other lymphocytes act as antigen-presenting cells informing B and T cells of their respective target's molecular makeup. B cells are responsible for producing immunoglobulins which exist on the membrane of these lymphocytes or as soluble antibodies which are secreted by active B-cells called plasma cells (Hoffman et al., 2016). These processes form part of humoral immunity. Contrastingly, T-cells govern cell-mediated adaptive immunity. The role of T-cell adaptive immunity and related cytokine functioning plays an important role in the manifestation of periodontitis (Silva et al., 2015). A type of T cell, T-helper 17, is known to be very active and largely responsible for the excessive IL-17 and IL-23 levels observed in periodontitis (Ohyama et al., 2009). Tissue destruction, such as that of gingival and bone degradation, is prompted by elevated levels of IL-17 (Shabgah et al., 2014). Knockout studies have provided insight into the extent of T cell involvement in periodontitis. Immunodeficient strains of mice have shown that T cell deletion results in reduced periodontal pathology, specifically reduced bone loss (Baker et al., 2002). Furthermore, T cells express a degree of crosstalk with osteoclasts and their precursors, and their osteoclastogenic role has been denoted (Weitzmann et al., 2001). They increase levels of receptor activator of nuclear factor-κβ ligand (RANKL) which acts as an agonist for osteoclastogenesis (Chen et al., 2014). The need for T cells for pathological bone loss and their ability to promote osteoclastogenesis highlights their involvement in periodontitis. This impactful role of T cells makes them a useful therapeutic target in relation to bone loss in periodontal disease.

It seems that a complex aberration of immune function, both from the innate and acquired compartments, is integral for periodontal pathogenesis. After all, periodontal pathogens are inflammophilic. It is intuition to thus assume that the influence of pathogenic microbes on

immune function in periodontitis is in part responsible for the manifestation of comorbidities. This emphasises the need, in order to fully treat a patient and account for possible comorbidities, to join disciplines in the context of oral health care professionals and general practitioners and associated specialists. An interdisciplinary approach to this pathological complexity has already been stipulated (Lyons and Darby, 2017) in order to reduce associated comorbidities such as CVD and neurodegeneration.

2.6. Periodontitis and its links with cardiovascular disease

For decades correlations between periodontal disease and CVD have been made. In the late 1980s Mattila *et al.* (1989) completed a study that evaluated the state of dental health in patients that have suffered a myocardial infarction. The researchers demonstrated that dental health was seemingly worse in the patients suffering from cardiovascular complications than healthy controls. Using 9760 participants, a study followed individuals for fourteen years in order to learn the risk of developing coronary artery disease (CAD) in healthy and periodontal disease inflicted individuals. Among the participants, those with periodontitis showed an overall increased risk for total mortality and a 25% increase in the risk of developing CAD (DeStefano et al., 1993). More noteworthy is the finding that among men aged 50 and younger, those with periodontitis exhibited a staggering 72% increase in the risk of developing CAD than the controls. The National Health and Nutrition Examination Survey (NHANES III) provided invaluable data relevant to periodontal comorbidities. Out of 5564 subjects assessed for cardiovascular health, the extent of periodontal pathology was linked to an odds ratio of 3.8 [95% CI (1.5–9.7)] in relation to periodontally healthy subjects (Arbes et al., 1999); this was after adjustment for confounding factors including smoking, diabetes mellitus, blood pressure, age, sex and gender. Late in the 1990s, when the association between periodontitis and CVD was disclosed, researchers correlated levels of subgingival bacteria – those implicated in periodontitis – with the risk of experiencing a heart attack (Stein, 1999).

In a meta-analysis of nine cohort studies, periodontitis was 19% more likely to be associated with a cardiovascular event such as a stroke or myocardial infarction (Janket et al., 2003). Once again, the degree of association was dependent on age. Individuals afflicted with periodontal disease that were under the age of sixty-five exhibited an increased risk of 44% as opposed to the total population's risk of 19%. Another comprehensive meta-analysis study completed in 2009 concluded a total risk for developing CVD estimated at 34% (Blaizot et al., 2009). The link between oral health and CVD, by this point, was more than convincing to negate it as coincidence. Interventional and causal studies provided more conclusive

inferences thus paving way for further investigation into the intimacy between periodontitis and CVD, two chronic inflammatory conditions.

In 2010, de Oliveira *et al.* (2010) investigated if routines of tooth brushing had a significant outcome on inflammation as measured by CRP levels, fibrinogen levels and cardiovascular health. Participants that reported poor dental hygiene exhibited an elevated risk of developing CVD and also presented with higher serum CRP and fibrinogen levels. As CRP and fibrinogen are acute-phase reactants whose levels increase during acute-phase responses (inflammatory cue), this study is suggestive of increased inflammation as a result of poor dental health. Although parameters of coagulation were not measured, an increase in fibrinogen levels is also indicative of a hypercoaguable state (Kattula et al., 2017). The concentration of serum CRP levels are positively correlated with the severity of periodontal disease (Podzimek et al., 2015). Furthermore, elevated CRP levels are deemed a major risk factor for the development of CVD (Fonseca and Izar, 2016). It would therefore provide some benefit to decrease CRP levels in periodontitis in order to prevent cardiovascular complications. In an intervention study, CRP levels, as well as another acute-phase reactant called haptoglobin , were decreased in patients with periodontitis by intervening with dental scaling, root planning and drug administration (flurbiprofen) (Ebersole et al., 1997). However, whether the ameliorative effects observed in this study were due to dental care is blurred by the presence of flurbiprofen, a non-steroidal anti-inflammatory drug. In another study, 120 patients with periodontitis were subjected to nonsurgical periodontal treatment and had their CRP levels measured 45 days after the first day of intervention; the results from this particular study concluded that dental treatment alone significantly reduces CRP levels (Kumar et al., 2013). In a diabetic population with poor oral health, periodontal treatment lowered the rate of CVD (Peng et al., 2017). Common to most assessments is the fact that periodontal intervention favours cardiovascular health. This establishes an intimate link between the two disorders and hence requires further attention.

Even with this data in hand, clinically targeting certain inflammatory markers such as CRP in periodontitis with the aim of preventing CVD has provided minimal benefit (Li et al., 2017, Offenbacher et al., 2009). Nonetheless, these studies in question favour correlation as opposed to causation; and the correlations made between oral health and CVD are significant enough to prompt action. This beckons for more comprehensive investigation – apart from overlapping inflammatory profiles – into the detailed pathogenesis of periodontitis – particularly the activity of periodontal pathogens – and how it may promote cardiovascular complications.

Relevantly, there are two prominent theories as to how periodontitis is causally linked with CVD (Hajishengallis, 2015). The first stipulates that periodontal lesions act as a gateway for oral pathogens to enter circulation and induce bacteraemia through systemic dissemination. This ideology is supported by the fact that bacteraemia is well documented in periodontal patients (Horliana et al., 2014, Silver et al., 1977, Tomas et al., 2012). These atopobiotic bacteria then contribute to further systemic inflammation and possibly act in an atherogenic manner. The next theory is infers that key microbes implicated in periodontitis are able to induce dysbiosis in the gut which subsequently causes systemic inflammation leading to CVD. This theory was supported by a mice model in which oral pathogens induced endotoxemia and systemic inflammation (Arimatsu et al., 2014); however, the intervening pathogens were undetectable in the gut thereby raising scepticism regarding the exact mechanism in question. Overall, cardiovascular health is eminently related to oral health; the next step is to determine the functionality of periodontal microbes in the cardiovascular system.

2.7. Periodontitis and its links with neurodegenerative disease

For several years now neurodegenerative disease has been correlatively tied to periodontal disorders (Cicciu, 2016, Ranjan et al., 2018, Rai et al., 2012). In the context of neuropathology, the focus here is directed on PD and AD – the two most common neurodegenerative disorders. While these two neurodegenerative diseases differ in molecular hallmarks and clinical symptoms, disease aetiology and pathophysiological mechanisms are somewhat similar (Xie et al., 2014). Neuroinflammation is an integral factor to the development and progression of these pathologies (Guzman-Martinez et al., 2019). Along with inflammation in the central nervous system (CNS), AD and PD also presents with a systemic inflammatory phenotype (Ferrari and Tarelli, 2011, Chiang et al., 2017) with a noted dysregulation of inflammatory biomarkers (Adams et al., 2019). In addition to the fact that periodontal disease has a pronounced inflammatory impact and is capable of leisurely causing systemic inflammation, periodontitis also manipulates the immune system in a manner that upregulates neuroinflammation (Teixeira et al., 2017). Focusing on driving factor of periodontal disease, oral pathogens are believed to – as with CVD – be at the root of this comorbidity between periodontitis and neurodegeneration. Proposed mechanisms of operation are (i) persistent bacteraemia; (ii) atopobiosis of oral pathogens into the CNS *via* the trigeminal nerve; and (iii) orchestration of pathological activity from the oral cavity, i.e. production of toxins and inflammatory molecules that enter the blood and/or nervous system (Teixeira et al., 2017).

In 2008, researchers sought to compare cognitive function among periodontally healthy and ill elderly individuals; their results reflect a similar trend with those of studies assessing links with CVD – cognitive defects (disease) is positively associated with periodontal disease (Yu and Kuo, 2008). After this period in time, a research branch designated to the implication of periodontal disease in neurodegeneration was well established. In recent years it was shown that individuals practicing regular dental hygiene exhibited less of a chance of developing PD (Chen et al., 2018a) – this is synonymous to dental hygiene and cardiovascular disease. Another study correlated the severity and duration of PD with oral pathology (van Stiphout et al., 2018). Ultimately, these data supports the notion that having periodontitis is associated with an increased risk of developing PD (Chen et al., 2017b, Kaur et al., 2016). The ability of periodontal disease to causes neuro- and systemic inflammation has lead researchers to propose oral pathology as 'the missing link' of Parkinson's pathogenesis (Kaur et al., 2016). However, whether systemic inflammation caused by oral pathogens is orchestrated solely from the oral cavity or whether they disseminate systemically and impose a nearer and more direct effect on the CNS and in PD is yet to be fully discerned. In fact, periodontopathic bacteria have not yet been identified in the CNS nor the blood of individuals afflicted with PD.

AD too has a significant relation to periodontal pathologies (Poole et al., 2015, Singhrao and Olsen, 2018, Chen et al., 2017a, Poole et al., 2013). AD and periodontitis share common inflammatory cytokines – typically IL-6 and TNF α (Cestari et al., 2016). This highlights the possibility of overlapping mechanisms accredited to inflammation (Bui et al., 2019). This notion is strongly supported (Teixeira et al., 2017, Ganesh et al., 2017, Singhrao and Olsen, 2019). Even with this data in hand, immune (dys)function is extremely complex and thus exact mechanisms are difficult to recognize. It is known that inflammatory reasons, accredited to pathogens, account in part for the association between periodontal, AD and PD; however, the identification of these oral pathogens or products in the CNS – at the scene of neuropathology – was lacking.

Poole *et al.* (2013) sought to identify periodontopathic bacteria, specifically their molecular trace, in brain tissue of Alzheimer's patients post-mortem. They were one of the first to demonstrate that LPS from chief periodontopathic bacteria is present in the AD brains (Poole et al., 2013). This finding fed the notion that virulent factors from oral pathogens can leave the oral cavity and infect other systems such as the brain. Even more so, LPS stimulates neuroinflammation, amyloid beta aggregation and causes cognitive impairment (Sheng et al., 2003, Zhao et al., 2019) – these factors, along with the finding of oral-derived LPS in AD brains, makes the contribution of periodontopathic bacteria in neurodegenerative disease convincing. The identification of atopobiotic pathogens and their products became a more convincing tie between oral health and neurodegeneration then speculation over inflammation.

Further studies were propelled in this direction. Mice studies have revealed the translocation and presence of periodontopathic bacteria in the brain (Poole et al., 2015, Ishida et al., 2017) and immunoglobulin analysis in human patients suffering from AD have found antibodies against oral pathogens (Laugisch et al., 2018, Sparks Stein et al., 2012). All of this data is greatly significant and support a tight relation between periodontitis and neurodegeneration, but it was only in 2019 that a breakthrough happened.

2.8. Chief periodontal pathogen discovered in the brains of Alzheimer's disease patients

There exist many species of pathogenic bacteria implicated in periodontal disease which may be expected to link with systemic pathology, but only a few are capable of prompting periodontitis in solitude. Stealing the spotlight from hundreds of other bacterial species, a complex of three bacteria – the red complex – is highlighted as the most implicative bacterial species and consortium in periodontitis (Socransky et al., 1998). The red complex comprises the species *Bacteroides forsythus, Treponema denticola,* and *Porphyromonas gingivalis,* with *P. gingivalis* being the most pathogenic of the trio (Mysak et al., 2014). It is predominantly the interplay of activities between these anaerobic bacteria that coordinate the induction of a dysbiotic polymicrobial profile capable of wreaking oral tissue detriment. With that being said, an emphasis is laid on the activity of *P. gingivalis* as this bacterium, apart from the fact that this anaerobe can single-handedly promote and drive systemic inflammation and periodontitis (Blasco-Baque et al., 2017, Bui et al., 2019), is the most implicated in systemic disease (Singhrao and Olsen, 2019) This study will thus focus on *P. gingivalis*.

While many associations have been made between periodontitis and other chronic inflammatory disorders such as cardiovascular and neurodegenerative pathology, there are very few that have directly identified the chief periodontal pathogen – *P. gingivalis* – in an atopobiotic manner such as in the brain or blood. However, to much avail, this scarcity was contested in 2019. *P. gingivalis* and its molecular signatures were recently discovered in the brains of individuals with overt AD (Dominy et al., 2019). Dominy et al. (2019) demonstrated the presence of DNA and virulent proteases originating from P. gingivalis in the cerebral cortex of Alzheimer's patients thereby directly implicating this bacterium at the scene of neuropathology; to take it even further, these researchers correlated the levels of these exclusive proteases with ubiquitin and tau pathology (Dominy et al., 2019). In a mouse model apart of the same study, oral P. gingivalis translocated to the brain where an increase in

amyloid β 1-42 was observed. When a gingipain-inhibitor was used as a therapeutic intervention, a reduction in neurotoxicity was eminent and therefore suggests that gingipains may be a significant contributor to neurodegeneration.

This is the most direct connection between this bacterium, periodontitis and neurodegeneration. While Parkinson's and Alzheimer's are individualised pathologies, the development of neurodegeneration is believed to share causative and driving factors; hence, it is fair to hypothesize that *P. gingivalis* will be identified in haematological tissue of both PD and AD patients. Prospective identification of this oral pathogen in PD, coupled to the recent discovery of *P. gingivalis* in AD brains, may provide further insight into the pathogenesis of not only PD but neurodegeneration as a whole. Along with a study that discovered *P. gingivalis* in organs, particularly brain and atherosclerotic tissue, exterior to the oral cavity in mice (Velsko et al., 2014), this bacterium is now suspected to travel and persist in the haematological system. Hence, the influence that this chief periodontopathic bacterium and its virulent factors have on coagulation – since clotting processes are impaired in both periodontal and neurodegenerative disease – is of particular importance and thus requires further comprehensive investigation.

2.9. *Porphyromonas gingivalis*

P. gingivalis is a gram-negative, non-motile, rod-shaped anaerobic bacterium that is infamous for its pathogenic potential in the oral cavity. This bacterium acts as a keystone pathogen in the context of periodontal disorders by causing bacterial dysbiosis in the oral cavity, manipulating immune function and thus orchestrating the pathogenesis of periodontitis (Darveau et al., 2012). Whilst *P. gingivalis* is present and homeostatically maintained in healthy states, it has the capacity to act opportunistically and induce the transformation of balanced microbiota into a pathogenic (dysbiotic) community (keystone pathogen). The severity of periodontitis exhibits a positive correlation to bacterial load (Lee et al., 2015, Ready et al., 2008). As periodontitis has been shown to associate with a number of systemic diseases, it is believed that *P. gingivalis* – the chief initiator and driver of periodontitis – has a prominent role to play in associated inflammation and the pathophysiology of comorbidities. Multiple studies have investigated and discovered possible roles played by this bacterium and its virulent factors in a list of diseases including cardiovascular pathologies (Yun et al., 2005, Zhang et al., 2015, Sheets et al., 2005, Sheets et al., 2006, Mougeot et al., 2017, Hussain et al., 2015, Brodala et al., 2005, Liccardo et al., 2019, Patrakka et al., 2019, Velsko et al., 2014),

neurodegeneration (Singhrao et al., 2017, Dominy et al., 2019, Poole et al., 2015, Poole et al., 2013, Kaur et al., 2016, Singhrao and Olsen, 2018), diabetes (Liccardo et al., 2019, Makiura et al., 2008, Ohtsu et al., 2019), rheumatoid arthritis (Jung et al., 2017, Okada et al., 2013, Mikuls et al., 2014) and cancer (Zhou and Luo, 2019). Hence, treatment options for periodontitis mostly work to reduce the activity and thus success of *P. gingivalis* (Puth et al., 2019, Kugaji et al., 2019, Sha and Garib, 2019) with the hope of reducing further bone loss and soft tissue damage as well as preventing systemic inflammation and the onset of comorbidities.

Eminent to the pathological potential of this prokaryote, *P. gingivalis* possesses an extensive inventory of virulent factors. Table 1 list the main virulent factors of *P. gingivalis* and briefly mentions their role in pathology. To sum up, *P. gingivalis* uses these endogenous molecules to enable survival and persistence by manipulating and evading the host immune system (Hajishengallis, 2011, Olsen et al., 2016, Slocum et al., 2016), altering host proteins *via* cleavage and citrullination (Engstrom et al., 2018, Marchant et al., 2013, Guo et al., 2010), inducing apoptosis of host cells (Desta and Graves, 2007, Inaba et al., 2018) and aiding in the survival and success of other pathogenic microbes (Karkowska-Kuleta et al., 2018, Olsen et al., 2017). Although all these virulent factors act in unison to cause dyshomeostasis, there are specific enzymes – called gingipains – that take credit for the majority of the pathological success of *P. gingivalis* (Zenobia and Hajishengallis, 2015) and thus constitute the focus of this study.

Table 1: Important virulent factors produced by *P. gingivalis* that enable its pathological success in human tissue.

Virulent Factors	Action and consequences	References
Gingipains	Degradation of host antimicrobial peptides, immunoglobulins and central complement component C3; prevents complement activation; nutrient acquisition *via* proteolysis; causes tissue damage and subsequent inflammation	(Popadiak et al., 2007, Grenier, 1992, Palm et al., 2013)
Peptidylarginine deiminase	Host and bacterial protein citrullination linked to arthritis; induction of autoantibodies in rheumatoid arthritis	(Maresz et al., 2013, Wegner et al., 2010)
FimA fimbriae	Binds CXCR4 and induces CXCR4-TLR2 crosstalk; cAMP signalling and inhibition of nitric oxide-dependent killing; required for oral colonization and infection gingival epithelial cells	(Hajishengallis et al., 2008, Hajishengallis and Lambris, 2011)
SerB (serine phosphatase)	Suppresses IL-8 production by dephosphorylation of NF-kB p65; prevents nuclear translocation and transcription	(Takeuchi et al., 2013)

Adapted from (Zenobia and Hajishengallis, 2015)

2.9.1. Gingipains – Major virulent factors of *P. gingivalis*

P. gingivalis is incapable of carbohydrate cleavage (asaccharolytic) and instead relies on proteolysis for the accession of nutrients (Lamont and Jenkinson, 1998). This beckons for the production and utility of proteolytic enzymes. Accordingly, *P. gingivalis* has evolved to synthesize trypsin-like proteases called gingipains that confer functionality meeting and extending past the need for nutrients.

Gingipains are cysteine proteases produced by *P. gingivalis* that account for roughly 85% of the proteolytic activity of this bacterium (Li and Collyer, 2011). There exist three forms: arginine-specific gingipain A (RgpA), arginine-specific gingipain B and lysine-specific gingipains (Kgp). These proteases are named according to their respective cleavage site e.g. Kgp cleaves at lysine-Xaa residues and Rgp at arginine-Xaa residues. These enzymes exist in monomeric forms (e.g. RgpA) or may be processed into a non-covalent protease complex such as the RgpA-Kgp complex. These enzymes are present on the membrane of this species, released extracellularly in a soluble form and also contained in outer membrane vesicles (Imai et al., 2005, Veith et al., 2014). As trypsin-like proteases, gingipains enable *P. gingivalis* to carry out life-sustaining activities which include adherence to host substrates such as fibrinogen, fibronectin, haemoglobin, laminin and erythrocytes (Pike et al., 1996, Belstrom et

al., 2011), degrading host proteins (Potempa et al., 2003) such as plasma proteins, extracellular matrix proteins, cytokines and proteins involved in the complement system thereby leading to immune manipulation and evasion (Abdi et al., 2017, Mezyk-Kopec et al., 2005, Slocum et al., 2016, Hajishengallis, 2011), causing agglutination and haemolysis of erythrocytes (Li and Collyer, 2011, Shah and Gharbia, 1989) and heme acquisition for auxotrophic reasons (Smalley et al., 2011, Lewis, 2010). Studies involving immunisation, knock-outs and inhibitors of gingipains demonstrate the necessity of these enzymes in the initiation and progression of periodontal pathologies (Table 2). As the fate of this bacterium's success is considerably determined by the functionality of these gingipains, the detection and inhibition of gingipains may be a vital key in combating its virulence and role in periodontal and systemic pathology.

Table 2: Previous experimental results demonstrating the necessity of gingipains for the pathological success of *P. gingivalis.* The studies referenced are limited to periodontal disease; the reason being is that *P. gingivalis* originates from the oral cavity and hence the pathological activity of gingipains precedes systemic infection. Furthermore, the discovery of *P. gingivalis* in physiological systems exterior to the oral cavity is scarce.

Mode of Experiment	Model Utilized	Result	References
Immunization with RgpA	Murine periodontitis model	Prevention of *P. gingivalis*-induced oral bone loss	(Gibson and Genco, 2001)
Immunization with a peptide domain of Rgp	Murine subcutaneous chamber model	Immunity against *P. gingivalis* infection	(Genco et al., 1998)
Immunization with RgpA-Kgp complex	Murine skin abscess model	Immunity against *P. gingivalis* infection	(O'Brien-Simpson et al., 2000)
Immunization with the RgpA-Kgp proteinase-adhesin complex	Rat periodontitis model	Resistance to *P. gingivalis* colonization and periodontal bone loss	(Rajapakse et al., 2002)
Immunization with wild type *P. gingivalis* gingipain-deficient mutants and re-infection	Murine skin abscess model	Protection against re-infection elicited by the wild-type strains devoid of gingipains	(Yoneda et al., 2003)
Inoculation of *P. gingivalis* W50 with Rgp-specific inhibitor	Murine subcutaneous chamber model	Inactivation of Rgp attenuates virulence	(Genco et al., 1999)
Passive immunization of human subjects	Anti-gingipain antibodies were applied into periodontal pockets	Treated group had reduced level of *P. gingivalis* and decreased bleeding on probing	(Yokoyama et al., 2007)

Since the invasion of the haematological and other systems by P. gingivalis is distinguished, and considering it is associated with increased cardiovascular risk (Ghizoni et al., 2012), the effect of its integral proteases on coagulation (specifically fibrinogen to fibrin conversion)

warrants further investigation. Additionally, inflammation brought about by *P. gingivalis* may also cause defective clotting.

2.9.2. Inflammation as a disturbance of coagulation in periodontitis – the possible role of *P. gingivalis*

As a defence mechanism, the human body allows inflammation to occur in order to identify harmful entities such as foreign substances and organisms, as well as to repair damaged tissue. Simply, there exist two types of inflammation: acute and chronic inflammation. Acute inflammation functions to remove organisms, toxins and repair acute injuries in a short period of time. Maladaptations in this operation may lead to dysregulated inflammatory cues that persist and lead to chronic inflammation. Chronic inflammation occurs over a long period of time and results from long-term exposure or failure to remove necessary organisms, toxins or endogenous molecules, autoimmune implications, and recurring episodes of acute inflammation (Pahwa and Jialal, 2019). Chronic inflammation is causative of pathological detriment and subsequent systemic disease (Straub and Schradin, 2016). Systemic disease encompasses disorders such as diabetes mellitus type II, neurodegeneration (Alzheimer's and Parkinson's disease), psoriasis, rheumatoid arthritis, cancers, systemic lupus erythematosus and periodontitis, all of which presents with a chronic inflammatory profile (Multhoff et al., 2011, Tsalamandris et al., 2019, Lotti et al., 2010, Munoz et al., 2010, Cekici et al., 2014). Interestingly, bacterial endotoxins have been implicated in these disorders (Gomes et al., 2017, Romani et al., 2013, Pretorius et al., 2017a, Jain et al., 2019).

Intimately related to chronic inflammation are aberrations in normal coagulative processes (Levi and van der Poll, 2010). The clotting system comprises of platelets, coagulation machinery (enzymes, cofactors) that enables functionality and obviously fibrinogen. Platelet function is considerably influenced by inflammation, especially in the chronic state (Senturk, 2010). Inflammatory markers such as cytokines are dysregulated during (chronic) inflammation and can modulate platelet receptors thereby causing platelet hyperactivity (Randeria et al., 2019) – a contributing factor to hypercoagubility. Bacterial endotoxins have also been implicated in the functionality of these clotting entities (Lopes Pires et al., 2017, Saluk-Juszczak et al., 2000). During (chronic) inflammation and an acute phase response, fibrinogen (an acute phase reactant) levels increase (over 7mg/ml) and act as a prominent cardiovascular risk factor (Fibrinogen Studies et al., 2005). The concentration of this plasma protein is a determinative factor for clotting efficiency; hence, increased levels of fibrinogen

leads to increased clot formation – a relation that leads to hypercoagubility and an increased risk for thrombosis.

A significant modulator of coagulation is inflammation (Foley and Conway, 2016). Inflammatory processes in periodontal disease contributes to excessive bleeding observed in gingival tissue (Dikshit, 2015). Oral pathogens greatly modulate immune function in order to ensure their survival and subsequent pathogenesis of pathology including periodontitis, atherosclerosis and Alzheimer's disease (Olsen et al., 2016). Predominantly, periodontopathic bacteria operate to evade immune function thereby leading to their persistence in the oral cavity. However, with that being said, the overall inflammatory response is not nullified but instead becomes dysregulated and pathological in function (Hajishengallis, 2011). Thus, this immune-modulation by oral pathogens starts off as an evasion strategy that inevitably results in pathological systemic inflammation characterised by cytokine dysregulation (Hajishengallis, 2015). This influence on systemic inflammation mediated by oral bacteria may underlie aberrant coagulation and downstream effects seen in periodontitis and associated comorbidities.

In the context of a more specific influence on the clotting system, gingipains exert more of a direct effect by targeting key role players in the coagulation system, predominantly fibrin(ogen).

2.9.3. The impact of gingipains on the coagulation system

Excessive bleeding of the gums – one of the hallmarks of periodontal pathology – is encouraged by gingipains (Imamura et al., 1995). Furthermore, patients with gingivitis or periodontitis exhibit increased levels of plasma proteins in salivary fluids, an indication of plasma leakage due to increased vascular permeability (Henskens et al., 1993). These proteases possess the ability to process proteins involved in the clotting cascade as well as modulate the activity and concentration of platelets in the blood (Benedyk et al., 2016). One prominent effect of gingipains on the clotting system is the ability to cleave fibrinogen and fibrin (Imamura et al., 1995). Hence, this presents with major implications for the efficacy of coagulation and terminal clot properties.

Results from experiments involving human plasma and exogenous RgpA has unveiled the ability of this specific gingipain to activate prekallikrein with ensuing increases in the levels of bradykinin, an inflammatory mediator that promotes vascular permeability enhancement (Imamura et al., 1994). Imamura *et al* (1994) speculated that this activation of the

kallikrein/kinin is a direct cause for the increase in gingival crevicular fluid (an inflammatory exudate) seen in periodontal disorders. The same researchers, whom have provided comprehensive insights into gingipains and their role in the coagulation system, also demonstrated RgpA-mediated activation of a number of clotting factors. Both RgpA and RgpB activate factor X in a dose- and time-dependent manner (Imamura et al., 1997); this coincides with lower prothrombin times measured in the same experiment. This suggests that Rgp-dependent factor X activation seems to promote faster clotting cascades (hypercoagulation). Rgps also activate factor IX (Imamura et al., 2001b) and prothrombin (Imamura et al., 2001a). Both the activation of factor IX and X leads to increases in thrombin – a factor which regulates the activation of other coagulation factors, catalyses the conversion of fibrinogen to fibrin and activates protein C (anticoagulant) when bound to thrombomodulin (Esmon, 2001). Increases in thrombin increase fibrin formation and results in densely arranged clots (Wolberg and Campbell, 2008), factors which can contribute to abnormalities in coagulation and subsequent cardiovascular implications (Kim et al., 2016, Kell and Pretorius, 2015).

Platelets are also manipulated by gingipains (Pham et al., 2002). In a mice model, researchers inoculated subjects with gingipains and observed a successive increase in both platelet activity and count (Benedyk et al., 2016). Gingipains act proteolytically on protease-activated receptors 1 & 4 on the membranes of platelets; this results in an intracellular influx of calcium ions and subsequent platelet aggregation comparable to that of thrombin (Lourbakos et al., 2001). Klarström Engström *et al* (2014) determined that Rgp is essential for the activation of platelets (Klarstrom Engstrom et al., 2015). Interestingly, the sensitivity of human platelets to epinephrine, an agonist of platelet activity (Shattil et al., 1989), is increased in the presence of RgpA (Nylander et al., 2008); there is speculation as to whether this may be a link between stress and cardiovascular disease.

Fibrinogen – the zymogen to fibrin, the structural constituent of clots – exerts significant binding affinity towards all three gingipains (Pike et al., 1996). It is speculated that this assists in attachment and adhesion to host surfaces as do gingipains with fibronectin, an extracellular matrix protein (Pike et al., 1996). In addition to simply binding fibrinogen, gingipains have fibrin(ogen)olytic potential (Imamura et al., 1995). Whilst the trio of gingipain enzymes degrade the α chain of fibrinogen synonymously, RgpA is more efficient in cleaving the β chain – a phenomenon attributed to differences in amino acid residues in the active site of the proteases (Ally et al., 2003). Degradation of fibrinogen decreases clot formation and will diminish the ability of the coagulation system to resolve bleeding. Consequently, it seems likely that gingipain-mediated fibrinogenolysis underlies the hallmark symptoms of excessive bleeding and inflammation observed in periodontal tissue.

Clinical significance of this fibrin(ogen)olytic capability may not be limited to periodontal disorders; additionally, the role of gingipains on systemic physiology and more specifically overall coagulation may pose of benefit to chronic inflammatory patients whom of which may present with simultaneous periodontitis. This ideology is supported by recent findings of gingipains in the hippocampal region of the brain of Alzheimer's patients (Dominy et al., 2019) as well as in human atherosclerotic tissue (Kozarov et al., 2005).

Even though the fibrinogen-degrading role of gingipains has been elucidated, a holistic approach using an array of modern techniques such as thromboelastography, rheometry, scanning electron microscopy and confocal microscopy will offer a better understanding of these proteases' effect on the kinetics of coagulation, the deformation and response of clots under shear force and the architecture of terminal fibrin networks. The presence of this microbe in diseased states also requires establishment, especially since their existence has been found in cardiovascular and neurological disease.

2.9.4. Lipopolysaccharide from *P. gingivalis*

LPSs are immunogenic membrane components of gram-negative bacteria. While there are intra- and inter-species differences between LPSs in terms of structure, function and serogenicity, common to this class of bacterial products is the prominent immune response they induce (Pulendran et al., 2001, Tan and Kagan, 2014). With relevance to the oral cavity, LPS PG (LPS from *P. gingivalis*) has been shown to increase the production of inflammatory cytokines including such as interleukin (IL)-1, IL-6, IL-8 and TNF-α from gingival cells (Imatani et al., 2001, Taguchi et al., 2015). Fibroblasts of the periodontal ligament have been noted to succumb to oxidative stress induced by LPS PG (Golz et al., 2014) and release inflammatory cytokines *via* TLR signalling (Wang and Ohura, 2002, Savitri et al., 2015), the major detection and signalling receptors of the innate immune system mentioned earlier. Through such immune-related means, inflammatory cytokines produced by LPS PG may contribute to an overall state of systemic inflammation common to periodontitis, CVD, AD and PD (Singhrao and Olsen, 2019, Deleon-Pennell et al., 2013).

Focusing on neurodegeneration, LPS PG favours serum amyloid beta peptide formation (Leira et al., 2019). This mechanism likely contributes to amyloid load in neuropathology and therefore the severity of AD (Lee et al., 2019). Using mice, TLRs have also been implicated in LPS PG-induced cognitive impairment, inflammatory cytokine dysfunction and neuroinflammation (Zhang et al., 2018). Comprehensively, it has also been shown that LPS

PG, with the involvement of cathepsin B, can in solitude cause AD-like phenotypes in mice (Wu et al., 2017b). It should be noted that LPS from other bacterial species are also capable of giving rise to an AD phenotype in mice (Zhan et al., 2018). Nonetheless, it is expected that LPS PG, due to its immunogenic capabilities, are important virulent factors used by *P. gingivalis* to cause systemic inflammation and thus dyshomeostasis.

In the context of coagulation, there is little research investigating the function of LPS PG in clotting processes. Studies conducted using other species' LPS have all provided similar results – that LPS causes increased clotting propensity (hypercoaguable state) and anomalous fibrin network formation (Pretorius et al., 2016, Pretorius et al., 2018b, Pernerstorfer et al., 1999). From the one study that does assess LPS PG, it was shown that LPS PG causes platelet activation *via* the Cdc42 receptor which causes platelet spreading and pseudopodia formation (Senini et al., 2019) – modulation of platelet activity *via* periodontal pathogens may be in part responsible for the prothrombotic phenotype observed in periodontitis. It would be expected that LPS PG prompts a similar response to other forms of LPS. What is of particular importance, in relation to proteolytic functionality in clotting, is the association of LPS with gingipains. These two inflammagens form a complex that is virulently functional (Takii et al., 2005); subsequently, the activity of gingipains while bound to LPS PG was noted to be altered. The researchers involved discovered that the LPS-gingipain complex inhibited the inflammatory response characteristic to LPS. This phenomenon has been postulated as one of *P. gingivalis'* potential immune evasive techniques (Takii et al., 2005). Furthermore, the proteolytic activity in the complex form deviated significantly from gingipains that were in their free (monomeric) form. In the frame of coagulation, this complex form can alter the outcome of the overall effect that gingipains impose on clot formation. As *P. gingivalis* exists by utilizing both LPS and gingipains, the co-incubation of these two bacterial inflammagens in experiments is warranted. Hence when assessing the role of gingipains in clotting experiments, co-incubation with LPS PG will offer considerable insight as both bacterial inflammagens are present in the physiological state. Such an intervention will help understand the impact that LPS PG in the presence of gingipains or the LPS-gingipain complex has on clot kinetics and fibrin formation.

2.10. Concluding remarks

Figure 3 depicts the hypothesized order of events beginning from oral dysbiosis to systemic inflammation and coagulation dysfunction. The influence of *P. gingivalis* on systemic health and disease is currently under scrutiny, especially after the study conducted by Dominy and colleagues (Dominy et al., 2019). As this bacterium has recently been found in Alzheimer's disease brains, atherosclerotic plaques and thrombi from stroke patients, the persistence and activity of this bacterium in the haematological system may be pose as a major link between periodontitis and systemic disease. Since cardiovascular disease – a comorbidity of periodontal and neurodegenerative disease (Firoz et al., 2015, Vogelgsang et al., 2018) – is the leading cause of global mortality and that defects in coagulation contribute significantly to this burden, the role that gingipains from *P. gingivalis* has on the coagulation cascade and overall fibrin synthesis warrants investigation.

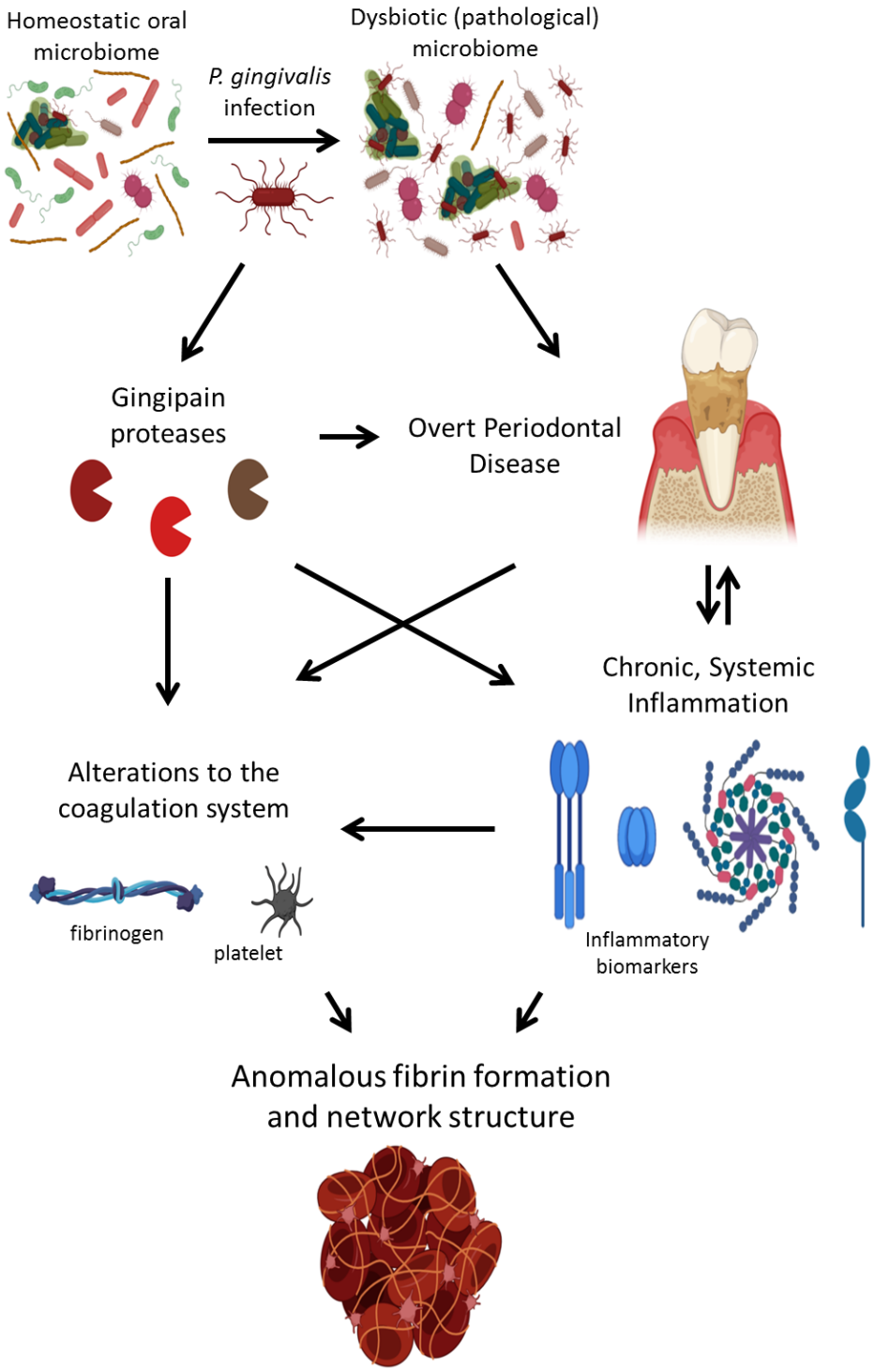


Figure 3: The sequence of events leading from oral dysbiosis, governed by *P. gingivalis*, to systemic inflammation and subsequent aberrations in the coagulation system. Typically, oral dysbiosis contributes significantly to inflammation. Ultimately, dysregulated immune function manifests as systemic inflammation. This proinflammatory state, along with gingipains from *P. gingivalis*, alters normal functioning of the coagulation system which results in impaired clot kinetics, formation and degradation.

Following this comprehensive literature review, the following chapters will further investigate the influence of gingipains and LPS of *P. gingivalis* on the haematological system, and also determine if it is indeed present in the circulation of PD and AD patients. For a quick recap, our research questions and hypotheses are again presented:

Research questions:

- Can bacterial membrane inflammagens from *P. gingivalis* be detected in blood samples of the two most common neuro-inflammatory conditions, namely PD and AD?
- How does inflammagens from *P. gingivalis* affect blood clotting?

Our hypothesis

We hypothesize that RgpA will be detected, for the first time, in PD blood samples using immunohistochemistry. Furthermore, the same technique will be applied to AD samples thereby identifying RgpA in the blood (not brain tissue) of these patients for the first time.

Furthermore, due to its fibrinogenolytic activity, fibrinogen incubated with RgpA will result in a much lower load of fibrin formed when exposed to thrombin. Plasma clot kinetics and formation will shift towards a hypocoaguable state when exposed to RgpA while the rheology of clots will be less pliable and stiffer. These effects of RgpA will be altered when co-incubated with LPS from the same species of bacteria, *P. gingivalis.*

CHAPTER 3: STUDY DESIGN AND SAMPLE COLLECTION

The present study takes the form of a cross-sectional study design conducted at the Department of Physiological Sciences within the Clinical Hemorheology and Coagulation Research Group.

3.1. Ethical Statement

Ethical clearance was obtained from the Health Research Ethics Committee (HREC) of Stellenbosch University (South Africa) (approval number HREC Reference #: N19/03/043) (Appendix A). Written informed consent was obtained following a verbal explanation of the study details and perusal of the written consent forms (Appendix B). Study participants received a unique number that was used to guarantee discretion by protecting their identity. Furthermore, researchers followed Good Clinical Practice and abided by ethical codes of conduct throughout this study. With that being said, the guidelines of the Declaration of Helsinki were abided by.

3.2. Study Population and Blood Collection

3.2.1. Control Samples

Forty-two healthy volunteers were recruited from the general population residing in Stellenbosch, Cape Town and surrounding areas. In this control population, 26 were female and 17 were male. Exclusion criteria for the control population included (i) inflammatory and/or coagulation disease (ii) infection (iii) smoking (iv) conflicting medication such as aspirin or hormone replacement drugs that may affect blood clotting processes. Blood was drawn in sodium citrate tubes by a registered medical biological scientist (Health Professions Council of South Africa (HPCSA)) and phlebotomist (MW: 0010782) from the Department of Physiological Sciences, Stellenbosch University.

3.2.2. Parkinson's Disease Samples

The PD samples were collected from n=40 patients visiting and receiving treatment at Tygerberg hospital in Bellville, Cape Town. The PD patients were n=15 female and n=25 male. PD volunteers were recruited with the following inclusion criteria: (i) a confirmed diagnosis by a neurologist of which includes the use of the Unified Parkinson's Disease Rating Scale (UPDRS), as well as the Hoehn and Yahr scale used to rate the relative level of the PD

disability, (ii) males and females of any age, (iii) devoid of any anticoagulant medication. Participants who were unable to provide written consent were excluded from this study.

3.2.3. Alzheimer's disease Samples (Obtained during 2015)

Ethical clearance was obtained from the Faculty of Health Science Research Committee of the University of Pretoria (Appendix C). Exclusion criteria included smoking, infectious disease, pregnancy, hormonal therapy and comorbid disease (vascular dementia).

Alzheimer's disease patients were diagnosed and treated by a neurologist that took caution to offer blood samples from those without vascular dementia. These patients were subjected to the Mini-Mental State Exam which is used to rate the severity of cognitive dysfunction. Blood collection was carried out in the same manner as previously stated – collected in citrate tubes.

3.3. Storage of Plasma Samples

After the blood withdrawal, the samples viable for coagulation testing were allowed to rest for 30 minutes at room temperature after which they were centrifuged at 3000g for 10 minutes. The plasma was then carefully collected with a Pasteur pipette and transferred into a 1.5mL microcentrifuge tube for storage at -80°C. When samples were required for testing, the sample tubes were allowed to thaw at room temperature.

5.4. Statistical Analysis

Statistical analyses performed in this study were conducted on GraphPad Pad (7.04). Shapiro-Wilk normality tests were performed on all data in order to determine whether parametric or nonparametric tests were done. Normally distributed data was then analysed with paired T-tests whereas nonparametric data underwent Mann-Whitney-Wilcoxon tests. Significance of results were determined at $p < 0.05$. Mean grey value of confocal images was measured on ImageJ 1.45s.

5.5. Demographics of the subject populations

The ages of healthy, PD and AD groups were subjected to a Shapiro-Wilk normality test of which the data passed as normally distributed. The age data is represented in Table 3 and visually depicted in Figure 4. The mean ages are 62, 68 and 73 for the healthy, PD and AD population, respectively. Hoehn and Yahr ratings of PD patients are also represented in Table

3, alongside the respective patient's age. Hoehn and Yahr ratings are used to classify the stages and severity of PD.

Table 3: The ages of all study participants plus the Hoehn and Yahr rating for each PD patient.

Control (40)	PD (40)		AD (10)
		Hoehn & Yahr	
69	58	2	76
72	69	2	65
61	53	2	68
79	68	3	79
49	59	2	86
50	66	3	81
77	59	2	82
81	59	3	58
74	59	2	59
74	69	2	78
77	68	3	mean = 73
72	75	2,5	
76	64	2,5	
66	65	1	
69	64	1	
66	66	4	
84	64	1	
72	63	2,5	
71	64	4	
39	72	3	
57	87	2	
59	70	2	
53	62	3	
59	71	4	
55	64	1,5	
47	63	3	
50	77	2	
51	71	1	
49	73	2	
51	87	3	
57	72	5	
56	65	2	
54	81	2,5	
50	52	1	
56	60	2,5	
58	72	1	
58	79	2,5	

58	83	2	
49	59	1	
57	84	3	
mean = 62	mean = 68		

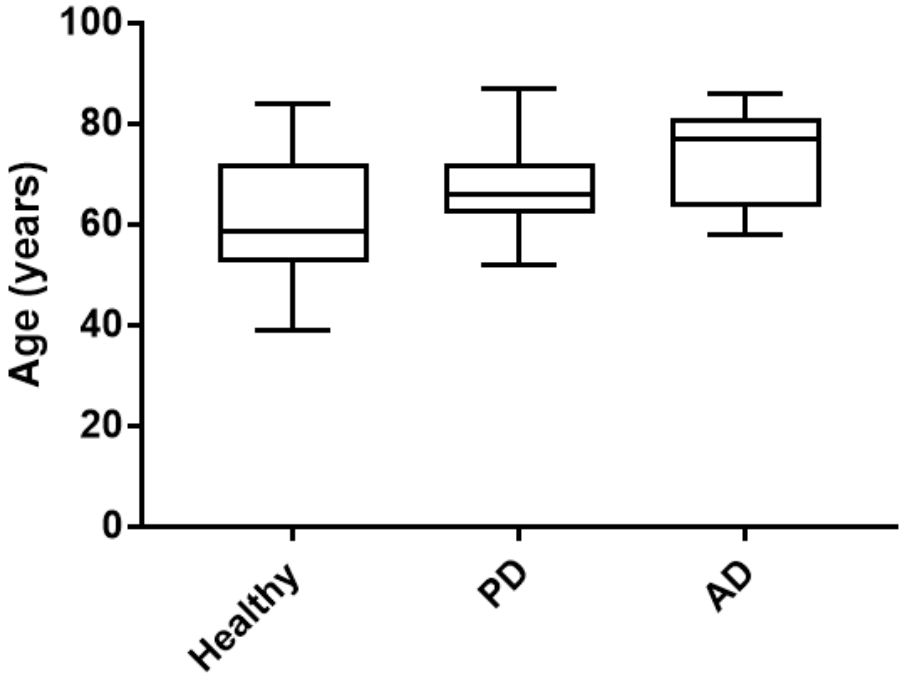


Figure 4: Box plots of the age differences among the healthy, PD and AD populations participating in this study.

Layout of the rest of the thesis

This study is further subdivided into three research chapters according to the type of model applied, namely:

- **Chapter 4: clinical blood model** in which AD and PD blood samples will be probed for the presence of RgpA;
- **Chapter 5: healthy blood model** in which the effects of recombinant RgpA on clotting kinetics and rheology will be tested on healthy whole-blood (WB) and platelet-poor plasma (PPP) samples; and
- **Chapter 6: purified fibrinogen model** in which the proteolytic effect of RgpA on terminal fibrin network structure will be analysed.

The last few chapters will discuss and conclude the results and issue relevant to this thesis:

- **Chapter 7: Discussion** of the experimental data obtained in this thesis with relevance to overall topic; and
- **Chapter 8: Conclusion**, bringing together the clinical relevance of this research thesis.

CHAPTER 4: CLINICAL STUDY

4. The detection of RgpA in the blood of PD and AD patients

4.1. Introduction

With the mounting evidence indicating a strong association of PD and AD with periodontitis, and with the recent discovery of DNA and gingipains from *P. gingivalis* in the brains of patients suffering from AD, this section seeks to identify RgpA in PD and AD blood. Here, we use PPP from n=40 PD individuals, n=10 AD individuals, and n=40 healthy controls whereby each sample is incubated with a fluorescent antibody specific for RgpA; after sample preparation is complete, confocal microscopy is used to detect – *via* fluorescent means – the presence of RgpA in a particular sample. The fluorescent intensity of each micrograph, although easily discernible *via* qualitative means, is also measured in a quantitative manner to enhance significance.

The research question we aim to address, by using immunohistochemistry and confocal microscopy, is: **'Can bacterial membrane inflammagens from *P. gingivalis* be detected in blood samples of the two most common neuro-inflammatory conditions, namely PD and AD?'**

4.2. Techniques

4.2.1. Immunohistochemistry and confocal microscopy

Immunohistochemistry is the utilization of antibodies for identifying target molecules (antigens); normally these are coupled to a fluorophore whose emission can be detected when excited. Confocal microscopy is an optimal imaging technique that, in this case, uses fluorescence to study target molecules and is a central tool with admirable resolution. For instance, in blood and purified fibrinogen models, our research group has detected the presence of amyloid, using probes (conjugated to a fluorophore) specific for amyloid character, within clots exposed to bacterial LPS and LTA (Pretorius et al., 2018b). Immunohistochemistry in this manner has been implemented to identify bacterial inflammagens, such as LPS (de Waal et al., 2018). Together, immunohistochemistry and confocal microscopy will be used to identify RgpA in PD and AD blood samples.

Here, the Zeiss LSM 780 confocal laser scanning microscope (Carl Zeiss Microscope, Munich, Germany) was used to view fluorescent signal.

4.3. Methods and materials

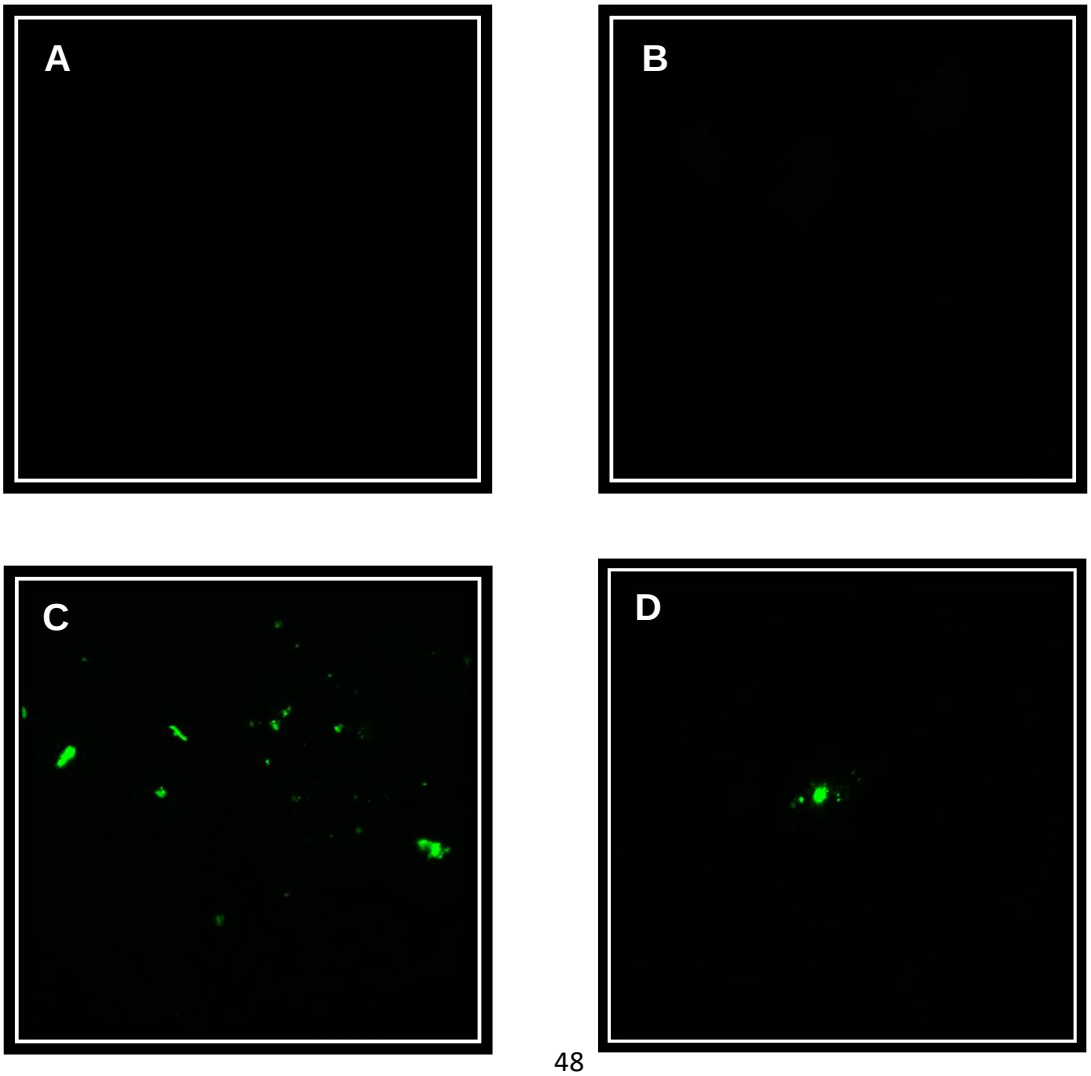
4.3.1 Immunohistochemistry and confocal microscopy

In order to detect the presence of RgpA in of healthy, PD and AD individuals, a rabbit polyclonal (derived from multiple B-cell lineages) antibody against RgpA, conjugated to fluorescein isothiocyanate (FITC), was purchased from Abbexa (catalogue number: abx107767). During the preparation, the antibody as well as goat serum was kept on ice. 5µL of PPP (PD) or WB (AD) was carefully pipetted onto a microscope slide and left to stand for 2 minutes. Thrombin solubilized in PBS containing 0.2% human serum albumin with a concentration of 20 $U \cdot mL^{-1}$ was obtained from the South African National Blood Service. 2,5µL of thrombin was pipetted directly into the centre of the sample on the microscope slide and carefully spread with the pipette tip in order to ensure fibrin network formation. After the addition of thrombin, the sample was left for five minutes to allow fibrin fibres and network to form and adhere to the slide. The sample was then fixed with 10% neutral buffered formalin for 10 minutes. After washing the sample three times with PBS (pH=7.4), a blocking step using 5% goat serum (diluted with PBS) was employed for five minutes. The sample was then incubated with the RgpA FITC polyclonal antibody at a ratio of 1:100 (diluted in PBS) for an hour in a dark room. After antibody incubation, the samples were then washed with PBS to rid of unbound antibody. A drop of Dako fluorescent mounting media was added and the sample was subsequently mounted with a coverslip for confocal imaging. The prepared samples were viewed on a Zeiss LSM 780 with ELYRA PS1 confocal microscope using a Plan-Apochromat 63x/1.4 Oil DIC objective. The gingipain R1 FITC antibody was excited at 488 nm, with emission measured at 508 to 570 nm. As a positive control, recombinant RgpA (Abcam; catalogue number: ab225982) was added at a final concentration of 500 $ng.L^{-1}$ to healthy PPP (devoid of signal when naïve) and left to rest for 30 minutes; then, this sample was incubated with the antibody and visualized to confirm specificity for RgpA.

4.4. Results

4.4.1. Positive control using the polyclonal RgpA antibody

In order to determine the specificity and reliability of binding between the RgpA polyclonal antibody and its target in plasma, a positive control experiment was performed (Figure 5). A healthy control whose plasma was devoid of fluorescent signal when incubated with the antibody received an exogenous dose of 500ng.L^{-1} recombinant RgpA. Following this addition of extrinsic RgpA, the antibody was then added to the plasma in a 1:100 concentration and viewed under confocal microscopy. The confocal image in Figure 5A is that of unstained plasma with the purpose of negating autofluorescence that may give rise to false positive results. When stained with the RgpA polyclonal antibody (Figure 5B), there was no signal detected indicating that this plasma sample is lacking a noticeable load of endogenous RgpA. Conversely, when exogenous RgpA is added to the plasma and probed with the antibody (Figure 5C), definitive signal was obtained. This infers that the antibody has bound to the added RgpA thereby ensuring the detection specificity for RgpA.



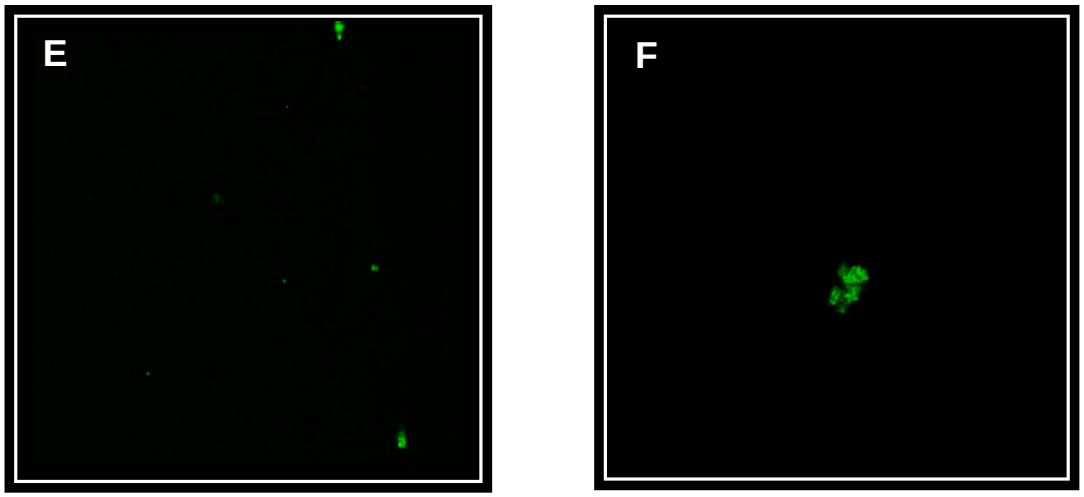
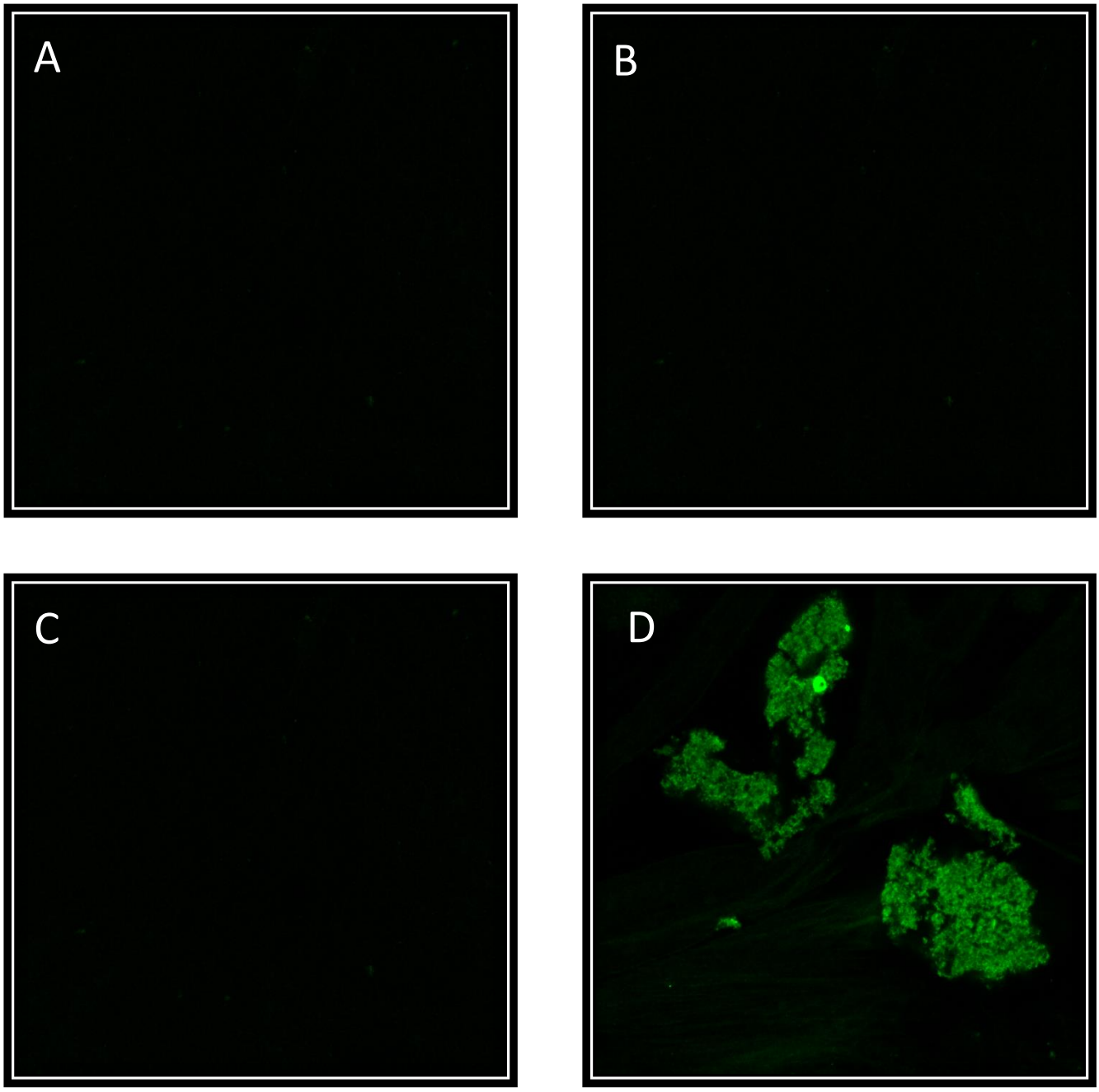
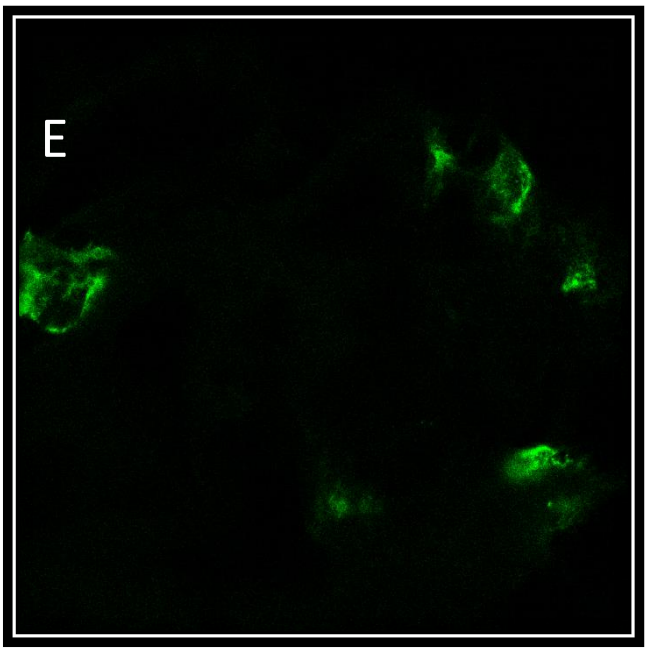


Figure 5: Positive control using the RgpA antibody in plasma samples. In this figure, a naïve (A) and RgpA antibody-stained (B) depiction of control 9 reflects no fluorescent signal, thereby negating the presence or detection of RgpA. Conversely, when control 9 is incubated with an exogenous load of recombinant RgpA (500ng.L^{-1}) and then stained with the antibody in question, noticeable fluorescence is observed (C-F). This serves to show that the specificity of the antibody is restricted to RgpA in plasma samples, and therefore detected fluorescence can be attributed to the presence of RgpA.

4.4.2. RgpA in Parkinson's disease plasma

Figure 6 shows – for the first time – the presence of RgpA in the haematological system of Parkinson's disease patients. Unstained control and PD plasma clots display undetectable fluorescent signal (A & C); these micrographs serve to discount autofluorescence that may lead to false positive results. When the control samples were stained with the antibody specific for RgpA (B), the micrographs yielded imperceptible fluorescent signal therefore demonstrating the absence of RgpA in healthy individuals. Conversely, the stained Parkinson's disease clots exhibited extensive fluorescent signal (D-F). This infers, *via* immunohistochemistry, that RgpA, the protease from the chief periodontopathic bacterium *P. gingivalis,* is present in the haematological system of individuals suffering with Parkinson's disease. In order to quantitatively support the observed difference between the control and PD populations, the mean grey value (fluorescent intensity) of the confocal images were calculated (Figure 7). The Parkinson's population exhibited a mean fluorescence three times that of the controls. Accordingly, a p-value less than 0.001 (****) was determined. This is the first evidence to demonstrate the presence of *P. gingivalis* in the form of its molecular signatures in the haematological system of individuals suffering from Parkinson's disease.





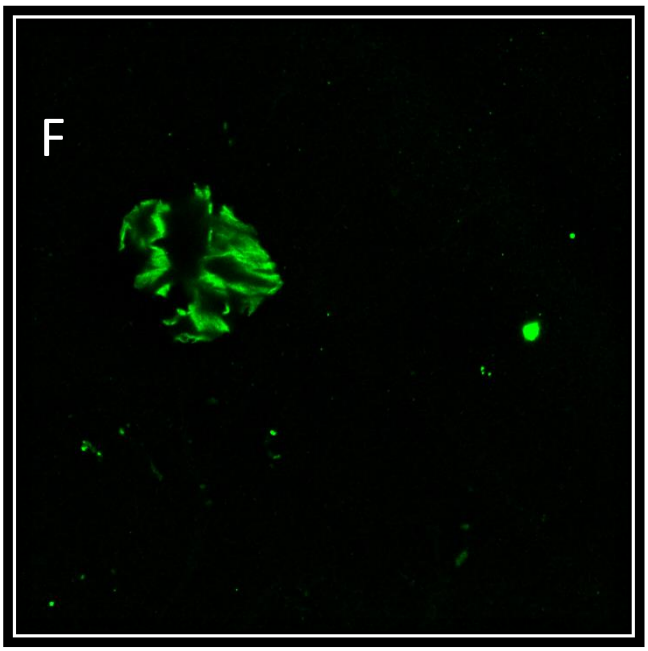
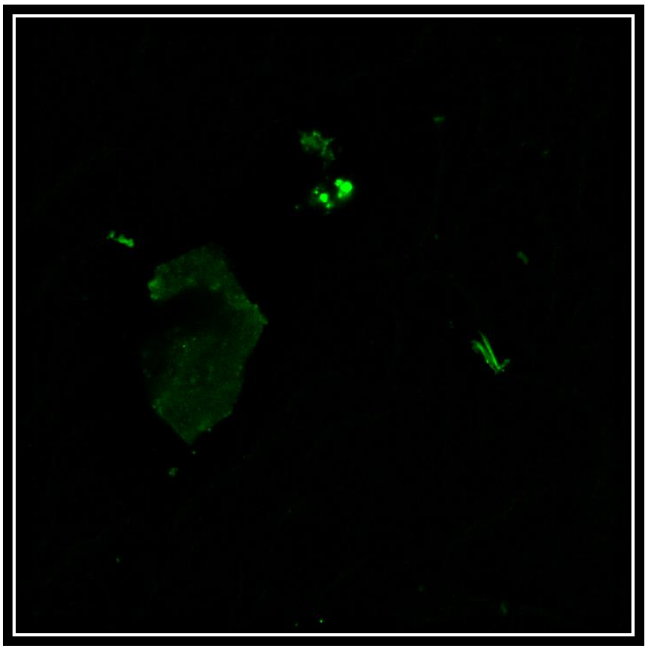
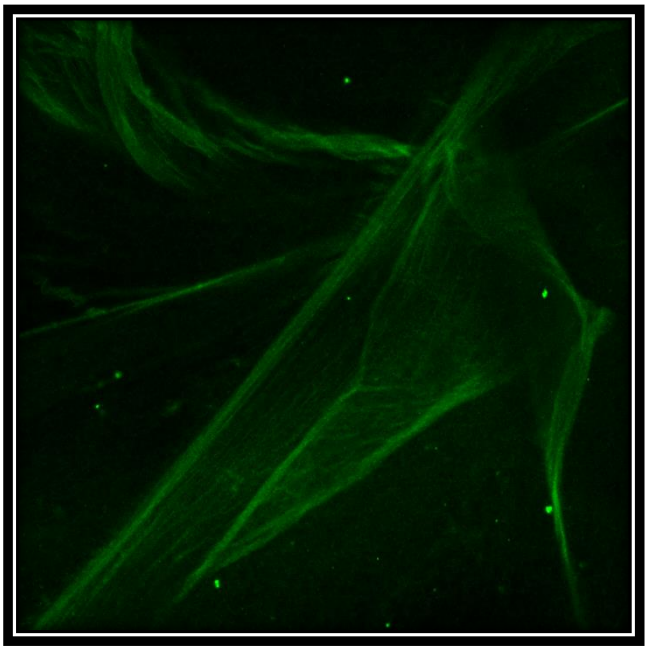


Figure 6: Confocal micrographs of control and Parkinson's disease plasma clots stained with the polyclonal FITC-conjugated gingipain R1 antibody. Control plasma clots, devoid of stain (A), shows no fluorescent signal and thus dismisses any presence of autofluorescence which may produce false positive results. The same control same is stained with the antibody (B) which subsequently lacks fluorescent signal, thereby indicating that the control is devoid of the protease. For the Parkinson's disease plasma clots, unstained samples were also viewed to account for any autofluoresence of which none was found (C). Conversely, stained Parkinson's disease clots exhibited significant fluorescence which indicates the presence of RgpA in the blood system of these diseased patients (D-H). C & D represents plasma of the same individual; D-H are different Parkinson's disease individuals.





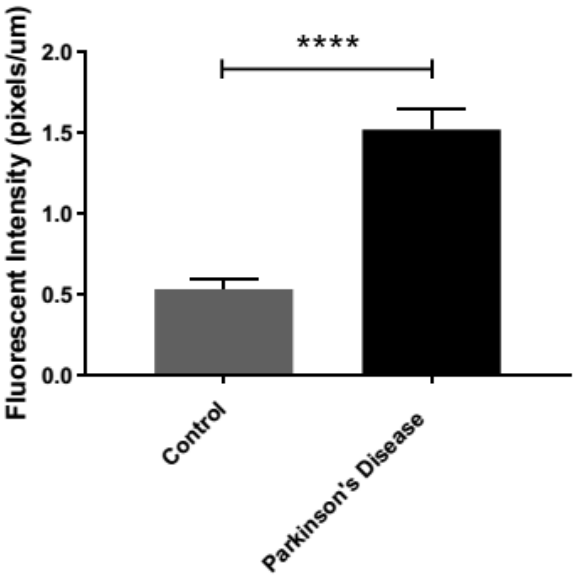


Figure 7: The difference in mean fluorescent intensity of confocal micrographs between healthy and PD plasma stained with the RgpA antibody. This measured fluorescent intensity between the two populations, as inferred the mean grey value, supports what is qualitatively expressed in Figure 9 – this quantitative measurement supports a significance difference (p < 0.0001) with the PD population yielding three times the fluorescent signal than the control group. (**** = p < 0.0001). Three repeats (micrographs) of each sample were measured to increase statistical power. Due to the fact that measurements of the confocal images in question record signal intensity in areas rich or absent in RgpA (large deviations), standard deviation is negated from the graphs. Significance was established at a p < 0.05. (**** = p < 0.0001)

4.4.3. Probing for RgpA in Alzheimer's disease whole blood

Control and Alzheimer's WB used to make RgpA antibody-stained clots are represented in Figure 8. In terms of fluorescence observed, the control samples demonstrated little to none (A-B) which highlights the absence or scarcity of RgpA in this population. One control subject, however, demonstrated strong fluorescent signal; while this individual may not be have Alzheimer's, it is possible that this subject may have periodontal disease and thus *P. gingivalis* infection. In comparison, the Alzheimer's population demonstrates considerable qualitative fluorescent signal (C-D) indicating the presence of a protease from *P. gingivalis.* This observed difference is quantitatively supported by the measurement of signal intensities (Figure 9) which yields a significant difference among the two groups (p < 0.0001). The Alzheimer's population,

albeit only ten subjects, is thus positively associated with the existence of virulent factors from *P. gingivalis* in their blood.

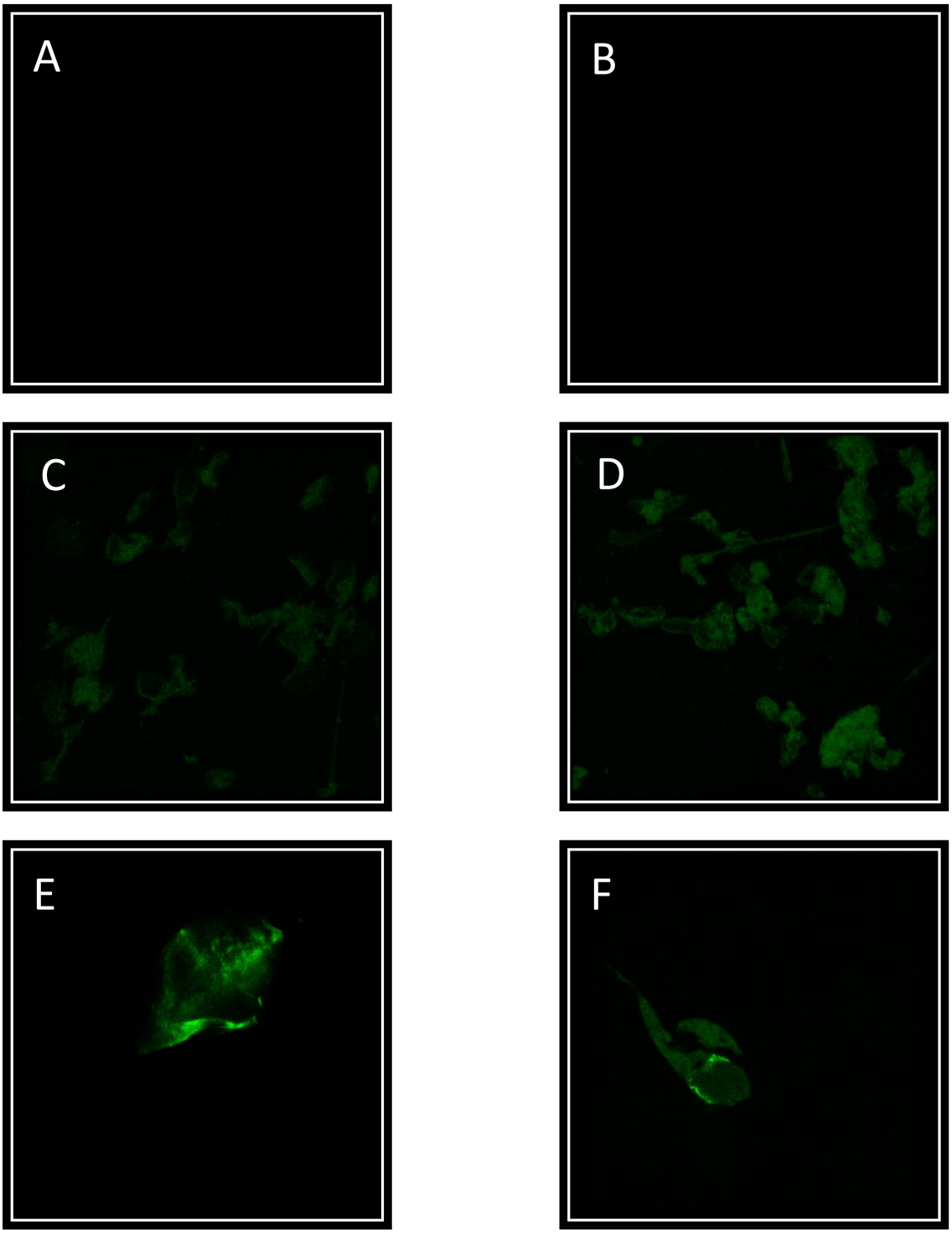


Figure 8: Confocal images of WB from healthy and Alzheimer's disease individuals after incubation with the RgpA polyclonal antibody at an exposure concentration of 1:100. A-B) As

a whole, control WB expressed little or no fluorescence. However, one subject in particular did demonstrate excessive signal. Alzheimer's WB samples (C-F) collectively show strong fluorescent signal when viewed.

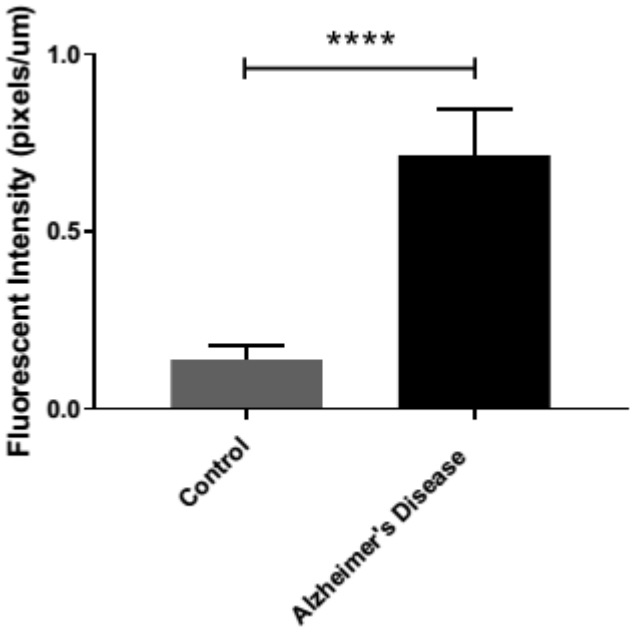


Figure 9: The mean fluorescent intensity between healthy and PD plasma exposed to the RgpA antibody. Quantitatively, the mean of signal intensity (E) supports a significant difference between the control ($\bar{x}$ = 0.139) and the Alzheimer's ($\bar{x}$ = 0.716) population (**** = $p < 0.0001$). Three repeats (micrographs) of each sample were measured to increase statistical power. Due to the fact that measurements of the confocal images in question record signal intensity in areas rich or absent in RgpA (large deviations), standard deviation is negated from the graphs. Significance was established at a $p < 0.05$.

4.5. Discussion

4.5.1. The detection of RgpA in the haematological system of PD and AD patients

There have been numerous studies depicting that poor oral hygiene and the presence of periodontopathic bacteria are major risk factors for the manifestation of these inflammatory conditions (Mattila et al., 1989, DeStefano et al., 1993, Schwarz et al., 2006, Tomas et al., 2012) and in terms of PD and AD, correlations with *P. gingivalis* are plentiful (Holmstrup et al., 2017, Chen et al., 2018a, Kaur et al., 2016, Sparks Stein et al., 2012, Singhrao and Olsen, 2018, Ranjan et al., 2018). Before the study in hand, this bacterium was yet to be discovered in physiological systems exterior to the oral cavity in PD patients. Recently, *P. gingivalis* and gingipains were discovered in the hippocampus of AD brains (Dominy et al., 2019). This is a significant link between periodontal disease and AD substantiating the associations made between the two disorders. The researchers also correlated the levels of gingipains to tau and ubiquitin pathology. Also highlighted is that the Dominy *et al.* (2019) study indicates that the atopobiosis of *P. gingivalis* is eminent, which predominantly occurs through entry into the blood and subsequent dissemination (Imamura et al., 2001b, Ambrosio et al., 2019, Horliana et al., 2014, Deshpande et al., 1998). This finding by Dominy *et al* (2019) led to this thesis' first hypothesis that suggests gingipains are present and persistent in the haematological system of PD as well as AD patients. Hence, the first aim of this study was to detect RgpA in the haematological system of individuals suffering from neurodegenerative disease, particularly PD and AD.

Accordingly, this study is the first to detect the presence of RgpA from *P. gingivalis* in the haematological system of PD and AD populations. Furthermore, it is the first study to show inflammagens from *P. gingivalis* present in an atopobiotic manner in PD patients – in the blood as opposed to the oral cavity. Using immunohistochemistry and confocal microscopy, both the PD and AD yielded a significant difference ($p < 0.0001$) in fluorescent intensities when compared to controls. Even without quantitative data, this difference was easily discerned in a qualitative manner. This finding corroborates the numerous associations of *P. gingivalis* and periodontitis with neurodegenerative disease. Here, we add more support to the theory that *P. gingivalis* is 'the missing link' between PD and periodontitis (Kaur et al., 2016). In an applicative manner, the significant difference between the presence of RgpA in PD and AD patients and healthy controls suggests that this particular gingipain possesses biomarker potential – perhaps as a diagnostic (or prognostic) biomarker. Nonetheless, more research is required to fully understand the role that this protease plays in PD and AD and how it can be utilized clinically. Ultimately, this finding of RgpA in the blood provides novel insight for the

pathogenesis, prevention, diagnosis, assessment and treatment of PD and AD, as well as other neurodegenerative disorders.

CHAPTER 5: HEALTHY BLOOD MODEL

5. The effects of bacterial inflammagens from *P. gingivalis* on coagulation kinetics and clot rheology

5.1. Introduction

Since *P. gingivalis* seems to be significantly implicated in inflammatory conditions as demonstrated by recent research, the effect that this bacterium's virulent factors may have on the coagulation system is of interest. After all, increased cardiovascular comorbidities are observed in most inflammatory conditions such as PD, AD, periodontal disease and T2DM, and defective coagulation – present in these disorders – is a contributing cardiovascular risk factor. Although the fibrinogenolytic effect of RgpA is noted, there remains the need to assess the effect of this protease, by using modern techniques, on different stages of coagulation, such as clot initiation time and stiffness. Hence, a holistic approach using thromboelastography and rheometry will offer more insight into the effects of bacterial inflammagens from *P. gingivalis* on certain parameters of coagulation.

In this chapter, by using thromboelastography and rheometry, the research question we aim to address here is: **'How does inflammagens from *P. gingivalis* affect blood clotting?'.** Specifically, this section deals with the impact of the bacterial inflammagens in question on coagulation kinetics (initiation of clotting cascade to final fibrin formation) and clot rheology.

5.2. Techniques

5.2.1. Thromboelastography

Thromboelastography (TEG) is a non-invasive method of measuring the state, efficiency and viscoelasticity of blood coagulation. This technique stands as a hallmark tool within our research group along with microscopy. While measuring biomarkers involved in coagulation such as fibrinogen concentration, platelet count, factor V and prothrombin time is extremely useful, TEG offers a comprehensive approach for assessing the kinetics of clotting as it measures the cascade from propagation to fibrin formation and then degradation. Discernment can be made as to whether an individual's state of coagulation is normostatic, hypercoaguable (rapid coagulation) or hypocoaguable (slow coagulation). This obviously has clinical utility and is appropriately utilized in healthcare; a hypercoaguable state infers increased thrombotic risk whereas a hypocoaguable state, in the more extreme cases, can be

used to detect bleeding disorders such as haemophilia. This technique is useful in surgery whereby drug administration is determined by TEG assessment. It must be noted that this system serves to monitor the intrinsic pathway of coagulation and not the extrinsic, tissue factor-activated pathway.

The TEG 5000 Thrombelastograph Hemostasis Analyzer was used in this study. WB, platelet-rich plasma (PRP) or PPP collected in sodium citrate tubes is eligible for testing by a thromboelastography machine. The operating principle of thromboelastography is depicted in Figure 10. In this test, 340mL of either one of the aforementioned sample types and 20mL of 0.2M calcium chloride ($CaCl_2$) for clot initiation is carefully pipetted into the oscillating TEG cup. As sodium citrate blood collection tubes function to sequester calcium ions (a cofactor for coagulation), this exogenous $CaCl_2$ is reintroduced into the blood or plasma to commence coagulation. As fibrin forms, or as the viscosity of the sample increases parallel to coagulation, a pin extending into the blood or plasma senses these changes and transfers the information to an electromagnetic transducer. A trace of the fibrin formation is then recorded and represented as a graph and numerical values.

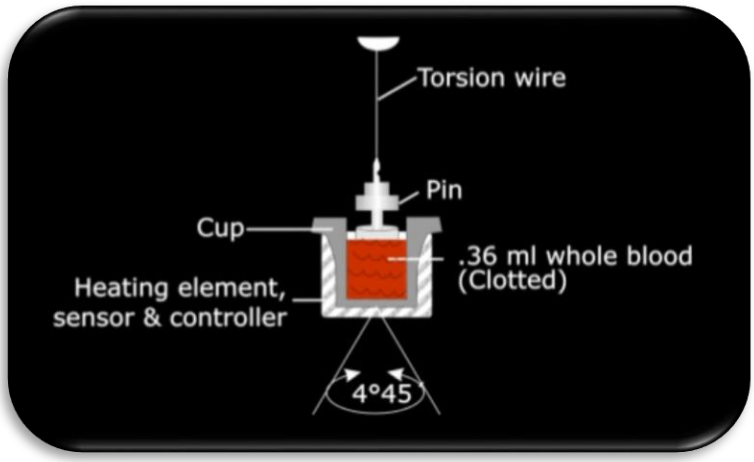


Figure 10: The working mechanism of a thromboelastograph. (Adopted from slideshare.net/drsumitg).

The thromboelastograph records certain parameters that relate to clot size, strength and predominantly kinetics. Table 4 contains the TEG parameters assessed in this study, their description and relative units. In addition, the table includes whether a larger or smaller value of each parameter indicates a hypercoaguable or hypocoaguable state.

Table 4: TEG parameters assessed and their description, along with the direction towards a hyper- or hypocoaguable state within each parameter.

Parameter Assessed	Description	Units	Hypercoaguable	Hypocoaguable
R-value	Latency time prior to initial fibrin formation (amplitude of 2mm)	Time (minutes)	↓	↑
α-angle	The speed of fibrin build up and cross linking (rate of clot formation); i.e. thrombin burst	Degrees (°)	↑	↓
Maximum Amplitude (MA)	Maximum stiffness of clot; i.e. overall stability of the clot	Millimetres	↑	↓
Maximum Rate of Thrombus Generation (MRTG)	The maximum velocity of clot growth observed or maximum rate of thrombus generation using G, where G is the elastic modulus strength of the thrombus in $Dyn.cm^{-2}$	$Dyn.cm^{-2}.s^{-1}$	↑	↓
Time to Maximum Rate of Thrombus Generation (TMRTG)	The time interval observed before the maximum speed of the clot growth	Time (minutes)	↓	↑
Total Thrombus Generation (TGG)	The clot strength: the amount of total resistance generated during clot formation (total area under the velocity curve during clot growth)	$Dyn.cm^{-2}$	↑	↓

5.2.2. Rheometry

Rheometry evaluates the rheological properties of a material. In the case blood, it is referred to as hemorheology. Due to the various constituents of blood, such as erythrocytes, platelets, white blood cells, plasma, and especially the clotting protein fibrin(ogen), the viscoelastic changes during and after the clot formation results in a unique profile when assessed with rheology. Accordingly, blood is classified as a non-Newtonian fluid – one whose viscosity varies according to stress applied. Discrepancies within the haematological system, such as increased fibrinogen concentrations during an acute phase response, alter the standard profile of blood clot rheology and can thus be compared to healthy controls. Surprisingly, data regarding blood with the addition of exogenous bacterial inflammagens is scarce or absent.

In a rheometer, the initiation of clot formation up until terminal fibrin formation is assessed in a kinetic manner and thus is synonymous to the TEG (this assessment will thus be negated). However, after the clot has been formed, various stressors are applied to the material to understand how the sample (clot) responds or deforms to the force applied. Blood clots, with a special emphasis on fibrin, have inherent viscoelastic properties enabling them to deform under particular forces and recoil into its original structure. This is known as the linear viscoelastic (LVE) range. Similarly, it is the range in which forces can be applied to the sample without leading to sample destruction. However, when this force exceeds the LVE range of a sample, the physical components within the sample begin to break and thus deems the sample unable to recoil to its original state (the elastic threshold of the sample has been surpassed) – this is known as the nonlinear viscoelastic (NLVE) range.

This study focuses on the response of clot networks to applied stress using a MCR 502 Anton Paar rheometer, which seems to be one of few assessing the effects of exogenous bacterial inflammagens on blood rheology. A rheometer is capable of various modes of testing. For our study, we performed oscillation tests whereby an increasing oscillatory stress is applied to the clots until molecular damage passes a certain threshold – data was obtained both within the LVE and NLVE range. Table 5 represents the rheology parameters utilized and their relevant descriptions. This section of the study was conducted in the Medical University of Vienna over the course of two weeks.

Table 5: Parameters assessed by the rheometer and their description as well as meaning if increased or decreased.

Rheometry parameter	Description	Meaning if increased	Meaning if decreased
G' initial (G'_{LVE})	Maximum strength/stiffness of fibrin clot., i.e. overall stability of the clot at rest	The material is stiffer; fibres could be thicker or network denser	The material is less rigid; possibly thinner fibres or looser fiber architecture
G'_{max}	Stiffness of the clot when the network is maximally elongated/deformed; fibrin networks stiffen when they are deformed	The clot is stiff at its maximum deformation	The clot is weak at its maximum deformation
Breakup Stress	Shear stress needed to break the clot	The clot can sustain higher stresses	The clot sustains lower stresses
Elastic Limit	Marks the end of the elastic behaviour of the clot; Beyond the elastic limit the network starts to stretch out and rearrangement takes place	The network allows higher deformations without experiencing a structural change	The network experiences a structural change at lower deformations
Linear viscoelastic behavior	Elastic behaviour of the clot; Reversible deformation occurs, there is no structural change	Higher clot stiffness	Lower clot stiffness
Shear stiffening ($G'_{max} – G'_{min}$)	All bending fluctuations are stretched out and the clot becomes less compliant; No more occurs the re-arrangement of the network, but increasing stretching of the network to a final value (G´max)	The clot stretches out well	The clot does not stretch out well
G´max – G´final	Individual fibers and/or network extend until the clot breaks	Slow breaking of the clot	Brittle breaking of the clot
G' = Storage modulus (the ability to store energy in an elastic manner)			

5.3. Material and Methods

5.3.1. Thromboelastography

Naïve healthy platelet-poor plasma was subjected to TEG analysis to determine the subjective state of coagubility in each individual. The subjects' PPP was then diluted with RgpA and immediately tested or incubated with recombinant RgpA for 30 minutes at an exposure concentration of 500ng.L^{-1}; coagulation was then measured by the TEG to determine the effect of recombinant RgpA (purchased from Abcam; catalogue number: ab225982) on the efficacy of the clotting cascade. In this test, the 324uL of the naïve samples were diluted with 36uL of PBS (vehicle) to match the circumstances of the intervention group. Then, 340uL of the diluted naïve plasma was then placed in the TEG with 20uL of calcium chloride ($CaCl_2$) and

measured. The $CaCl_2$ returns calcium to the sample which is removed by the sodium citrate tube and required as a cofactor for a number of the relevant reactions in the coagulation cascade. Conversely, the intervention group (matched to the naïve TEG group) received an addition of 36uL of 5µg/L RgpA solution (in PBS) in 324uL of PPP. As mentioned, this was left to stand for thirty minutes before pipetted into the TEG machine with the addition of $CaCl_2$. Following the addition of the cofactor required to commence coagulation, the test is promptly started in order to include the entire lag phase prior to clot formation (R-value).

5.3.2. Rheometry

Whole blood (WB) and PPP were subjected to rheometry analysis on an Anton Paar MCR 502 with a plate-plate measuring system in order to determine the flow behaviour and deformation of samples. Kinetic as well as oscillation tests were run on all the samples at 37°C. A shear rate of 0,1% at a frequency of 1Hz was applied to WB and PPP samples during the kinetics test. In the oscillation analysis, an increasing shear rate of 1-5000Pa at 1rad/s was applied to all samples. For WB and PPP samples, 0,2M $CaCl_2$ was used to induce coagulation. Samples received the addition of $CaCl_2$ immediately before the sample was placed on the measuring plate and subjected to the kinetic test. The intervention groups, matched to the controls, were incubated with varying concentrations of RgpA and/or LPS (purchased from Sigma-Aldrich; catalogue number: SMB00610) from *P. gingivalis*. RgpA was exposed at 100ng.L^{-1} and 250ng.L^{-1} whereas LPS was exposed at 20ng.L^{-1} alone or in combination with 100ng.L^{-1} RgpA to WB and PPP samples.

5.4. Results

5.4.1. TEG of healthy plasma exposed to RgpA

TEG results of naïve and RgpA-exposed PPP is shown in Table 6. The results of the control group, albeit normal, serve as a basis for comparison with the intervention group. Naïve and RgpA-exposed samples are paired, i.e. subject X's naïve run is compared with subject X's intervention run.

The effect exerted by this protease on viscoelastic parameters of clotting is substantial as all six parameters assessed exhibited significance. Overall, the protease in question shifted the state of coagulation to a more hypocoaguable one; this is reflected in each time-dependent parameter – namely the R-value (**), α-angle (**), MRTG (***), and TMRTG (**) – all of which

are increased (longer time taken). RgpA inhibits the ability of clots to initiate (increased R-value) and cross-link (decreased α-angle) and also inhibits the rate of enzymatic function during fibrin synthesis (decreased MRTG & increased TMRTG). Therefore, it appears that a lagging of clotting processes in the presence of RgpA occurs.

Other than time-dependent parameters, the TEG also assesses physical clot attributes such as relative stiffness and size. TTG is decreased in the presence of RgpA. This indicates a decreased clot strength and size – a result linked to the fibrin(ogen)olytic and hence fibrin-diminishing ability of the protease. The RgpA group, surprisingly, exhibited clots with an increased MA – total stability and stiffness of clot. This is deemed unexpected as lower fibrin loads (due to fibrinogenolysis) would be expected to result in lower clot stability and stiffness. RgpA-induced biochemical changes in fibrin and network arrangement likely attribute to this enigma. While the increase in MA under RgpA exposure is a confusing result to explain, overall clot kinetics is slowed by RgpA prompting a state a hypocoagubility. The clots formed, albeit it stiffer, were also smaller and weaker than naïve clots.

Table 6: TEG® results of naïve control PPP vs. 500ng.L^{-1} RgpA exposed PPP (matched).

Parameter	**Control**	**RgpA**	***p*-value**
R (min)	10.02 ± 3.12	11.97 ± 4.57	0.0011 (**)
α angle (°)	63.59 ± 7.79	59.63 ± 8.89	0.0014 (**)
MA (mm)	24.57 ± 6.21	29.37 ± 7.35	0.0211 (*)
MRTG (Dyn.cm^{-2}.s^{-1})	4.2 ± 1.72	3.37 ± 1.73	0.0001 (***)
TMRTG (min)	11.56 ± 3.44	13.84 ± 5.29	0.0032 (**)
TTG (Dyn.cm^{-2})	167.7 ± 56.79	149.8 ± 43.28	0.0224 (*)

Statistical significance was established at $p < 0,05$ (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). Due to technical implications, only 20 K-values were recorded. Data is represented as the mean ± standard deviation.

5.4.2. Rheometry of healthy blood samples exposed to RgpA and LPS

Table 7 shows the rheological data obtained from naïve and inflammagen-exposed whole-blood and platelet-depleted plasma (PDP) samples. In PDP, both the exposures of RgpA and LPS from *P. gingivalis* enhanced the linear viscoelastic shear modulus (G' initial and max). This indicates that these two bacterial factors interfere with the biochemical nature of clots thereby resulting in a rheological profile of increased stiffness (G'). Shear stiffening was

increased by both interventions, with LPS exhibiting a more prominent effect. Conversely, LPS results in clots that break at larger shear stresses (breakup stress) while RgpA significantly lowers the breakup threshold. RgpA also lowers the elastic limits of clots. This decrease in the breakup stress and elastic limit can be explained by the fibrin(ogen)olytic ability of RgpA whereby a decrease in clot size and mass will result in a weaker, smaller clot; this (decrease in breakup stress and elastic limit) is synonymous to TTG measured by the TEG (Table 6).

In the WB samples, LPS forms less rigid and less stiff clots in WB whereas the opposite effect is observed in PDP. LPS decreased G' initial at both concentrations while G' max was decreased compared to the naïve samples at 20µg·L^{-1} and increased compared to the naïve samples at 20µg·L^{-1}. Conversely, RgpA increased both G' initial and max thereby resulting in stiffer clots; the same effect was observed in PDP. This effect is somewhat unexpected as the fibrin(ogen)olytic effect of RgpA would be expected to result in a smaller clot (lower fibrin load due to decrease in fibrinogen); this correlates to the MA (Table 6) values in which RgpA-exposed clots exhibited increased stiffness. RgpA also decreased the breakup stress and elastic limit in WB samples. The detection of increased clot stiffness in the presence of RgpA by both the TEG and rheometer strengthens this reliability of this result, as it does with the breakup stress and elastic limit with TTG. With regards to breakup stress both concentrations of LPS lowered the breakup stress of clots.

Table 7: Rheological data of WB and PDP samples.

Sample	G' initial [Pa]	G' max [Pa]	Breakup Stress [Pa]	Elastic Limit [Pa]	Shear stiffening (G' max – G' min) [Pa]
Whole-blood					
Control 1	174.12 ± 25.29	207.54 ± 49.07	441.47 ± 192.88	17.75 ± 2.67	54
Control 1 + RgpA (100 ng·L^{-1})	208.63 ± 13.35	227.48 ± 16.22	339.25 ± 113.95	16.55 ± 5.36	41
Control 2	315.87 ± 21.48	607.28 ± 48.81	1239.07 ± 417.61	26.13 ± 3.32	331
Control 2 + PG LPS (20 ng·L^{-1})	291.02 ± 26.74	653.41 ± 77.02	1202.23 ± 154.12	17.12 ± 2.12	396
Control 2 + PG LPS (20 µg·L^{-1})	253.99 ± 37.74	577.73 ± 69.65	1041.16 ± 122.53	16.09 ± 3.37	349
Platelet-depleted plasma					
Control 1	40.55 ± 2.07	551.77 ± 30.02	912.36 ± 193.61	2.89 ± 0	511
Control 1 + RgpA (250 ng·L^{-1})	45.34 ± 7.5	570.52 ± 64.78	737.63 ± 156.51	2.52 ± 0.32	525
Control 3	48.86 ± 5.3	561.67 ± 113.36	757.54 ± 246.59	3.39 ± 1.81	513
Control 3 + PG LPS (20 ng·L^{-1})	71.19 ± 5.84	762.39 ± 52.03	901.7 ± 0.57	3.35 ± 0.4	691

Data expressed as mean ± SD.

5.5. Discussion

5.5.1. The effects of RgpA on the kinetics of coagulation

Coupled to PD and AD are abnormalities in coagulation predominantly characterised by hypercoagulability (Adams et al., 2019, Bester et al., 2015). This is a major contributing mechanism to the prothrombotic state observed in PD and AD and contributes to an overall risk of cardiovascular mortality. Furthermore, periodontal disease too exhibits a similar phenotype in the clotting system (Dikshit, 2015, Senini et al., 2019). The question that arose is if whether periodontopathic bacteria and their inflammagens may be responsible, in part, for the aberrations in clotting processes seen in PD, AD and periodontal disease. Even more so, in light of the new data presented in this thesis that shows the presence of RgpA in the blood of PD and AD patients, the effects that *P. gingivalis* and its inflammagens have on clotting is warranted. Thus, the second component of this literature seeks to determine the impact of

RgpA on the clot kinetics, rheology and terminal (at the point of a stabilised clot, before fibrinolysis) fibrin load and network architecture.

In terms of clot kinetics, RgpA shifts the normostatic state of coagulation to a more hypocoaguable one as reflected by the four time-dependent TEG parameters (R-value, α-angle, MRTG and TMRTG) in Table 6. Lagging of clotting processes is believed to be induced by the fibrin(ogen)olytic activity of RgpA. To explain further, as exogenous RgpA is added to a blood sample, fibrinogen cleavage occurs subsequently resulting in less substrate available for fibrin synthesis and thus clot formation; since the TEG records values only when the clot has reached a certain size (e.g. 2mm for the R-value), a lower load of fibrinogen in a sample (here, due to RgpA activity) will lead to a longer time taken to achieve a certain clot size and hence register a value for a given TEG parameter. Supporting this ideology, exogenous RgpA yields a clot that is smaller in size and mass compared to naïve clots (TTG). This is consistent with the process of fibrinogen cleavage whereby increased fibrinogenolysis results in decreased fibrin synthesis and thus lower clot size and mass. Inhibiting haemostasis in this manner aids *P. gingivalis* in heme acquisition, entry and dissemination into physiological systems and contributes to increased bleeding tendencies observed in periodontitis. Interestingly, this hypocoaguable-promoting effect of RgpA is in contrast to the hypercoaguable-favouring effects of LPS (Pretorius et al., 2016).

Apart from fibrinogen, RgpA has a plethora of targets in plasma, some of which include plasma proteins and platelets receptors (Imamura et al., 2001b, Imamura et al., 1997). The modulation of platelets *via* RgpA (Lourbakos et al., 2001) can prompt aberrant function and impede the efficiency of coagulation. It is possible that RgpA also acts on certain clotting factors which lead to disturbances in the efficiency of clot formation. When analysing the α-angle the time taken to achieve a certain magnitude of crosslinking is lengthened (smaller °) by RgpA. Factor XIII is responsible for coordinating the crosslinking of fibrin and may be modulated by RgpA. However, there seems to be little or no data on RgpA and its functionality with factor XIII. The activation of factors IX and X as well as the release of thrombin from prothrombin by RgpA (Imamura et al., 2001b, Imamura et al., 1997, Imamura et al., 2001a) is noted and can alter the enzymatic balance of the clotting cascade; but contradictory to the overall effect, increases in these enzymes, especially thrombin which is a catalyst for fibrin synthesis, would be expected to prompt a hypercoaguable state. Although such an effect may occur, it is most likely masked and overpowered by RgpA-mediated fibrinogenolysis – the most probable cause of the observed shift of plasma samples to a hypocoaguable state.

The MA parameter produced unexpected results. RgpA-exposed plasma exhibited a higher MA value (29.37) than the control group (24.57) with a significance difference of $p < 0.05$ (*).

This indicates that the plasma incubated with RgpA yields a clot that is stiffer and more stable than naïve clots unexposed to the protease. Rheological analysis of clots also exhibited and supports this stiffening in the presence of RgpA (Table 7). As mentioned, RgpA acts on clotting factors and platelets – two factors which determine the physical properties of clots. Manipulation of these two factors by RgpA may alter the biochemical arrangement and structure; for example, RgpA may alter the function of factor XIII and result in abnormal fibrin crosslinking characteristic of a stiffer, more stable clot. Nonetheless, this result is difficult to explain, and requires further biochemical investigation.

5.5.2. The effects of RgpA and LPS on clot rheology

This study is one of few assessing the role of bacterial products on rheological properties of blood clots. Here, it is shown that RgpA-exposed clots are stiffer, break at lower stresses and are less deformable under stress when compared to control clots. These effects were consistent in both WB and PDP samples. RgpA seems to impede the inherent elastic capacity of clots. This will result in clots that are less responsive (deformable) and undergo destruction under certain forces. In a clinical perspective, this prompts bleeding tendencies observed in periodontal disorders (Page et al., 1978) as inadequate clot formation, strength, deformability and stability hinders effective hemostasis. Supporting the inconspicuous MA result obtained by the TEG, rheological data of RgpA-exposed clots also exhibits an increase in clot stiffness. While speculations regarding RgpA and its possible modulation of clotting factors and platelets are put forth, this finding requires comprehensive biochemical investigation.

Interestingly, the effects imposed on rheological properties of clots by *P. gingivalis'* LPS differ according to sample type, e.g. PDP & WB. PDP clots exposed to LPS are stiffer and break under higher stresses when compared to controls whereas LPS-exposed WB clots are less stiff compared to controls. In WB, the mean breakup stress is lower in LPS-clots which relates to decreased stiffness. LPS-exposed PDP clots also present with a larger degree of elasticity than controls, a result in contrast to that of LPS-exposed WB clots RgpA-exposed clots. These differences observed between WB and PDP can be attributed to the presence (and response of) erythrocytes and platelets in WB. Erythrocytes increase spaces within fibrin networks and possess inherent pliability and a significant deformability capacity; this contributes to a decreased integrity and 'stiffness' of clots and likely has a role to play the result of decreased stiffness in LPS WB clots. Additionally, erythrocytes bind fibrinogen (Litvinov and Weisel, 2017) and influence clot properties in this manner. Platelets may also act as contributors to decreased stiffening observed in WB. Platelets play an important role in orchestrating clot formation and architecture. They secrete factor XIII, the clotting factor responsible for forming

crosslinks. While this factor functions to increase the integrity of fibrin networks, the presence of LPS and its modulation of platelet receptors (Saluk-Juszczak et al., 2000, Lopes Pires et al., 2017, Senini et al., 2019) may induce atypical function. For example, a study concluded that LPS reduced the levels of clotting factor XIII and additionally noted alterations to this protein (Pabst et al., 2008). Furthermore, abnormalities in platelets and subsequent factor XIII handling results in smaller, weaker and less stiff clots. These perturbations in platelet function and factor XIII induced by LPS could account for decreased stiffness observed in WB clots. Nonetheless, without red blood cells, platelets and other WB factors, LPS-exposed PDP clots are more densely packed which typically will result in increased clot integrity as indicated by increased G' and breakup stress values. Ultimately, compared to RgpA, LPS-exposed clots are more capable of deforming in response to a stress and according to sample type (WB & PDP) exhibit different trends with regards to stiffness.

Changes that occur to the viscoelastic and rheological properties of blood clots induced by RgpA and LPS are substantial. While the two techniques employed to study these changes have derived insightful data, further coagulation studies, preferably in animal models, involving these virulent factors is warranted.

CHAPTER 6: PURIFIED FIBRINOGEN MODEL

6. The effects of bacterial inflammagens from *P. gingivalis* on fibrin load and network structure

6.1. Introduction

RgpA, as a proteolytic enzyme, is capable of cleaving a number of target proteins. With relevance to the coagulation system, RgpA exerts a noted fibrin(ogen)olytic effect. This cleavage of fibrin(ogen) may contribute to fibrinogen dyshomeostasis and defective coagulation seen in inflammatory conditions, particularly those that exhibit associations with periodontal disease. The proteolysis of fibrin(ogen) by RgpA will thus be assessed, by microscopy, in this section.

In this chapter, purified fibrinogen is used in order to assess the effects of bacterial inflammagens on the clotting constituent without any confounding coagulation factors such as platelet and clotting factor interference. Purified fibrinogen is exposed to RgpA for 30 minutes prior to clot formation via thrombin-catalysis; the networks are then assessed *via* microscopy. Since LPS from *P. gingivalis* forms a complex with RgpA (Li and Collyer, 2011) and since these two molecules are present in a system at the same time, the clotting-related effects of RgpA in the presence of LPS will also be studied in order to match realistic physiological scenarios.

In this chapter, by using fluorescent fibrinogen and confocal microscopy, and SEM, the research question we aim to address here is: **'How does inflammagens from *P. gingivalis* affect blood clotting?'** Specifically, this section deals with the impact of the bacterial inflammagens in question on fibrin load and terminal network structure.

6.2. Techniques

6.2.1. Confocal Microscopy

With relevance to coagulation, fluorophore-conjugated fibrinogen allows researchers to study a purified fibrinogen model whereby the network structure and architecture of fibrin is fluorescent and easily observed. This greatly aids the understanding of how molecules (inflammagens) affect fibrin(ogen) and resulting clot formation. Relevant to the aim of this section, the load of fibrin and network formed can be discerned as well. Recently, using confocal techniques, it was demonstrated that LPS leads to abnormal clot formation

characteristic of dense matter deposits (Pretorius et al., 2018b). The effect on (fluorescent) fibrin clots formed from fibrinogen exposed to inflammagens from *P. gingivalis* can be studied in this regard.

6.2.2. Scanning Electron Microscopy

Ultrastructural changes to constituents of blood are favourably studied with electron microscopy. As we study the clotting component of blood (protein), transmission electron microscopy is negated; SEM thus poses as the desired option to study the structure and network arrangement of fibrin. Here, a focused beam of electrons are emitted onto a sample coated with carbon. The topography of the sample is recorded and due to the electron-based nature of this technique, the resultant micrographs are of impeccable resolution.

It has been noted that inflammatory molecules, bacterial LPS, cigarette smoking and glucose can alter the morphology of RBCs, platelets and fibrin (Pretorius, 2013, Pretorius et al., 2013, Bester and Pretorius, 2016, Pretorius et al., 2018b). Although other microscopy techniques are adequate to study such changes, SEM stands as the primary mode of assessment in terms of ultrastructural analysis. Furthermore, results obtained by TEG and confocal microscopy are translatable to SEM micrographs (e.g. hypercoagulability in TEG and hyperactivated platelets in confocal), provided the same sample is used.

A Merlin Field Emission Scanning Electron Microscope (FE-SEM) from Carl Zeiss was utilized in order to view the ultrastructural changes that occur to fibrin fibres and networks with the addition of bacterial products from *P. gingivalis*.

6.3. Methods and materials

6.3.1. Confocal microscopy using fluorescent fibrinogen

Purified human fluorescent fibrinogen (Alexa 488) (ThermoFisher; catalogue number: F13191) was prepared to a final concentration of 2mg.mL^{-1}. Thrombin, obtained by the South African National Blood Service, was solubilized in PBS containing 0.2% human serum albumin to obtain a concentration of 20 U.mL^{-1}. In order to form clots, 5mL of fibrinogen received 2,5mL of thrombin (2:1 ratio) to stimulate the formation of fibrin networks. The sample was then fixed with 10% NBF and then washed with PBS. The sample was then blocked with 5% goat serum (diluted with PBS) and incubated with gingipain R1 polyclonal antibody (1:100 in goat serum)

for one hour at room temperature. The samples were washed with PBS and a coverslip was mounted along with Dako fluorescence mounting medium. The recombinant protease was exposed at two different concentrations, 100 and 500 ng.L^{-1} (final exposure), and incubated for 30 minutes. Clots were also viewed with the confocal microscope and fluorescent fibrinogen was excited at 488 nm, with emission measured at 508 to 570 nm. As the gingipains antibody used above has the same excitation and emission as the purified fibrinogen, we could not trace the added gingipains by using this antibody.

6.3.2. SEM of purified fibrinogen exposed to RgpA and LPS

Purified fibrinogen received the addition of RgpA and LPS at exposure concentrations of 500ng.L^{-1} and 10ng.L^{-1}, respectively, and left to incubate with these inflammagens for 30 minutes prior to SEM preparation.

10µL of purified fibrinogen (exposed or unexposed) was pipetted onto a 1cm diameter glass cover slip. The pipette tip is then removed, forcefully yet carefully bent and then used to create a smear on the circular glass slip. 5µL of thrombin was then added into the drop of PPP/fibrinogen and carefully mixed. The sample was left to stand for five minutes to allow to adhere to the glass surface. The slip is then lifted using tweezers and translocated to a 24-well plate. The sample was then washed using PBS and left to rest for ten minutes. After PBS removal, 10% NBF – enough to cover the sample – is added to the well as a fixative using a Pasteur pipette and left for 20 minutes. After removing the fixative, the sample was washed three times with PBS. Working in a fume hood, a glass pipette was used to add 2-3 drops of osmium tetroxide – a potent fixative optimal for SEM – to sample wells and left to incubate for 30 minutes. After discarding of the osmium tetroxide, the samples were again washed with PBS 3 times. The samples were then dehydrated with increasing concentrations of ethanol – 30%, 50%, 70%, 90% and 100% – each for three minutes. After removing the last of the 100% ethanol, hexamethyldisilazane (HMDS) is added as the terminal dehydrating agent. Three drops of HMDS were added to each sample well and left for 30 minutes. The slips were carefully lifted slightly to prevent adhesion to the plate-surface during HMDS incubation. Lastly, slips are removed from each well and placed onto a glass microscope slide ready for subsequent carbon coating and SEM analysis.

6.4. Results

6.4.1. Confocal micrographs of fibrin networks exposed to RgpA and LPS

Confocal micrographs displaying fibrin networks formed from naïve or inflammagen-exposed purified fibrinogen (Alexa 488) is displayed in Figure 11. Naïve fibrin networks (A-B) demonstrate the normostatic arrangement of clot architecture (healthy clot). There are consistent distances between fibres and synonymous branching points. However, when purified fibrinogen is incubated with recombinant RgpA prior to thrombin exposure, the fibrin network formed is almost non-existent (C-D). Fibrinogenolysis mediated by RgpA explains this observation. There seems to be no visible network but rather small fragments of fibrin possibly formed from fibrinogen uncleaved by RgpA. Conversely, LPS exposure results in a substantial clot formed (E-F). However, the clot deviates from what is considered normal and healthy; the fibrin network is more densely packed with less spaces between fibres resembling dense matter deposits. This phenomenon is also credible to LPS from other species of bacteria (Pretorius et al., 2016). When RgpA and LPS are co-incubated with purified fibrinogen (G-H) preceding thrombin catalysis, the effect of LPS (causing dense matter deposits) is manifested more as opposed to RgpA's deleterious effect on fibrin formation. The fibrinogenolytic activity of RgpA is thus hindered and negated in the presence of LPS; LPS seems act on fibrin architecture as if RgpA was absent in the sample.

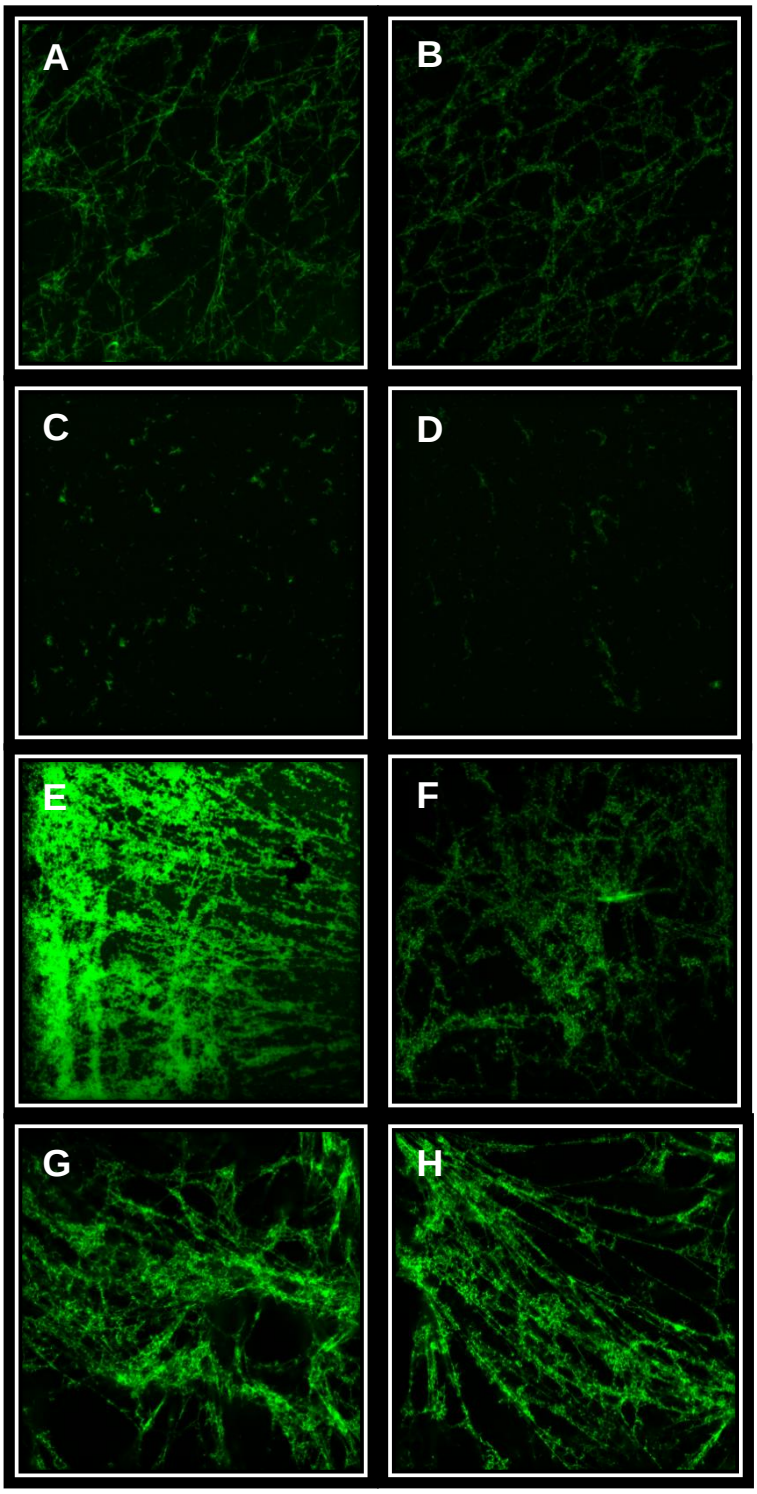


Figure 11: Confocal images (63x magnification) of 2mg/mL purified fibrinogen with and without the addition of bacterial inflammagens from *P. gingivalis.* A-B) Naïve fibrin architecture representative of healthy clots. C-D) Clot formed form purified fibrinogen exposed to 500ng.L^{-1} RgpA. E-F) Purified fibrinogen exposed 10ng.L^{-1} LPS. G-H) Fibrin network after purified fibrinogen was co-incubated with 500ng.L^{-1} RgpA and 10ng.L^{-1} LPS.

6.4.2. SEM micrographs of fibrin networks exposed to RgpA and LPS

Ultrastructure of fibrin networks analysed by SEM are shown in Figure 12. Naïve fibrin architecture (A-B) is representative of healthy clots. Again, as with the confocal micrographs, spaces between fibres and the nature of branching points is consistent, forming a 'spaghetti-like' network. When exposed to purified fibrinogen, RgpA results in little or no fibrin formed (C-D) after the zymogen undergoes thrombin catalysis. Consistent with the confocal results obtained (Figure 11), the fibrin(ogen)olytic effect of RgpA is prominent. The particles present in the micrographs may be residual particles of fibrin(ogen) similar to the fluorescent fragments observed in the confocal micrographs in Figure 11 (C-D). Again, synonymous to the confocal results, co-incubation of RgpA with LPS yields a fibrin clot (E-F), thereby negating the (complete) fibrinogenolytic effect of the protease. The fibrin clot formed is extremely aberrant in form and reflects a clot characteristic of dense matter deposits. The effect of LPS thus dominates whereas the proteolytic activity of RgpA is masked as reflected in the confocal data. To explain this inhibition of RgpA's proteolytic activity, an association between the two bacterial inflammagens during co-incubation must occur; alternatively, LPS may bind fibrinogen molecules (de Waal et al., 2018) with a higher affinity than RgpA thereby disabling the protease from reaching the zymogen.

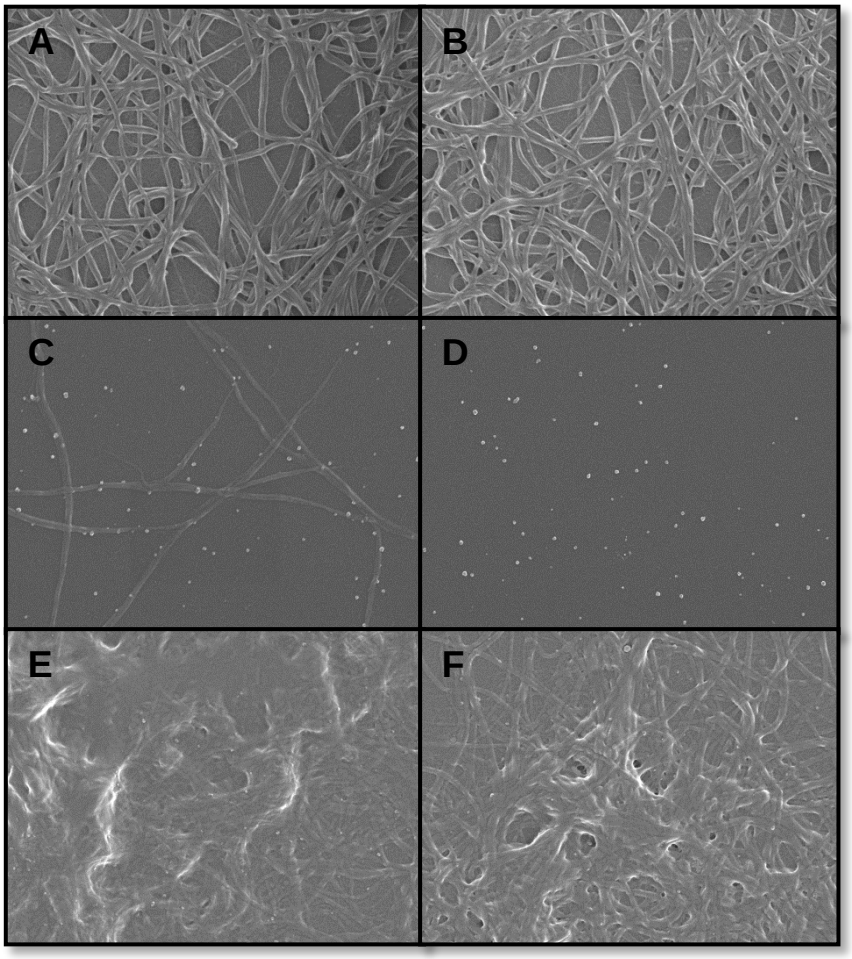


Figure 12: SEM micrographs of fibrin clots formed by thrombin-mediated catalysis of purified fibrinogen incubated with or without inflammagens from *P. gingivalis.* A-B) Naïve fibrin clots. C-D) Fibrin clots formed from fibrinogen exposed to 500ng.L^{-1} RgpA prior to thrombin addition. E-F) Fibrin clots formed from fibrinogen exposed to 500ng.L^{-1} RgpA and 10ng.L^{-1} LPS prior to thrombin addition.

6.5. Discussion

6.5.1. The effects of exogenous RgpA on fibrin load and network formation using purified fibrinogen

Although fibrinogen cleavage by RgpA is a defined process, the impact that this proteolysis has on fibrin network load and arrangement using modern techniques such as SEM and confocal microscopy is yet to be discerned. Here, a purified fibrinogen model was used to determine the influence that *P. gingivalis'* key protease has on the outcome of a thrombin-catalysed clot. Since LPS from the same bacterial species forms complexes with RgpA and even alters its structure (Takii et al., 2005), co-exposure of these two bacterial products

enables the discernment of any inhibition or enhancement promoted by LPS on RgpA-mediated fibrin alterations.

Thrombin-catalysed fibrin formation is prevented when the zymogen is incubated with 500ng.L^{-1} RgpA (Figure 11 & 12). Fibrinogen cleavage as a result of RgpA exposure depletes the substrate available for fibrin synthesis thereby reducing the load of clot formed. As seen in Figure 11B & 12B (500ng exposure), a clot network is absent. Hence, RgpA exhibits great fibrin(ogen)olytic ability. In the confocal micrographs, very few fluorescent fragments are present; these are likely strands fibrin formed from naïve (unreached by RgpA) fibrinogen remaining in the sample. These residual fragments can also be seen in the SEM micrographs. This abrogation of clot formation by RgpA decreases hemostatic capabilities and contributes to bleeding and inflammation seen in periodontal disease (Imamura et al., 1995). In WB and plasma, however, this fibrin depletion and clot absence is not as prominent (as inferred by TEG and rheological oscillatory tests). Inhibitors of gingipains in plasma, such as endogenous protease inhibitors and other target proteins such as albumin (Imamura et al., 1995), likely account for this difference observed between purified fibrinogen and blood samples.

In solidarity, RgpA hinders the formation of fibrin in purified fibrinogen samples. However, co-incubation of RgpA with LPS yields opposing results in the same sample. For instance, co-incubation of these two inflammagens allows a clot to form (Figure 11 G-H & Figure 12 E-F) whereas RgpA incubation alone abolishes visible clot formation. This indicates that LPS hinders the ability of RgpA to cleave fibrinogen and reduce fibrin load. This likely occurs due to binding and complex formation of these two factors from *P. gingivalis* (Takii et al., 2005); biochemical changes that occur to RgpA due to LPS binding may render some its proteolytic activates redundant. Whether RgpA has a stronger binding affinity for LPS as opposed to fibrinogen is questionable and relevant. Furthermore, the clot that does form is characteristically abnormal and resembles dense-matter deposits – a clot structural variant that poses significant cardiovascular risk and is induced by other types of LPS (Pretorius et al., 2018b). It therefore seems that when these two inflammagens are co-exposed to fibrinogen, the fibrin(ogen)olytic effect of RgpA is masked by LPS which instead is chiefly responsible for determining the outcome of the clot e.g. dense matter deposit instead of fibrin-depletion induced by RgpA.

CHAPTER 7: CONCLUSION

For quite some time now, the role of microbial dysbiosis in PD and AD has received much attention (Wu et al., 2017a, Roy Sarkar and Banerjee, 2019, Dutta et al., 2019, Friedland and Chapman, 2017, Ilievski et al., 2018). While the gut microbiota remains the focal microbial residence investigated in most neurodegenerative studies, the influence of the oral microbiome on PD and AD is being unravelled and shifting the spotlight from gut to oral microbes. *P. gingivalis* – the keystone pathogen of the oral cavity – is largely responsible for oral dysbiosis and is capable of prompting periodontal disease in solitude. It has also been shown that *P. gingivalis* has a significant effect on systemic inflammation (Arimatsu et al., 2014, Zhang et al., 2018) which is believed to orchestrate systemic dyshomeostasis associated with periodontal disease, and thus the manifestation of comorbidities such as PD and AD. Relevantly, *P. gingivalis* is significantly associated with both PD (Kaur et al., 2016, Chen et al., 2017b) and AD (Ding et al., 2018, Chen et al., 2017a, Carter et al., 2017, Ganesh et al., 2017) thereby suggesting a more intimate role of this periodontopathic anaerobe in neurodegenerative pathology. Relevantly, recent findings of DNA and gingipains from *P. gingivalis* in the brains of AD patients substantiate the associations between periodontal disease and neurodegeneration and hence inspired the ideology of this thesis – identifying RgpA in the blood of PD and AD populations.

This study is the first to discover the presence of RgpA in the blood (or a system exterior to the oral cavity) of patients afflicted with PD. This finding substantiates numerous associations found between periodontal disorders and PD (Kaur et al., 2016, Chen et al., 2017b) and offers novel insight into disease pathogenesis. A significant level of the protease was also detected in the haematological system of AD individuals. This result along with the finding of *P. gingivalis* in the brains of Alzheimer's patients (Dominy et al., 2019) suggests that this pathological bacterium persists in circulation and enters other physiological systems such as the central nervous system where pathological processes can ensue. With confidence, it seems plausible that *P. gingivalis* stands as the direct link between periodontal disease and neurodegeneration. Relevantly, inhibitors of gingipains are being tested and implemented for therapeutic reasons (Dominy et al., 2019, Kariu et al., 2017, Singh et al., 2017) with the desire of disease prevention and amelioration. The biomarker potential of RgpA, in terms of diagnostics and prognostics, is highlighted. Further research is required to reveal the degree of impact that this bacterium has on the manifestation of not only neurodegenerative disease, but also the cohort of chronic inflammatory to which periodontitis is associated.

Periodontal disease, as well as PD and AD, present with abnormalities in the functioning and efficacy of the coagulation system that typically manifest as a hypercoagulable and thus prothrombotic state. The question that arose, especially in light of *P. gingivalis* being found in the brains of AD patients (Dominy et al., 2019) and in the blood of PD and AD patients (thesis in hand), is whether periodontopathic bacteria can have a significant effect on clotting processes and thus can contribute to overall increased cardiovascular risk observed in inflammatory conditions. The effects of RgpA on the kinetics of clotting, rheology of clots, and terminal fibrin load and network architecture is thus of interest.

The effect that RgpA exerts on the coagulation system is extensive. In a reductionist-type purified fibrinogen model, RgpA completely diminishes clot formation to the point of little or no fibrin detection as assessed by confocal and scanning electron microscopy. Hence, RgpA promotes hypocoagubility. Interestingly, this effect is masked in the presence of LPS PG where instead it imposes its effects of anomalous clot formation characteristic of dense matter deposits. In WB and plasma samples, however, clot formation still occurs as indicated by TEG and rheometry analysis. This suggests that a degree of inhibition of RgpA-mediated fibrin(ogen)olysis originates from plasma, possibly enforced by albumin. Nonetheless, RgpA induces significant viscoelastic changes in WB and plasma and causes normostatic coagulation to deviate towards a state of hypocoagubility. In the presence of this protease the kinetics of clot formation is hindered, fibrin load and clot size is smaller, and the deformation capacity of clots is diminished due to a stiffer network. These significant viscoelastic changes promoted by RgpA can contribute to coagulation defects in diseased states if the bacterium is present. Hence, whole-blood clotting efficiency in the presence of bacterial inflammagens from *P. gingivalis* warrants further investigation as does the identification of this bacterial species and its virulent factors in periodontal and systemic disease.

Overall, the pathogenesis of PD and AD is complex (Figure 13). Environmental and genetic factors contribute to the development and manifestation of neurodegeneration (1). Distinctive hallmark characteristics of PD and AD are amyloid β and α-synuclein accumulation, respectively, along with shared defects such as mitochondrial dysfunction, decrease in synapses and neuronal loss (2). Cardiovascular complications are widespread among PD and AD and as a result, cardiovascular events are a common mode of mortality among these patients. Hence, CVD is an important pathophysiological association to PD and AD (3). As mentioned, gut dysbiosis is prevalent in PD and AD (4) and highlights the impact that bacteria may contribute to pathology. Inflammation is an integral mechanism in the pathogenesis of PD and AD (5). The immune system expresses a notable degree of over activity and dysregulation which manifests as systemic inflammation, a factor which drives pathophysiological processes. What is relevant to this literature, however, is the implication of

bacteria and their inflammagens and their potential as novel biomarkers. RgpA, according to the results obtained in this thesis, is significantly present and both PD and AD populations and therefore may serve as a useful biomarker in terms of diagnostics and prognostics, or even as the target of therapy – more research is required to determine optimal application. Nonetheless, bacterial inflammagens, with their impact on inflammation and oxidative stress, may contribute to implications seen in the haematological system such as erythrocyte eryptosis, platelet hyperactivity, fibrin(ogen) dyshomeostasis and hypercoagulation. With the our novel findings that RgpA from *P. gingivalis* is present in the blood of PD and AD patients and that it exerts a prominent effect on clotting process, more insight is provided into the complex pathogenesis of both these disorders and highlights the role and biomarker potential of bacterial inflammagens, such as RgpA.

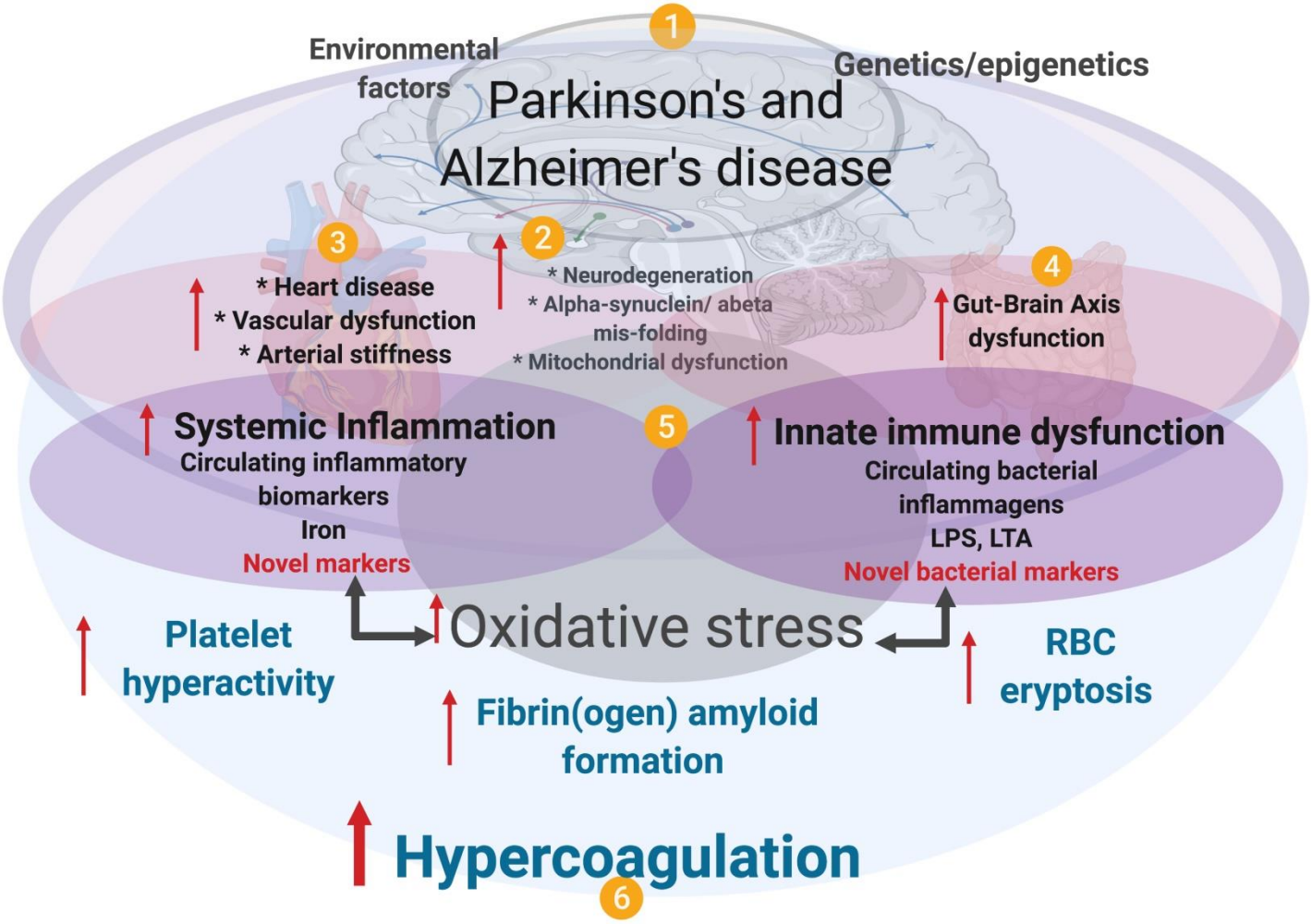


Figure 13: PD and AD express a complex pathophysiological profile with the presence of bacterial inflammagens. Environmental and genetic factors (1) lead to the development of PD and AD (2) whilst comorbidities such as CVD (3) soon manifests. The gut microbiome (4) becomes dysbiotic and contributes to CNS dysfunction and systemic inflammation (5). Ultimately, in relation to the clotting system, an overall state of hypercoagulability is induced.

Ultimately, with relevance to clinical prospects, RgpA has been identified, for the first time, in the haematological system of both PD and AD populations; this offers novel insight for the aetiology, diagnosis and treatment of both neurological disorders. Ultimately, in terms of the bioactive effects of this gingipain in the coagulation system, RgpA exerts a hindering effect on clot kinetics, reduces total clot size and alters clot rheology to form stiffer, less pliable clots. RgpA also diminishes and abrogates fibrin formation and network development – this effect was dampened in the presence of LPS from *P. gingivalis* suggesting a role of LPS in nullifying the proteolytic effect of RgpA.

Appendices

Appendix A: Ethical Clearance Form (2019)

Please note you can submit your progress report through the online ethics application process, available at: Links Application Form Direct Link and the application should be submitted to the HREC before the year has expired. Please see *Forms and Instructions* on our HREC website (www.sun.ac.za/healthresearchethics) for guidance on how to submit a progress report.

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

Provincial and City of Cape Town Approval

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

We wish you the best as you conduct your research.

For standard HREC forms and instructions, please visit: Forms and Instructions on our HREC website https://applyethics.sun.ac.za/ProjectView/Index/9521

If you have any questions or need further assistance, please contact the HREC office at 021 938 9677.

Yours sincerely,

Mrs. Melody Shana ,

Coordinator

HREC1.

National Health Research Ethics Council (NHREC) Registration Number:

REC-130408-012 (HREC1)•REC-230208-010 (HREC2)

Page 1 of 2

Federal Wide Assurance Number: 00001372
Office of Human Research Protections (OHRP) Institutional Review Board (IRB) Number:
IRB0005240 (HREC1)•IRB0005239 (HREC2)

The Health Research Ethics Committee (HREC) complies with the SA National Health Act No. 61 of 2003 as it pertains to health research. The HREC abides by the ethical norms and principles for research, established by the World Medical Association (2013). Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects; the South African Department of Health (2006). Guidelines for Good Practice in the Conduct of Clinical Trials with Human Participants in South Africa (2nd edition); as well as the Department of Health (2015). Ethics in Health Research: Principles, Processes and Structures (2nd edition).

Appendix B: Ethical Clearance Form (2015)

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

28/09/2016

Prof Resia Pretorius
Department of Physiology
University of Pretoria

Dear Prof Resia Pretorius

RE: Ethics and move to University of Stellenbosch

Your e-mail of 21 September 2016 refers.

The Faculty of Health Sciences Research Ethics Committee, University of Pretoria take note that you will be relocating to Stellenbosch University.

The ethics approvals for studies in which you are involved will continue to be valid as per ethics approval certificates. Please note that the specifications in the approved study protocols remain binding in all respects including from where participants may be recruited unless an amendment is approved by the Faculty of Health Sciences Research Ethics Committee. Recruitment of participants and study procedures executed at the Stellenbosch University will require ethics approval there.

Yours sincerely

pp.

Professor Werdie (CW) Van Staden
MBChB MMed(Psych) MD FCPsych FTCL UPLM
Chairperson: Faculty of Health Sciences Research Ethics Committee

☎ 012 356 3084 deepeka.behari@up.ac.za http://www.up.ac.za/healthethics
✉ Private Bag X323, Arcadia, 0007 - Tswelopele Building, Level 4, Room 60, Gezina, Pretoria

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

26/02/2015

Approval Certificate
Amendment
(to be read in conjunction with the main approval certificate)

Ethics Reference No.: 81/2013

Title: Erythrocytes and hypercoagulability in Alzheimer's disease

Dear Mrs Janette Bester

The **Amendment** as described in the documents received on 20/01/2015 was approved by the Faculty of Health Sciences Research Ethics Committee on the 25/02/2015.

Please note the following about your ethics amendment:
- Please remember to use your protocol number (**81/2013**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committe may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics amendment 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).

♦ Tel:012-3541330 ♦ Fax:012-3541367 Fax2Email: 0866515924 ♦ E-Mail: fhsethics@up.ac.za
♦ Web: //www.healthethics-up.co.za ♦ H W Snyman Bld (South) Level 2-34 ♦ Private Bag x 323, Arcadia, Pta, S.A., 0007

Appendix C: Blood-donor Consent Form

UNIQUE ETHICS NUMBER: 9521
Left-over blood will be stored and used for similar studies, e.g. clearance numbers: 6399; 1952; 6983; 6592;

Control (mark with x)

NAME OF CONDITION_____

Inflammatory disease (mark with x)

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT:
Circulating inflammatory biomarkers and dysregulated coagulation in inflammatory conditions

REFERENCE NUMBER: 9521

PRINCIPAL INVESTIGATOR: Prof E Pretorius

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

CONTACT NUMBER: 0829295041

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

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

What is this research study all about?

You are invited to participate in research study conducted by Prof Resia Pretorius. This information leaflet is to help you to decide if you would like to participate. Before you agree to take part, you should fully understand what is being involved in this research study. Your medical practitioner and/or phlebotomist will explain the reason for the drawing of blood. As part of this current research study, 4 blood tubes will be drawn from you. First tube will be sent to pathology laboratory or analyzed by ourselves, to determine your inflammatory status by looking at inflammatory markers like CRP levels. (An increase in CRP levels may influence the shape of the red blood cells, platelets and fibrin). The other 3 tubes will be used for our laboratory for haematological (blood) analysis, using specialized microscopes and specialized equipment for biochemical blood molecule (biomarker) analyses.

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

Why have you been invited to participate?

We are recruiting either healthy controls or individuals with inflammation and/or cardiovascular disease.
OR
Your medical practitioner wishes to obtain an individualized biomarker analysis of your inflammatory profile. He/she will discuss the results with you.

What will your responsibilities be?

You will donate 4 tubes of blood (20 ml). If you choose not to participate in this research study, you will still receive the standard care form your medical practitioner. You have no obligation to participate.

Will you benefit from taking part in this research?

There are no personal benefits but results generated from this study will allow researchers to determine the physiology of healthy clotting and red blood cell structure and will be used to test novel nanobiosensors for blood clotting analysis, constructed by the members of the Faculty of Engineering, Stellenbosch University. If your medical practitioner has requested the analysis, via our SUNguis blood laboratory, he/she will share the results with you. We as SUNguis blood laboratory will not be involved in any diagnoses. We will simply provide your medical practitioner with results generated in our laboratory using our point-of-care blood analysis devices or state-of-the-art research equipment.

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

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

the procedures will be performed under sterile conditions by your medical practitioner or by a trained phlebotomist.

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

none

Who will have access to your medical records?

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

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

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

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

No you will not be paid to take part in the research study.
If your medical practitioner requests the personalized biomarker and coagulation analysis, payment will be done via the SUNguis system.

Is there anything else that you should know or do?

- You can contact the Health Research Ethics Committee at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor.
- If you request it, you will receive a copy of this information and consent form for your own records.

Age:
Gender
HbA1c levels:

Cholesterol levels:

Other medication used:

Declaration by participant

By signing below, I ……..…… agree to take part in a research study "**Circulating inflammatory biomarkers and dysregulated coagulation in inflammatory conditions**
OR because my medical practitioner requested a personalized biomarker and/or coagulation analysis via the SUNguis incubator blood laboratory.

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

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

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

Declaration by investigator

I *(name)* ……..…… declare that:

- I explained the information in this document to ……..
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above

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

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

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

Declaration by interpreter

I *(name)* ……..…… declare that:

- I assisted the investigator (*name*) ……. to explain the information in this document to (*name of participant*) ……..…….. using the language medium of Afrikaans/Xhosa.

- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

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

....
Signature of interpreter **Signature of witness**

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