THE FUNCTION OF ADIPSIN AND C9 PROTEIN IN THE COMPLEMENT SYSTEM IN HIV-ASSOCIATED PREECLAMPSIA

By

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PREFACE

This study represents original work by the author and has not been submitted in any other form to another University. Where use was made of the work of others, it has been duly acknowledged in the text.

The research described in this dissertation was carried out in the Optics and Imaging Centre, Doris Duke Medical Research Institute, College of Health Sciences, University of KwaZu1uNatal, Durban, South Africa, under the supervision of Professor T. Naicker and Professor J. Moodley.

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DECLARATION

1, Mikyle David (216000603) declare that:

(i) The research reported in this dissertation is my original work, except where otherwise stated.

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(a) Their words have been paraphrased, but the general information attributed by them has been referenced.

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DEDICATION

To my parents,

You have educated and supported me throughout all my endeavours

Thank you both

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2. Publication funds, Prof T Naicker

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ETHICAL APPROVAL

This study was approved by the Biomedical Research Ethics Administration, University of KwaZulu-Natal, Durban, South Africa. Ethics No: BCA 338/17.

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LIST OF ABBREVIATIONS

HDP…… Hypertensive Disorders of Pregnancy

HIV……. Human immunodeficiency virus

PE…… Preeclampsia

WHO…… World Health Organization

EOPE…… Early onset preeclampsia

LOPE……. Late onset preeclampsia

EVT……... Extravillous trophoblast cells

sEng……... Soluble endoglin

sFlt-1…….. Soluble fms-like

TH1…… T-helper 1

TH2…… T-helper 2

TNF-α…… Tumour necrosis factor

IL……... Inter-leukin

PIGFs……. Placental growth factors

VEGFs…… Vascular endothelial growth factors

NK…….. Natural killer

HAART…….. Highly active antiretroviral therapy

PIs…… Protease inhibitors

CP…… Classical Pathway

LP…… Lectin Pathway

AP…… Alternative Pathway

MAC…… Membrane attack complex

BMI……. Body mass index

Tregs…… Regulatory T cells

DCs……. Dendritic cells

gp…… Glycoprotein

MBL……... Mannose-binding lectin

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CHAPTER 2

ABSTRACT

Background

Hypertensive disorders of pregnancy (HDP) and non-pregnancy related diseases (HIV, TB) are the most common causes of maternal mortality in South Africa (SA). Preeclampsia (PE), a complex medical disorder, accounts for the majority of maternal deaths emanating from HDP. In SA, the prevalence of HIV infection in pregnancy is high. The complement system, a part of our innate response, may be altered in the synergy of HIV infection and PE development. Complement activation at the maternal-interface may exacerbate inflammation, tissue injury, apoptosis as well as vascular leakage, in the complicated state of PE. The complement proteins, adipsin and C9, activates the body's natural defence against HIV infection. C9 functions as a pore-forming component of the membrane attack complex. Moreover, the high rollout in SA of antiretroviral therapy may affect the immune response in HIV infected women. This study investigates the concentration of the complement proteins, Adipsin and C9 in the duality of HIV-associated PE.

Method

Samples of 38 normotensive and 38 preeclamptic patients, stratified further by HIV status were collected from a regional hospital. Thereafter, analysis of analytes via the Bio-plex Multiplex immunoassay occurred.

Results

Maternal weight did not differ (p = 0.1196) across the study groups. The concentration of adipsin was statistically different between the PE *vs* normotensive pregnant groups, irrespective of HIV status (p = 0.0439). There was no significant difference in adipsin concentration between HIV-negative *vs* HIV-positive groups, irrespective of pregnancy type (p = 0.6290). Additionally, there was a significant difference in adipsin concentration between HIV negative normotensive *vs* HIV negative preeclampsia ($p < 0.05$), as well as a difference between HIV negative preeclampsia *vs* HIV positive preeclampsia ($p < 0.05$). C9 protein expression was not statistically different between the normotensive and PE groups, regardless of HIV status (p =0.5365). No statistical significance in C9 expression was found between HIV positive *vs* HIV negative groups, regardless of pregnancy type (p = 0.3166). Similarly, no statistical significance was noted across all study groups (p= 0.0774).

Conclusion

This study demonstrates a significant up-regulation of adipsin in PE compared to normotensive pregnancies; this finding correlates with the exaggerated inflammatory milieu of PE. Complement C9 protein was similar between pregnancy types. This similarity may emanate from properdin dysregulation or a genetic polymorphism. The concentration of adipsin and C9 proteins were not affected by HIV status due to the immune reconstitution effect of antiretroviral therapy. Furthermore, the up-regulation of adipsin in placental sites and urinary levels of PE in previous studies, in tandem with our findings, indicate the possibility of adipsin as a predictor value for PE development.

ABSTRACT IN ISIZULU

Ingemuva

Ukuphazamiseka ngokweqile kokukhulelwa (i-HDP) kanye nezifo ezingahlobene nokukhulelwa (i-HIV, i-TB) yizimbangela ezivame kakhulu zokufa komama eNingizimu Afrika (SA). I-Preeclampsia (PE) inkinga eyinkimbinkimbi yezokwelapha, ilandisa ngokufa komama abaningi okuvela ku-HDP. ENingizimu Afrika, izinga lokutheleleka nge-HIV ngesikhathi sokukhulelwa liphezulu. Uhlelo lokuncoma, oluyingxenye yokuphendula kwethu okungokwemvelo, lungaguqulwa ekubambisaneni kokutheleleka nge-HIV kanye nokuthuthuka kwe-PE. Gcwalisa ukusebenza ku-interface yomama kungakhuphula ukuvuvukala, ukulimala kwezicubu, i-apoptosis kanye nokuvuza kwemithambo, esimweni esiyinkimbinkimbi se-PE. Amaprotheni agcwalisa, i-adipsin kanye ne-C9 kusebenze ukuzivikela kwemvelo komzimba ekuthelelekeni nge-HIV. Imisebenzi ye-C9 njengengxenye yokwakha i-pore yenkimbinkimbi yokuhlasela kwe-membrane. Ngaphezu kwalokho, ukukhishwa okuphezulu eSA kwemishanguzo kungathikameza amasosha omzimba kwabesifazane abane-HIV. Lolu cwaningo luphenya ukugxila kwamaprotheni ahambisanayo, i-Adipsin kanye ne-C9 kubumbili be-PE ehlobene ne-HIV.

Amasampula

ezingama-38 ze-normotensive kanye nama-38 preeclamptic, ahlukaniswe ngokwengeziwe nesimo se-HIV aqoqwa esibhedlela sesifunda. Ngemuva kwalokho, ukuhlaziywa kwama-analytics nge-Bio-plex Multiplex immunoassay kwenzeka**.**

Imiphumela

Isisindo somama asihlukile (p = 0.1196) kuwo wonke amaqembu okufunda. Ukuhlushwa kwe-adipsin kwakwehluke ngokwezibalo phakathi kwamaqembu e-PE vs normotensive abakhulelwe, kungakhathalekile isimo se-HIV (p = 0.0439). Kwakungekho mehluko ophawulekayo ekugxileni kwe-adipsin phakathi kwamaqembu angenayo i-HIV noma i-HIV, kungakhathalekile uhlobo lokukhulelwa (p = 0.6290). Ngokwengeziwe, bekunomehluko omkhulu ekugxileni kwe-adipsin phakathi kwe-HIV negative normotensive vs HIV negative preeclampsia (p <0.05), kanye nomehluko phakathi kwe-HIV negative preeclampsia vs HIV positive preeclampsia (p <0.05). Isisho seprotheyini se-C9 sasingafani ngokwezibalo phakathi kwamaqembu we-normotensive nama-PE, ngaphandle kwesimo se-HIV (p = 0.5365). Akunakubaluleka kwezibalo ekuvezweni kwe-C9 okutholakele phakathi kwamaqembu ane-

HIV noma amaqembu angenayo i-HIV, ngaphandle kohlobo lokukhulelwa (p = 0.3166). Ngokufanayo, akukho ukubaluleka kwezibalo okwaphawulwa kuwo wonke amaqembu okufunda (p = 0.0774).

Isiphetho

Lolu cwaningo lukhombisa ukulawulwa okuphezulu kwe-adipsin ku-PE uma kuqhathaniswa nokukhulelwa okujwayelekile, lokhu kutholakala kuhambelana nendawo evuthayo yokuvuvukala ye-PE. Ukugcwalisa amaprotheni we-C9 ayefana phakathi kwezinhlobo zokukhulelwa. Lokhu kufana kungavela ekushayweni kwe-efaneledin, noma i-polymorphism yofuzo. Ukuhlungwa kwamaprotheni e-adipsin nama-C9 akuzange kuthinteke isimo se-HIV, ngenxa yomphumela wokwakhiwa kabusha kwamasosha omzimba okwelashwa ngezidambisigciwane. Ngaphezu kwalokho, ukulawulwa okuphezulu kwe-adipsin kumasayithi we-placental namazinga we-urinary we-PE ezifundweni ezedlule, ngokuhambisana nokutholakele kwethu, kukhombisa ukuthi kungenzeka yini ukuthi i-adipsin njengenani lesibikezelo sokuthuthuka kwe-PE.

CHAPTER 1

LITERATURE AND BACKGROUND REVIEW

1.1 HYPERTENSIVE DISEASE OF PREGNANCY AND PREECLAMPSIA

Hypertensive disorders of pregnancy (HDP) and non-pregnancy related diseases (HIV infection, TB) are the most common causes of maternal mortality in South Africa (Moodley *et al.*, 2019). Hypertensive disorders of pregnancy account for approximately 18% of all maternal deaths in South Africa (Moodley *et al.*, 2018). In developed countries, the prevalence of HDP is low (5-10%) compared to developing countries (Naicker *et al.*, 2019). Preeclampsia (PE) is a complex HDP that accounts for 500000 fetal and neonatal deaths and approximately 70000 maternal deaths worldwide (Gathiram and Moodley, 2016). The World Health Organisation (WHO) reports the prevalence of PE to be 1.6% in developed countries compared to 1.8-16.7% in developing countries (WHO., 2017). Preeclampsia is diagnosed by new-onset hypertension (BP≥140mmHg or ≥90mmHg diastolic) after 20 weeks of gestation, with/without proteinuria (≥300mg) (Brown *et al.*, 2018). It is accompanied by maternal organ dysfunction and severe maternal complications that may include-the liver, kidney, etc. and hemolysis, elevated liver enzymes, and low platelet count referred to as the "HELLP" syndrome (Phipps *et al.*, 2016; Brown *et al.*, 2018)

Preeclampsia is a heterogeneous disease and may be divided into two main subtypes, *viz.*, early and late-onset PE (Staff *et al.*, 2013). The clinical signs for early-onset PE (EOPE) occur before 33 gestational weeks of pregnancy, and late-onset PE (LOPE) appears after 34 weeks of gestation. Late-onset PE constitutes the majority (>80%) of preeclamptic cases (Gathiram and Moodley, 2016). The early-onset type is associated with abnormal placentation and correlates with high maternal and fetal morbidity and mortality rates (Von Dadelszen *et al.*, 2003).

The pathogenesis of PE is not fully understood as yet. However, a common implication is the inadequate spiral uterine artery remodelling due to the inadequate migration of extravillous trophoblast (EVT) cells (McNally *et al.*, 2017). During normal pregnancy, the trophoblast begins migration from the tips of anchoring villi through the decidua and the myometrium accompanied by spiral artery remodelling (Maître, 2017). The migration or proliferation of the EVT cells depends on the oxygen gradient established during placental development (Huppertz *et al.*, 2009). In PE, this shallow trophoblast invasion and the failed transformation of the myometrial spiral arteries emanates from elevated apoptosis of trophoblast cells (Naicker *et al.*, 2013). This inadequate remodelling leads to the reduced diameter of spiral arteries and,

therefore, lack of nutrients and oxygen supply to the placenta and developing fetus (Li *et al.*, 2015). As a result, the placenta becomes ischemic and releases anti-angiogenic factors such as soluble endoglin (sEng), soluble fms-like (sFlt-1) and inflammatory mediators into the general circulation (Tomimatsu *et al.*, 2017).

During normotensive pregnancies, multiple adaptations of the innate and adaptive immune systems are implemented to establish survival of the fetal allograft (Pierik *et al.*, 2019), whilst simultaneously protecting mother and fetus from various pathogens (Hsu and Nanan, 2014).

Crucial immune adaptations are required to prevent fetus rejection and to provide protection against invading pathogens. In normal pregnancies, this is accomplished by causing a shift of the maternal response from T-helper 1 (TH1) type to a T-helper 2 (TH2) type immune response, which favours an immuno-tolerant microenvironment (Alrahmani, 2018; Perez-Sepulveda *et al.*, 2014).

In PE, there is deficient development of maternal tolerance to the fetus or an altered maternal immune response due to excessive activation of neutrophils and monocytes (Saito *et al*., 2007). Notably, monocytes synthesise considerable amounts of pro-inflammatory chemokines and cytokines (Laresgoiti-Servitje, 2010; Perez-Sepulveda *et al.*, 2014). Additionally, CD8+, CD4+ and dendritic cells also escalated the pro-inflammatory response (Jabbour *et al.*, 2009).

Cytokines are immune modulators, and evidence suggests it plays a vital role in placentation, ovulation, implantation, and parturition (Bowen *et al.*, 2002). Additionally, they are mediators of numerous cell signalling pathways and are synthesised by (Th1)-type and (Th2)-type immune cells, which exert positive and negative effects during pregnancy (Sargent *et al.*, 2006). They assist in proliferation, angiogenesis, and placental invasion (Keelan and Mitchell, 2007) and are classified as pro-inflammatory (TNF-α, IL-6) and anti-inflammatory (IL-4, IL-10) cytokines (Sargent *et al.*, 2006). The pro-inflammatory cytokines increase vascular permeability and also induces apoptosis of trophoblast cells (Chen *et al.*, 2010). Furthermore, they also activate and damage endothelial cells, exacerbating the exaggerated inflammatory response seen in PE development (Granger, 2004; Kharfi *et al.*, 2003). Off note, anti-inflammatory cytokines are essential for the functioning of (Th2) and regulatory T cells, and this helps ensure a successful pregnancy (Thaxton and Sharma, 2010). Any imbalance in the levels of pro-inflammatory or anti-inflammatory cytokines may affect several immunological and apoptotic pathways, resulting in PE and other pregnancy-related disorders (Roberts, 2003).

1.2 Angiogenesis

Angiogenesis is the process by which endothelial cells form new blood vessels from pre-existing blood vessels by means of differentiation, migration and development (Kubis and Levy, 2003). Angiogenesis is initiated by placental growth factors (PIGFs) and pro-angiogenic vascular endothelial growth factors (VEGFs) (Steinberg *et al.*, 2009), which function to encourage proteolysis of extracellular matrix and increase the vessel permeability, resulting in endothelial cell proliferation (Mutter and Karumanchi, 2008).

During angiogenesis, there are several factors that are believed to disrupt this process, *viz*., activation of natural killer (NK) cells and macrophages, and oxygen tension (Alain and Raouf, 2010). This activation causes a shift in the balance of angiogenic versus anti-angiogenic factors that culminate in endothelial dysfunction (Wallace *et al.*, 2014). Aberrations in the angiogenic balance contribute to inadequate trophoblast invasion observed in PE (Maynard and Karumanchi, 2011).

According to Helmo *et al.*, (2018), an increase in the expression of sFlt-1 kinase receptor and soluble endoglin with a concomitant decrease in the expression of VEGF and PIGF, impairs angiogenesis and vasculogenesis in PE. In HIV-1 infected patients, the process of angiogenesis is also dysregulated (Paydas *et al*., 2009). Adverse birth outcomes are elevated and have been associated with antiretroviral therapy (ART), compared to HIV-uninfected women (Chen *et al*., 2012). In a study carried out by Powis *et al*., (2013), angiogenesis was evaluated in preeclamptic women who began HAART administration during pregnancy. Women who developed PE, show an increase in anti-angiogenic factors, prior to HAART usage. Notably, Conroy *et al*., (2017), found a correlation between altered angiogenesis and ARV usage in the second and third trimester as a progenitor of preterm birth, stillbirth and small for gestational age, factors maternal factors characteristic of PE development.

1.3 HIV Infection

Human immunodeficiency virus is a retrovirus that invades a host and impairs cellular immunity, thereby increasing the hosts' susceptibility to opportunistic pathogens (Maartens *et al.*, 2014). HIV infection remains a global pandemic, with 37.9 million people living with and 1.7 million people newly infected with HIV at the end of 201. South Africa has a population of 7.97 million people (13.5%) infected with HIV (SA Stats, 2019), of which 4.7 million are

women in their reproductive age (UNAIDS, 2020). Around 30% of South African women who are pregnant are infected with HIV (Kalumba *et al.*, 2013), with the province of KwaZulu-Natal having a prevalence of approximately 40%. The association between HIV infection and PE in pregnancy originates from opposing immune responses, as PE induces an exaggerated immune response, whilst HIV dampens the immune response (Kalumba *et al.*, 2013). However, this neutralisation is prevented by anti-retroviral therapy (ARVs) usage.

1.3.1 Highly Active Anti-Retroviral Therapy (HAART) in PE

The province of KZN has the highest ARV enrolment in the world (Kalumba *et al*., 2013). Exploiting the life cycle of HIV has led to the development of a combination of at least three ARV drugs, one of which is HAART (Reshi and Lone, 2010). Once HAART is initiated, the virus in the CD4+ T cells is suppressed in two phases: the early rapid phase occurring after 2-3 months of HAART usage and a slow delayed phase (Reshi and Lone, 2010). This causes a drop in the plasma HIV viral load count.

During pregnancy, HAART has the ability to decrease maternal viral replication and mother to child transmission, by reducing the plasma viral load in pregnant women (Siegfried *et al.*, 2011). This suggests that HIV may neutralise the hyper-reactivity of the immune system associated with PE, hence reduce the risk of PE development (Govender *et al.*, 2013). However, HAART re-establishes the immune response, thus increasing the risk for PE development (Landi *et al.*, 2014).

HIV suppresses the immune response, and thus neutralises the hyper-inflammatory response of PE (Govender *et al.*, 2013). In contrast, a greater risk for PE development can arise from the chronic arterial dysfunction with endothelial damage, associated with HIV infection (Fourie *et al.*, 2011). Nonetheless, the endothelial damage and vascular dysfunction caused by HAART administration have been reported to predispose patients to develop hypertensive disorders (Kline and Sutliff, 2008).

In the study conducted by Sansone *et al*., (2016) to determine the risk of PE in HIV-infected pregnant women receiving HAART, it was reported that HIV-infected women and those receiving HAART, had a higher risk for PE development. However, more research needs to be done on this, as there are contradicting results on the risk of PE development on HAART administration (Kline and Sutliff, 2008).

The prevalence of PE in HIV-infected pregnancies is lower; however, HAART administration may cause an upregulation of anti-angiogenic factors, endothelial dysfunction, and decreased nitric oxide (Aouache *et al.*, 2018). Additionally, HIV infected pregnant women on HAART, showed an increase in the Th1 immune response (Maharaj *et al.*, 2017), thereby increasing the risk of PE development increases (Kalumba *et al.*, 2013; Sansone, 2016). The reasoning behind the higher risk of PE development by HAART usage is not yet fully understood. However, it is plausible that HAART may induce PE due to a direct toxic effect on the liver and kidney (Mawson, 2003). Additionally, HAART usage is documented to improve maternal immune remodelling (Suy *et al.*, 2006), by restoring the mother's immune response to fetal antigens and subsequently making HIV-infected women more likely to develop PE (Mol *et al.*, 2016).

Protease inhibitors (PIs) are potent anti-angiogenic factors. They deter HIV aspartyl protease and, the production of HIV, to promote immune restoration. Anti-retroviral therapy was also shown to lead to endothelial dysfunction by decreasing nitric oxide, resulting in induced endothelial oxidative stress (Chai *et al.*, 2005), observed during the pathophysiology of PE development (Aouache *et al.*, 2018).

1.4 The complement system

The complement system forms part of the innate immune system and consists of numerous plasma and cell membrane proteins (Merle *et al.*, 2015). The complement system is initiated by three main pathways (figure 1.1) *viz.*, the classical pathway (CP), the lectin pathway (LP), and alternative pathway (AP), which culminates into one common pathway (Noris and Remuzzi, 2013). The function of the complement system is the opsonisation of target surfaces, induction of pro-inflammatory responses, and the lysis of cells and pathogens. It is also vital in defence of the host by clearing apoptotic cells, injured tissue, and immune complexes, thus contributing to maintaining homeostasis (Lokki *et al.*, 2014).

The CP is activated due to binding of recognition molecules C1q to IgG or IgM immune complexes bound on the surface of microbes or other structures (McDonald *et al.*, 2015). This causes a configurational change, resulting in the activation of C1r and C1s (Noris and Remuzzi, 2013). Thereafter, C1s cleaves C4 and C2 to form a C4bC2A complex, which is a C3 convertase (Regal *et al.*, 2015). The LP is activated by recognition molecules, *viz.*, mannose-binding lectin (Conroy *et al.*, 2017; Noris and Remuzzi, 2013) when they bind to mannose and sugar molecules on the surface of numerous microorganisms (Killick *et al.*, 2018). This

activates the LP serine protease MASP-1 and MASP-2, which then cleaves C4 and C2 to form C3 convertase, C4bC2a (Regal *et al.*, 2017). The AP is different from the CP and LP, as it is continuously activated at low levels in the body (Merle *et al.*, 2015). It is activated by the spontaneous hydrolysis of C3 to form C3(H2O), resulting in binding of factor B and cleavage of factor D to form C3 convertase, C3bBb (Schmidt *et al.*, 2016).

All pathways of the complement system create C3 convertase, which split C3 into components, C3a, and C3b (Denny *et al.*, 2013). C3b binds to the activating surface of its target and contributes to the activation of C5 convertase. C5 convertase cleaves C5 into C5a and C5b. C5b forms together with C6, C7, C8, and C9 to form the membrane-attack-complex (MAC), as seen in figure 1, to cause cell lysis (Denny *et al.*, 2013; Tincani *et al.*, 2010).

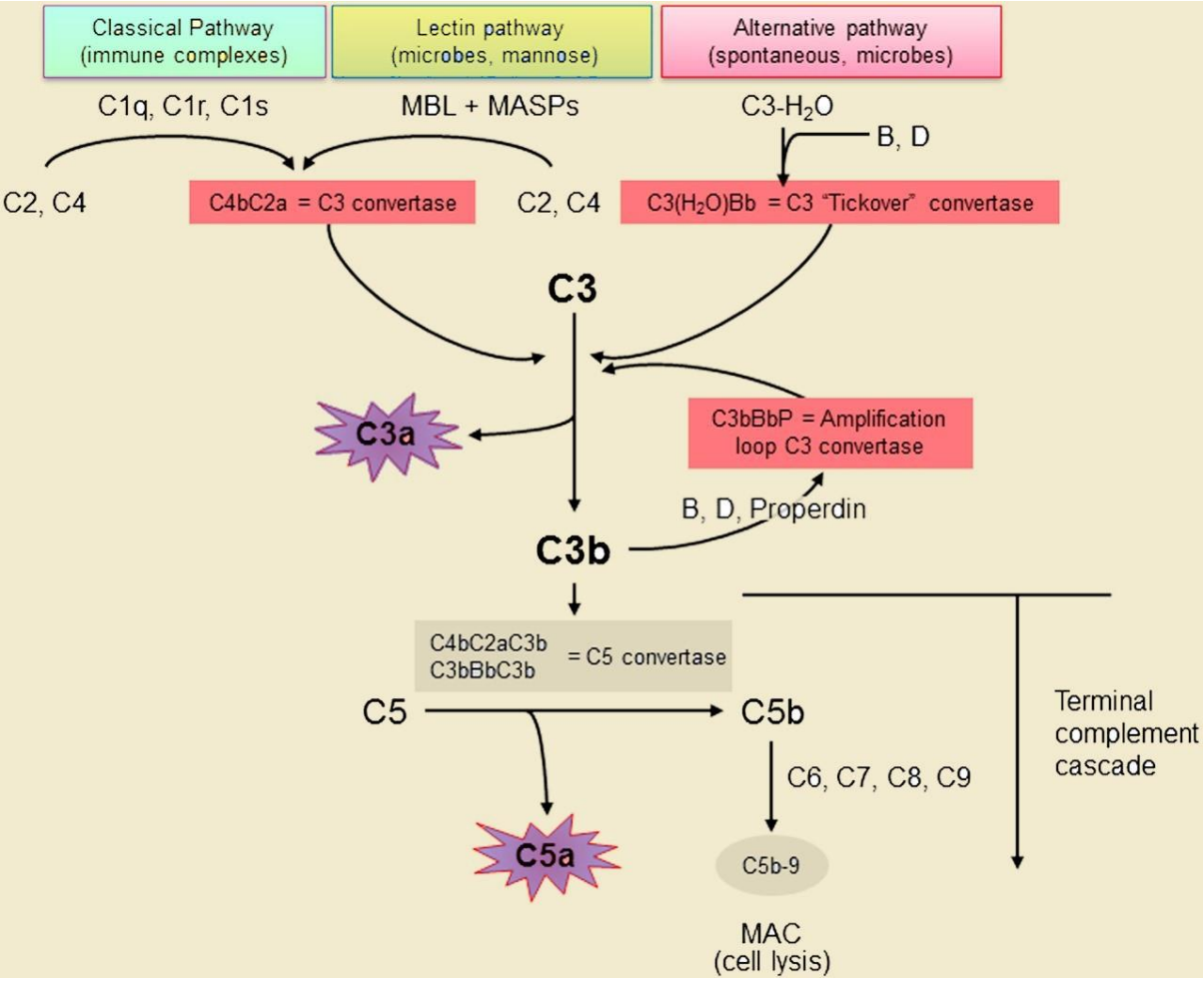

Figure 1.1: Schematic Overview of the complement system, illustrating the three pathways. Activation of the complement cascade occurs via three pathways *viz.*, the classical, lectin and alternative pathway. All three pathways result in C3 convertase being produced, which cleaves C3 into C3a and C3b. Adapted from (De Vriese *et al.*, 2015)

1.4.1 Complement regulation

Complement regulation is mediated by specific molecules called; complement regulators, which function by preventing the activation or initiation of their degradation (McDonald *et al.*, 2015). These components consist of many soluble and membrane-bound molecules, and they include C1-INH (C1 inhibitor), C4BP, and Factor H (Noris and Remuzzi, 2013). C1-INH inactivates C1r and C1s of CP and mannan-binding lectin serine protease (MASP-1), MASP-2 of the LP from binding, therefore preventing these pathways from being activated. C4BP binds to C4b and prevents the formation of C3 convertase (C4bC2a), while Factor H degrades the AP C3 convertase (C3bBb) (Regal *et al.*, 2017). Excessive complement activation during pregnancy is controlled by three regulatory proteins on the plasmalemma of trophoblast cells, *viz*., CD46, CD55, and CD59 (Noris and Remuzzi, 2013). The complement system is also known to initiate cell death in non-infected healthy cells (Ziporen *et al.*, 2009). Complement regulation is known for protection from maternal complement attack by establishing an immune tolerance needed for embryo development and survival (Regal *et al.*, 2017).

Normal placentation is accomplished due to local expression of complement regulators (CD55, CD59, and CD46) and by inhibiting C3 and C5 convertases (Girardi, 2018). Excessive complement activation leads to damage of invading extravillous trophoblasts and placental dysfunction, associated with pregnancy complications, including PE (Lokki *et al.*, 2014).

Activation of the complement system also causes pro-inflammatory and chemotactic anaphylatoxins release, which have the potential to exacerbate inflammation, thrombosis, and vascular leakage. Inadequate regulation of the complement system may also cause tissue damage by pro-inflammatory lesions and an increase in apoptosis (Ito *et al.*, 2015; Rampersad *et al*., 2008).

1.4.2 Complement activation

Complement activation at the maternal-fetal interface, recruits' neutrophils, which increase TNF-α while decreasing VEGF, thereby leading to abnormal placentation (Gelber *et al.*, 2015). Blocking the complement system by the use of inhibitors, viz., serpin, Factor 1, and C4-BP, specifically targets the sites of complement activation, results in neutrophil depletion whilst blocking of TNF-α improves the spiral artery remodelling (Qing *et al.*, 2011). A study conducted by (Lillegard *et al.*, 2013) established a link between hypertension decline in pregnancy and complement activation using a rat model.

Complement activation results in the release of chemotactic anaphylatoxins and pro-inflammatory cytokines, which may cause inflammation, thrombosis and vascular leakage (Rampersad *et al.*, 2008). Aberrant complement activation can also result in tissue damage distinguished by inflammatory lesions and increased apoptosis on placental villi (Ito *et al.*, 2015).

1.4.3 Complement dysregulation

Evidence demonstrates a dysregulation of the complement component within both the maternal circulation and the placenta (Regal *et al.*, 2017). Lynch *et al.*, (2016) showed dysregulation of the AP activation fragment Bb in PE, with the highest levels occurring early in pregnancy, highlighting a crucial role for the AP in PE development. In contrast, increased levels of Bb have been reported in maternal and umbilical venous blood in PE (Hoffman *et al.*, 2014).

Obesity causes complement components to increase and are positively correlated with body mass index (BMI), whilst decreases with weight loss (Moreno-Navarrete and Fernández-Real, 2019). Complement proteins are created primarily by the liver, but they can also be generated by other organs and tissues such as adipose tissues, therefore complement the complement system is activated, and complement components are increased in obesity and increasing BMI (Nilsson *et al.*, 2014).

The risk of PE development rises with an increase in body mass index (BMI) (Roberts *et al.*, 2011). This statement was confirmed by illustrating that bariatric surgery done to reduce obesity, can reduce PE occurrence (Galazis *et al.*, 2014). Maternal obesity, with its circulating factors such as fatty acids, may cause the excess of lipid accumulation in the placenta (Jarvie *et al.*, 2010). This may impede placental development, including angiogenesis and trophoblast, as well as deficient nutrient transfer between mother and fetus (Jarvie *et al.*, 2010). An increase in inflammation caused by pro-inflammatory mediators, such as interleukin (IL6) and tumour necrosis factor (TNF-α), and oxidative stress, occurs at the maternal-fetal interface and may lead to placental injuries, observed in PE (Saben *et al.*, 2014).

The complement system has been linked to excess adipose tissue and PE development (Nilsson *et al.*, 2014).

1.4.4 The complement system in normal and preeclamptic pregnancy- a summary

During normal pregnancy, immune adaptations are made by the complement system to prevent the rejection of the fetus by the mother, whilst protecting against pathogens (Lannaman *et al.*, 2017). The complement system achieves this by causing a shift in the maternal response from T-helper 1 (Th1) to T-helper 2 (Th2) type immune response (Perez-Sepulveda *et al.*, 2014). Trophoblast cells secrete the complement components, C3, C4 and C1q (Bulla *et al.*, 2012). C1q is synthesised by decidual endothelial cells, implying a correlation between trophoblast cells and spiral artery endothelial cells (Madhukaran *et al.*, 2016). Additionally, Singh et al., (2011), that C1q plays a role in trophoblast migration and spiral artery remodelling, as C1q deficient mice displayed features of PE such as hypertension and proteinuria.

Complement component C3 activation was also shown to contribute to normal phagocytic activity in mouse trophoblast cells, and this suggests that C3 may play a role in trophoblast invasion (Regal *et al.*, 2015). The increase in C5a deposition in macrophages and C5aR appearance in the placental trophoblasts of PE women also demonstrated. C5a inhibited vessel tube formation and migration of trophoblasts, hence leading to inadequate trophoblast invasion (Ma *et al.*, 2018).

Regulatory T cells (Tregs) are a subpopulation of T cells that modulate the immune system, prevent autoimmune diseases and maintain fetal tolerance during pregnancy (Jiang *et al.*, 2014). A decrease in Tregs is observed in PE, and forkhead box P3 (Foxp3+) regulatory T cells develop in the absence of C5aR1 activation (Strainic *et al.*, 2013). The stimulation of C5aR1 induces pro-inflammatory T cells; therefore, production of C5a during pregnancy may reduce Treg numbers with a concomitant increase in inflammatory T cells, exacerbating the inflammatory state (Drouin *et al.*, 2006).

Lynch *et al*., (2008), observed that pregnant women with increased Bb plasma levels during early pregnancy, are at a higher risk of developing PE later on in pregnancy. This suggests that in PE patients, abnormal complement activation may be present during the first trimester (10-15 weeks). Most studies of complement activation have been focused after the onset of PE and not during the early stages or first trimester (Denny *et al.*, 2013).

According to He *et al*., (2020), the abnormal regulation of the complement system by the alternative (increased levels of complement factor B and complement factor H) and classical (elevated C1q) pathways, were observed in the first trimester of patients with PE later in pregnancy. It was reported by Denny *et al*., (2013) and Burwick *et al*., (2018), that levels of

C3a, C5a and sC5b-9 are increased in PE patients, suggesting that abnormal complement regulation plays a role in two stages of PE development (Girardi *et al.*, 2006).

In stage 1 (placenta formation), abnormal complement regulation was found to affect the placental formation, which causes the onset of PE later in pregnancy (Girardi *et al.,* 2006; Rampersad *et al.,* 2008). In stage 2 (after the onset of PE), the complement system becomes activated by local placental ischemia and hypoxia, resulting in a cascade of reactions that contribute to the rapid development of PE (Lillegard *et al.*, 2013; Burwick *et al.*, 2018).

1.4.5 The complement system and angiogenic imbalance

Several studies have shown an increase in the incidence of PE women, who had increased levels of sFlt-1 and sEng in maternal circulation; therefore, a potential relationship between complement dysregulation and angiogenic imbalance is observed (He *et al.*, 2016). A study by Banadakoppa *et al.,* (2018), reported the in vitro activation of the complement system at sub-lethal levels on human trophoblast cells caused by the up-regulation of sFlt-1. Additionally, it was also suggested that C3a increased the levels of mRNA sFlt-1, causing MAC to be released.

1.4.6 The complement system in the duality of HIV infection and Preeclampsia

The complement system is vital during viral infection and is the first barrier to manage and maintain the spread of HIV. It also aids in the clearance and neutralisation of HIV (Posch *et al.*, 2011). When HIV-1 enters the host, it activates the complement system via its two glycoproteins, gp41 and gp120, without the need for HIV-specific antibodies and hence it becomes coated with complement fragments (Hladik and McElrath, 2008). Thereafter, adaptive immunity is activated, and specific anti-HIV-1 antibodies and activated T cells are generated (de Jong and Geijtenbeek, 2010).

In the HIV budding process, HIV acquires regulators of the complement system such as CD55, CD59 and factor H (figure 1.2). Therefore; the virus is protected from complement-mediated lysis (Roberts *et al.*, 2010). Additionally, it was observed that the complement system or together with dendritic cells (DCs) are involved with priming of antiviral T cell immunity, suggesting the complement system activates lysis and mediates an adaptive immune response (Posch *et al.*, 2011).

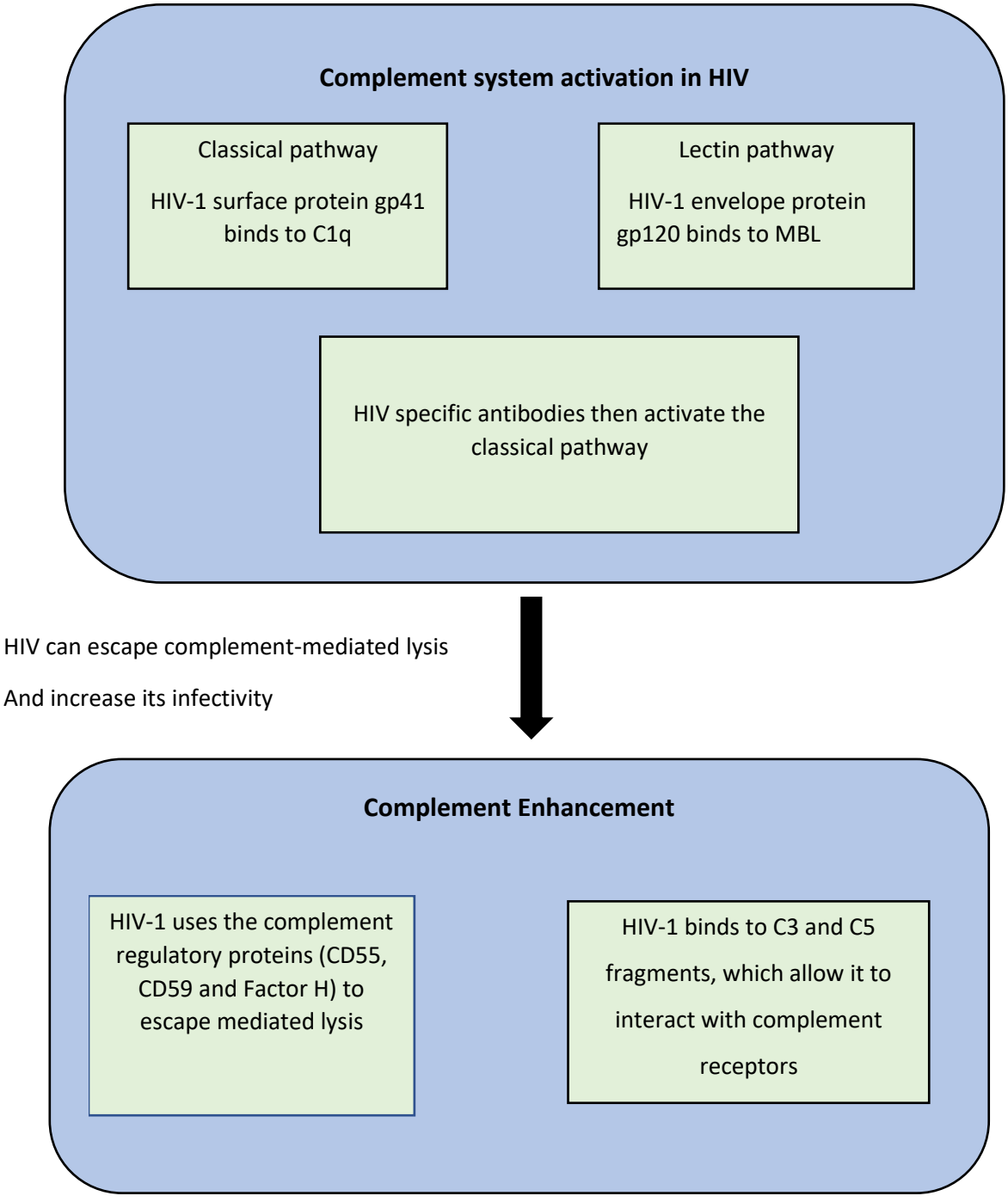

Figure 1.2: Schematic diagram on the role of the complement system during HIV infection. It is activated by HIV-1 virus binding to gp41 and gp120. HIV-specific antibodies also contribute to complement activation. HIV-1 uses the regulatory proteins to evade complement-mediated lysis.

Banki et al., (2010), demonstrated that failure to produce efficient T cell responses without the presence of complement may be attributed to DCs, which engulf C3-coated pathogens to prime CD8+ T cells, resulting in efficient activation of adaptive immunity. Moreover, in HIV-infected individuals, the NK cell response is impaired, with subsequent defects of cytotoxic activity of NK cells. This impairment begins early after infection and continues during HIV progression (Alter, 2005).

During the early stages of HIV-1 infection, the complement system is activated. The protein gp41 on the surface of HIV-1 binds to C1q and activates the CP (Yu and Qin, 2010), while HIV-1 envelope protein gp120 binds to mannose-binding lectin (Conroy *et al.*, 2017), to neutralise the virus (Eisen and Klein, 2008). MBL then triggers activation of the LP, to increase phagocytic uptake and to inhibit viral entry to susceptible cells (Eisen and Klein, 2008). Despite activation of the complement function during HIV-1 infection. HIV-1 is still able to evade complement-mediated lysis (Yu and Qin, 2010). It accomplishes this by using the complement regulatory proteins (CD 55 and CD59) from the host during its budding process. Factor H also binds to HIV-1, to provide extra protection from complement attack (Liu *et al.*, 2014).

Dysregulation in complement activation can shift the balance from a normal state to an inflammatory state (Ricklin and Lambris, 2013). C3a and C5a are complement components that are up-regulated in PE (figure 1.3) and are able to induce inflammation, recruit macrophages and dendritic cells (Harman *et al.*, 2016) for HIV-1 infection (Derzsy *et al.*, 2010). An increase of pro-inflammatory cytokines such as IL-6 and TNF-α were occurred in the presence of C5a, to promote HIV-1 infection (Popko *et al.*, 2010). Therefore, the chronic inflammatory state observed in HIV and PE may be aggravated further due to excessive complement activation.

In PE, different complement components are upregulated, suggesting an increase in complement activation, and this can lead to the opsonisation and greater number of HIV virions in HIV-infected women (Alqudah and Yaseen, 2016). The increase in inflammatory cytokines by pro-inflammatory T cells and a decrease in anti-inflammatory and regulatory cytokines, observed in PE, creates an imbalance leading to chronic immune activation (Rossheim *et al*., 2016). HIV infection also creates a similar chronic immune activation with the use of HAART (Harman *et al.*, 2016). Excessive or dysregulated complement activation can lead to inflammatory pathology (Rossheim *et al.*, 2016). Excessive or dysregulated complement activation enlists leukocytes (Lynch and Salmon, 2010), which release pro-inflammatory

cytokines and anti-angiogenic mediators, resulting in an inflammatory state observed in PE (Rossheim *et al.*, 2016).

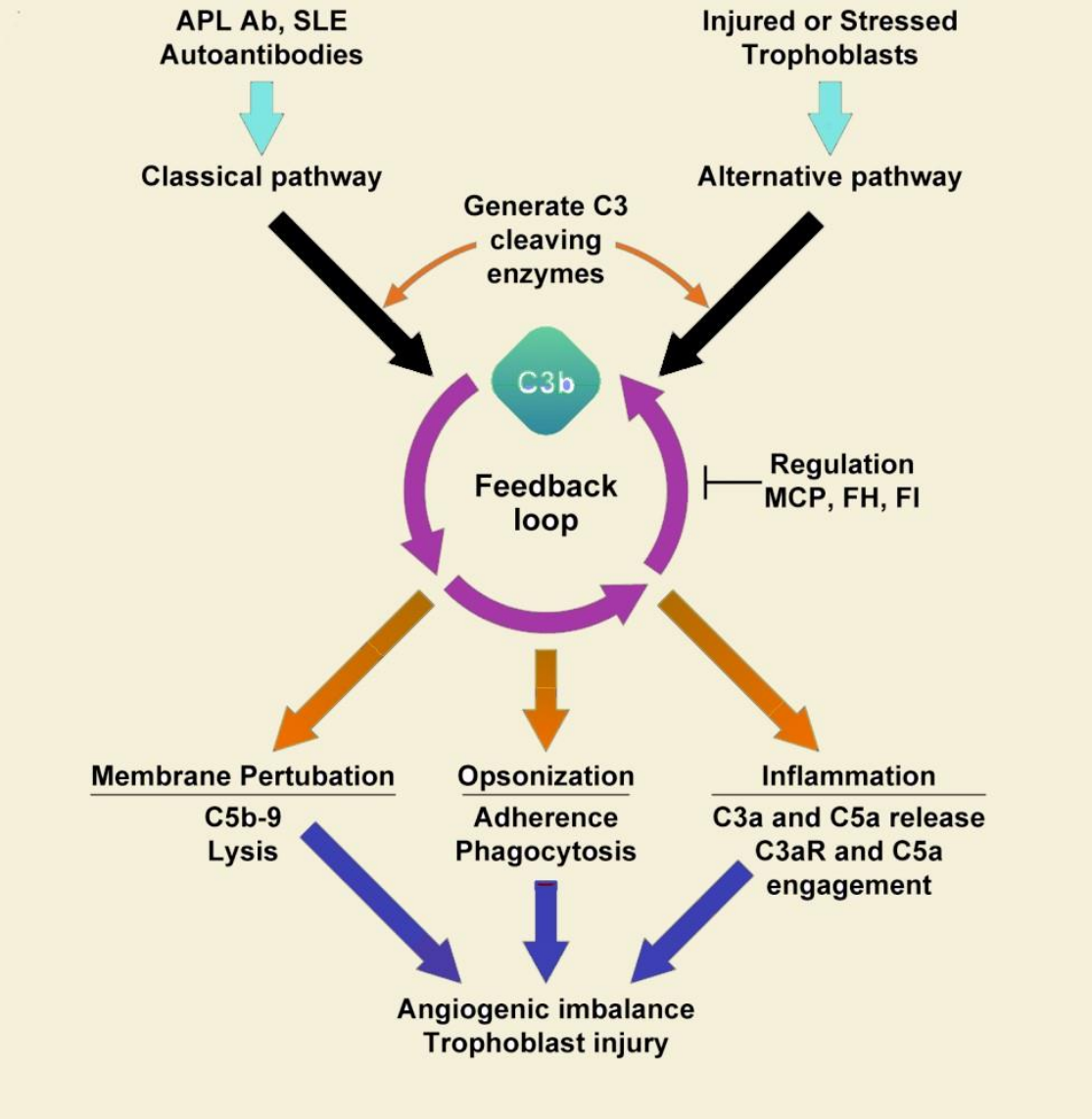

Figure 1.3: Schematic diagram on the role of the complement system in preeclampsia. The pathogenesis of preeclampsia is not fully elucidated; however, there is a potential link between complement dysregulation and angiogenic imbalance. Complement dysregulation leads to an elevated release of complement components, which cause excessive inflammation, and this can lead to angiogenic imbalances and trophoblast injury. Adapted from (Lynch and Salmon, 2010)

1.5 Adipsin

Adipsin, commonly known as Complement Factor D, is a serine protease synthesised and released by adipose tissue. It activates the alternative pathway of the complement system, thus enabling the body's natural defence against infections (Poveda *et al.*, 2016). Adipsin activates complement factor B and causes C3bBb formation (Morgan and Holmes, 2000).

More specifically, complement component (C3) is cleaved by spontaneous hydrolysis of C3 convertase enzyme complex in C3b and C3a (Podos *et al*., 2018). Lynch et al., (2012) correlated the increase of C3, together with obesity as a higher risk factor for developing PE. C3b forms a complex with factor B. Adipsin cleaves Factor B into Ba and Bb fragments. Factor Bb binds to C3b to form the C3bBb complex (figure 1.4) (Godahewa *et al*., 2016). C3bBb complex, acts as C3 convertase, to amplify the complement cascade (Podos *et al.*, 2018). Additionally, Adipsin plays a part in the formation of the MAC and generation of numerous signalling molecules of the complement system (Gómez-Banoy *et al*., 2019).

Adipsin is insulin-sensitive and was found to increase insulin levels in response to glucose and bring about homeostasis in adipose tissue (Lo *et al*., 2014). Several studies have revealed that Adipsin plays an important role in sepsis (Dahlke *et al*., 2011) and ischemic reperfusion (Stahl *et al*., 2003). These conditions cause excessive inflammation and releasing pro-inflammatory cytokines, similarly observed in PE (Ouchi *et al*., 2011). This will therefore cause the complement system to be activated, to regulate the excess inflammation (Derzsy *et al*., 2010), thus Adipsin levels will be increased.

Adipsin (Factor D)

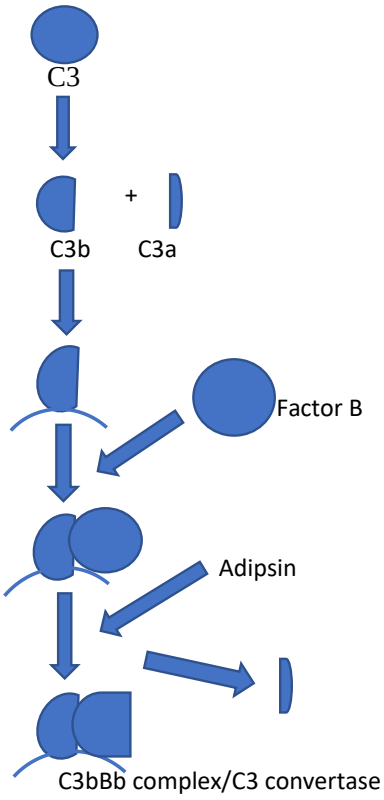

Figure 1.4: Illustration of the action of Adipsin in the complement system. C3 is spontaneously cleaved to form C3a and C3b. Adipsin cleaves Factor B to Ba and Bb. Factor Bb binds to C3b and forms the C3bBb complex, which acts as C3 convertase, to amplify the complement cascade. Adapted from Ruiz-Ojeda., (2018).

Plasma Adipsin levels were elevated in obese individuals, positively correlated to BMI and insulin resistance (Maslowska *et al.*, 1999). An increase in BMI leads to a greater risk of developing PE (Roberts *et al*., 2011) and obesity. Additionally, obesity results in an increase in complement components (Moreno-Navarrete and Fernandez-Keal, 2019), Adipsin being one such. In a study by Sivakumar et al., (2013), elevated Adipsin levels were observed in obese pregnant women. Furthermore, the study showed Adipsin positive areas in particular cells (Hofbauer cells), spread broadly in the placenta, specifically the perivascular area.

In the study conducted by Takeshita et al., (2010), changes in Adipsin concentrations in placentas of mouse abortuses was determined. Adipsin immunoreactivity was recorded to be elevated at the junctional and labyrinth zones of the abortion placenta sites. This evidence suggests that Adipsin may play a role in the up-regulation of complement activity in the placenta, and this up-regulation is implicated in complement dysregulation in PE (Kusakabe *et al.*, 2008).

Since there is no cure for PE, early diagnosis and treatment are crucial for sustaining pregnancy. A simple, rapid, and inexpensive assay is needed. This test should also be specific and sensitive for PE diagnosis. Urinalysis is one such test that meets these requirements, and Wang *et al.*, (2014) identified urinary adipsin protein as a potential biomarker for PE diagnosis. Urinary Adipsin was found to be best correlated with the 24-hour urine protein. Urinary Adipsin concentrations were elevated in preeclamptic women compared to non-preeclamptic and healthy non-pregnant women. This evidence suggests that urinary Adipsin could be used as a rapid diagnostic test and biomarker for PE. Additionally, it was reported that in some pregnant women who had high Adipsin concentrations, the babies were delivered prematurely and newborn babies tended to be lower in body weight, factors associated with PE (Wang *et al.*, 2014).

1.6 Complement component C9

Complement component 9 (C9) is a membrane protein used in the hosts' defence against pathogens. It is also involved in the pathogenesis of a number of liver disorders, liver injury and repair (Joller *et al.*, 2011; Wagner and Frank, 2010). C9 functions as the pore-forming component of the membrane attack complex (MAC), (figure 1.5). The MAC comprises of C5, C6, C7, C8, and C9 components and targets bacteria and envelops parasites and viruses (Dudkina *et al.*, 2016). C9 is also able to form poly-c9 structures, which closely resemble the MAC pore. During MAC formation, numerous copies of C9 are recruited in sequence to membrane-associated C5b8, to form a pore and thus to cause lysis (Spicer *et al.*, 2018).

The MAC also causes numerous physiologic changes, including apoptosis and the release of pro-inflammatory cytokines (Vlaicu *et al*., 2013). The release of pro-inflammatory cytokines (TNF-α, IL-2), causes excessive inflammation resulting in greater complement activation

(Regal *et al*., 2017). Brewster *et al*., (2008) showed increased levels of the C5b-9 MAC in PE, indicating excessive complement activation and hyper inflammation, like that in PE.

According to Sun et al, (2017), the complement signalling pathways were observed to be down-regulated in HIV co-infected patients. This is most likely due to the dampening effects of HIV on the immune system (Kalumba *et al.*, 2013).

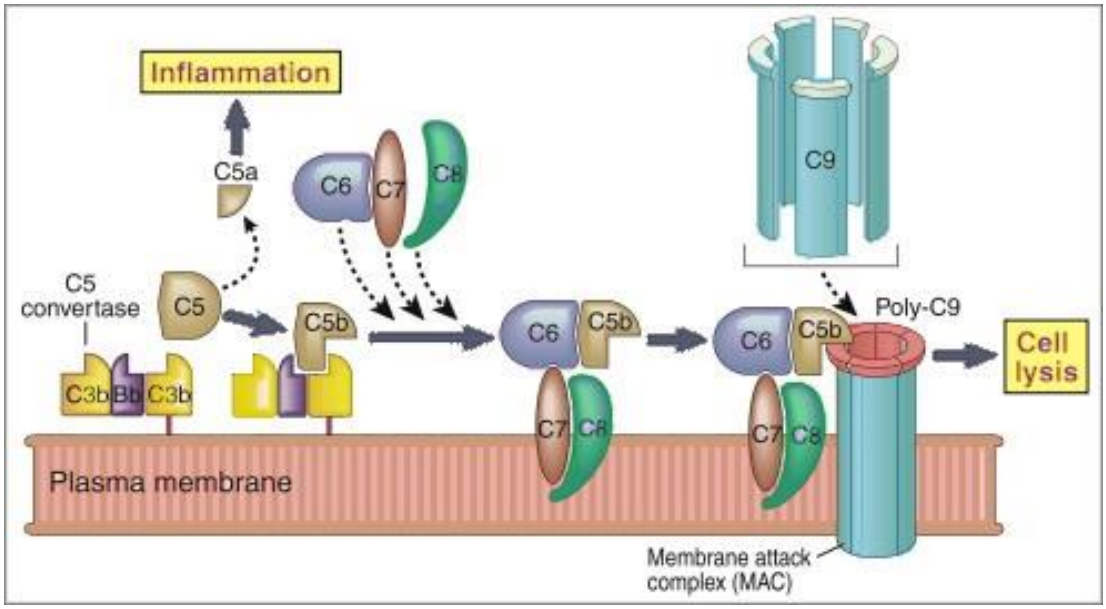

Figure 1.5: Schematic diagram on the function of complement component C9. C9 recruits complement components C5-8 to form a pore in the membrane attack complex, which causes lysis to occur. Adapted from (Andrade *et al*., 2017).

The synthesis and release of excessive amounts of biologically active products during the complement cascade activation due to PE may cause tissue damage via the MAC (Girardi *et al*., 2006). The fetal-maternal interface is potentially a target of the complement system, where its physiological function may cause damage (Le Bouteiller *et al.*, 2003). The vascular changes to the spiral arteries in PE, favours complement activation, with the possibility of placental damage (Bulla *et al*., 2012). Several studies demonstrated the late complement components (C5, C6 and C9) are found in the placenta (Girardi *et al*., 2006). The study by Rampersad *et al*., (2008), also reported that C5b-9 MAC on trophoblasts was related to fibrin deposits in the sites of villous injury in PE. Complement C9 was found on the trophoblast's membrane (Romero *et al*., 2007); this suggests that C9 and the complement system may be activated

during the vascular remodelling process in PE. There is still insufficient evidence on C9 and its function to HIV-associated PE.

Therefore, this study examines the concentration of Adipsin and C9 in the duality of HIV infection and PE stratified by pregnancy type and HIV status.

1.7 Hypothesis

Adipsin and C9 would be altered by HIV infection and preeclampsia

1.7.1 Aims of study

To determine the immunoexpression of Adipsin and C9 in the duality of HIV and Preeclampsia

1.7.2 Specific objectives

2. This study will compare the concentration of Adipsin and C9 in normotensive pregnancies compared to preeclamptic pregnancies irrespective of HIV status, using the Bioplex immunoassay procedure.
3. This study will compare the concentrations of Adipsin and C9 by HIV status regardless of pregnancy type, using the Bioplex immunoassay procedure.
4. This study will compare the concentration of Adipsin and C9 across the study population.
5. This study will compare patient demographics with Adipsin and C9 concentration across the study population

CHAPTER 2

Citation**:**

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Human Immunology

THE FUNCTION OF ADIPSIN AND C9 PROTEIN IN THE COMPLEMENT SYSTEM IN HIV-ASSOCIATED PREECLAMPSIA

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Abstract:	Objective In preeclampsia, there are excessive complement components expressed due to increased complement activation; therefore, this study investigated the concentration of adipsin and C9 in HIV-associated preeclampsia. Method The study population (n=76) was stratified by pregnancy type (normotensive pregnant and preeclampsia) and by HIV status. Serum was assayed for the concentration of adipsin and C9 using a Bioplex immunoassay procedure. Results Maternal weight did not differ ($p = 0.1196$) across the study groups. The concentration of adipsin was statistically different between the PE vs normotensive pregnant groups, irrespective of HIV status ($p = 0.0439$). There was no significant difference in adipsin concentration between HIV-negative vs HIV-positive groups, irrespective of pregnancy type ($p = 0.6290$). Additionally, there was a significant difference in adipsin concentration between HIV negative normotensive vs HIV negative preeclampsia ($p < 0.05$), as well as a difference between HIV negative preeclampsia vs HIV positive preeclampsia ($p < 0.05$). C9 protein expression was not statistically different between the normotensive and PE groups, regardless of HIV status ($p = 0.5365$). No statistical significance in C9 expression was found between HIV positive vs HIV negative groups, regardless of pregnancy type ($p = 0.3166$). Similarly, no statistical significance was noted across all study groups ($p = 0.0774$). Conclusion This study demonstrates that there is a strong correlation between the up-regulation of adipsin and PE and that adipsin is a promising biomarker to use as a diagnostic tool for PE.
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THE FUNCTION OF ADIPSIN AND C9 PROTEIN IN THE COMPLEMENT SYSTEM IN HIV-ASSOCIATED PREECLAMPSIA

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Abstract:

Objective: In preeclampsia, there are excessive complement components expressed due to increased complement activation; therefore, this study investigated the concentration of adipsin and C9 in HIV-associated preeclampsia.

Method: The study population (n=76) was stratified by pregnancy type (normotensive pregnant and preeclampsia) and by HIV status. Serum was assayed for the concentration of adipsin and C9 using a Bioplex immunoassay procedure.

Results: Maternal weight did not differ (p = 0.1196) across the study groups. The concentration of adipsin was statistically different between the PE *vs* normotensive pregnant groups, irrespective of HIV status (p = 0.0439). There was no significant difference in adipsin concentration between HIV-negative *vs* HIV-positive groups, irrespective of pregnancy type (p = 0.6290). Additionally, there was a significant difference in adipsin concentration between HIV negative normotensive *vs* HIV negative preeclampsia ($p < 0.05$), as well as a difference between HIV negative preeclampsia *vs* HIV positive preeclampsia ($p < 0.05$). C9 protein expression was not statistically different between the normotensive and PE groups, regardless of HIV status (p =0.5365). No statistical significance in C9 expression was found between HIV positive *vs* HIV negative groups, regardless of pregnancy type (p = 0.3166). Similarly, no statistical significance was noted across all study groups (p= 0.0774).

Conclusion: This study demonstrates that there is a strong correlation between the up-regulation of adipsin and PE and that adipsin is a promising biomarker to use as a diagnostic tool for PE.

Keywords: Preeclampsia, HIV, Adipsin, C9

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Introduction

Hypertensive disorders of pregnancy (HDP) and non-pregnancy related infections (HIV infection, TB) are the most common causes of maternal mortality in South Africa (Moodley *et al.*, 2019). Hypertensive disorders in pregnancy account for approximately 18% of all maternal deaths in South Africa (Moodley *et al.*, 2018). In high-income countries, the prevalence of HDP is 5-10%, the frequency being higher in low-middle-income (LMIC) countries (Naicker *et al.*, 2019). Preeclampsia (PE) is a complex pregnancy-specific disorder, accounting for 500000 fetal and neonatal deaths and approximately 70 000 maternal deaths worldwide (Gathiram and Moodley, 2016). It is diagnosed by new-onset hypertension (BP≥140mmHg or ≥90mmHg diastolic) after 20 weeks of gestation, with/without proteinuria (≥300mg) (Brown *et al.*, 2018). Preeclampsia is accompanied by multiple organ dysfunction and severe maternal complications that may include-the liver, kidney, etc. and hemolysis, elevated liver enzymes, and low platelet count referred to as the "HELLP" syndrome (Phipps *et al.*, 2016) (Brown *et al.*, 2018). In South Africa, the prevalence of PE is 12% (Onyangunga *et al.*, 2019). The World Health Organization (WHO) reports a significant disparity in the prevalence of PE in high-income countries compared to LMICs [1.6% versus 1.8-16.7% respectively] (WHO., 2019).

Preeclampsia is a heterogeneous disease and may be divided into two main subtypes, viz., early and late-onset PE (Staff *et al.*, 2013). The clinical signs for early-onset PE (EOPE) occur before 33 gestational weeks, whilst those of late-onset PE (LOPE) appears after 34 weeks of gestation. Late-onset PE constitutes the majority (>80%) of cases (Gathiram and Moodley, 2016). The early-onset type is associated with abnormal placentation and correlates with high maternal and fetal morbidity and mortality rates (Von Dadelszen *et al.*, 2003). The pathogenesis of PE is not fully understood as yet. However, it is generally accepted that inadequate myometrial spiral artery remodelling due to deficient migration of extravillous trophoblast (EVT) cells is involved in the pathophysiology of PE. (McNally *et al.*, 2017). During normal pregnancy, the trophoblast begins migration from the tips of anchoring villi through the decidua and the myometrium accompanied by spiral artery remodelling (Maître, 2017). In PE, there is shallow trophoblast invasion of the maternal myometrial spiral arteries, which leads to a reduction in the diameters of spiral arterioles resulting in decreased blood flow and subsequent placental hypoxia and fetal growth restriction (Li *et al.*, 2015).

The human immunodeficiency virus (HIV) is a retrovirus that invades a host, impairing cellular immunity and increasing one's susceptibility to opportunistic pathogens (Maartens *et al.*,

2014). Human immunodeficiency virus infection is a global pandemic, with 38 million people living with and 1.7 million people newly infected with HIV at the end of 2019 (UNAIDS, 2020). South Africa has a population of 7.97 million people (13.5%) infected with HIV (SA STATS, 2019), of which 4.7 million are women in their reproductive age (UNAIDS, 2020). Around 30% of South African pregnant women are infected with HIV, with the province of KwaZulu-Natal having a prevalence of approximately 37% (Kalumba *et al.*, 2013). The association between HIV infection and PE originates from opposing immune responses, as PE induces an exaggerated immune response (Than *et al.*, 2008), whilst HIV dampens the immune response (Kalumba *et al.*, 2013).

The complement system forms part of the innate immune system and consists of numerous plasma and cell membrane proteins (Merle *et al.*, 2015). The complement system is initiated by three main pathways viz., the classical pathway (CP), the lectin pathway (LP), and the alternative pathway (AP), each culminating in one common pathway (Noris and Remuzzi, 2013). The function of the complement system is the opsonization of target surfaces, induction of pro-inflammatory responses, and the lysis of cells and pathogens. It is also vital in the defence of the host by clearing apoptotic cells, injured tissue, and immune complexes, thus contributing to physiological homeostasis (Lokki *et al.*, 2014).

During normal pregnancy, for the mother to tolerate the semi-allogenic fetus, the immune system mediates a T helper 2 (Th2) response modulated by cytokines such as IL-10 (Saito *et al.*, 2007). Simultaneously, a T helper 1 (Th1) pro-inflammatory response is mediated by cytokines such as TNF-α and IL-2 (Otun *et al.*, 2011). Th1/Th2 response is critical for trophoblast invasion, parturition and protection against pathogens (Osman *et al.*, 2003) and the balance between Th1 and 2 response is essential to ensure a successful pregnancy. However, during pregnancy, complications such as PE, a shift from Th2 to Th1 occurs (Perez-Sepulveda *et al.*, 2014). This is due to cell-mediated immune responses that contribute to an overall increase in the maternal systemic inflammatory response (Saito and Sakai, 2003).

Adipsin, commonly known as Complement Factor D, is a serine protease that activates the AP. Adipsin is synthesized and released by adipose tissue. Adipsin levels are higher in PE compared to normotensive pregnant women (Poveda *et al.*, 2016). This upregulation of adipsin levels elicits complement activation, leading to an exaggerated inflammatory response (Regal *et al.*, 2017).

Similar to adipsin, complement component 9 (C9) is a membrane protein used as a defence against pathogens. Complement component 9 functions as the pore-forming component of the membrane attack complex (MAC). During MAC formation, numerous copies of C9 are recruited in sequence to membrane-associated C5b8, to form a pore and thus to cause lysis (Spicer *et al.*, 2018). Moreover, the complement signalling pathways are downregulated by HIV infection (Guha and Ayyavoo, 2013). This is most likely due to the HIV attacking the CD4+ T cells and thus dampening the immune system (Balasubramaniam *et al.*, 2019).

Notwithstanding this observation, there is a lack of research with regards to the function of adipsin and C9 in the duality of HIV infection and PE.

Therefore, this study examines the expression of adipsin and C9 in the duality of HIV infection and PE stratified by pregnancy type and HIV status, using the Bioplex multiplex immunoassay procedure.

Materials and methods:

This study was approved by the Biomedical Research Ethics Administration, University of KwaZulu-Natal, Durban, South Africa. Ethics No: BCA 338/17.

This was a prospective study that utilized retrospectively collected samples stored at -80°C. The study population (n=76) was used to determine a moderate effect size of 0.66 between the groups. The study population was recruited from a large regional hospital in Durban, South Africa. Two groups of normotensive pregnant and preeclamptic women were stratified by their HIV status into, HIV negative normotensive pregnant women, and HIV positive normotensive pregnant women, HIV negative PE and HIV positive PE women. To detect a large effect size of 0.95, a sample size of 19 was required in each group.

Bioplex immunoassay:

A MILLIPLEX MAP^{TM} Human Complement Panel one was performed according to manufacturer's instructions (Millipore by Sigma-Aldrich, catalogue no: HCMP1MAG-19K). The standards were prepared in a 1:100 dilution series. In a 96 well plate, adipsin and C9 capture antibody-coupled magnetic beads were added to each well and washed twice. Standards, samples and blanks were then added into their designated wells and left to incubate before washing three times. Thereafter, a biotinylated detection antibody was pipetted into each well and allowed to incubate. The plate was then washed three times before adding streptavidin-phycoerythrin throughout the wells. Finally, the plate was washed for a further three times, before resuspending each well with assay buffer. The plate was then ready to be placed into the Bio-Plex^{TM} system for reading. The Bio-Plex1MAGPIXTM Multiplex Reader (Bio-Rad Laboratories Inc., USA) was utilized to read the experiment plate. Bio-Plex Manager^{TM} software version 4.1 was used to obtain the data from the multiplex analysis.

Statistical analysis

Data was analysed using GraphPad Prism 5.00 for Windows (GraphPad Software, San Diego California USA). Results are represented as the median and interquartile range (IQR). A T-test for normality revealed parametrically distributed data. A Mann-Whitney U test was used to determine statistical significance according to pregnancy type (normotensive *vs* PE) and HIV (negative *vs* positive). Statistical significance was determined across all groups, using a one-way ANOVA test, a Kruskal-Wallis test in combination with Dunn's multiple comparison *post hoc* test was used. Statistical significance was reported as $p < 0.05$.

Results

Patient demographics and clinical characteristics

The patient demographics and their clinical characteristics are shown in table 1. No statistical significance was reported in weight (p = 0.1196) across the study groups. Statistical significance was reported for gestational age, systolic blood pressure (BP) and diastolic BP (p < 0.0001), as well as parity and maternal age (p < 0.05) across study groups.

Table 1: Demographic data and clinical profile of participants across all study groups.

	Normotensive HIV Negative n=19	Normotensive HIV Positive n=19	Preeclamptic HIV Negative n=19	Preeclamptic HIV Positive n=19	*p*-value
Maternal Body Weight (kg)	74 (22)	81 (28)	90 (46)	83 (36)	0.1196
Gestational Age (weeks)	37 (9)	25 (14)	24 (10)	23 (10)	0.0008 ***
Parity	1 (1)	2 (1)	1 (2)	2 (1)	0.0085 *
Systolic blood pressure (mmHg)	109 (20)	112 (16)	146 (14)	147 (20)	<0.0001 ***
Diastolic blood pressure (mmHg)	65 (13)	72 (14)	92 (9)	97 (13)	<0.0001 ***
Maternal Age (years)	25 (4)	31 (11)	29 (16)	34 (14.5)	0.0304 *

Patient demographics amongst study groups (n=76). Results are represented as the median (IQR), * p < 0.05 *** p < 0.001

Serum concentrations of adipsin

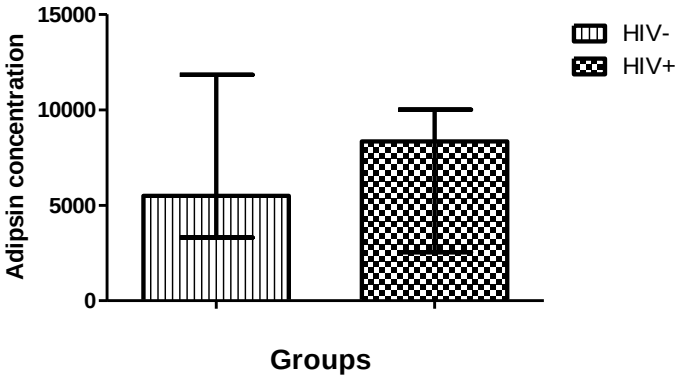
Pregnancy type: The concentration of Adipsin was statistically different between the PE group (median=10239 pg/mL, IQR=6632 pg/mL) and normotensive group (median=8335 pg/mL, IQR=5485 pg/mL), irrespective of HIV status (Mann-Whitney U=652, p = 0.0439; figure 1A)

HIV status: There was no significant difference in adipsin concentrations between HIV-negative (median=5498 pg/mL, IQR=8532 pg/mL) *vs* HIV-positive groups (median=8345 pg/mL, IQR=7453 pg/mL), irrespective of pregnancy type (Mann-Whitney U=675, p = 0.6290; figure 1B).

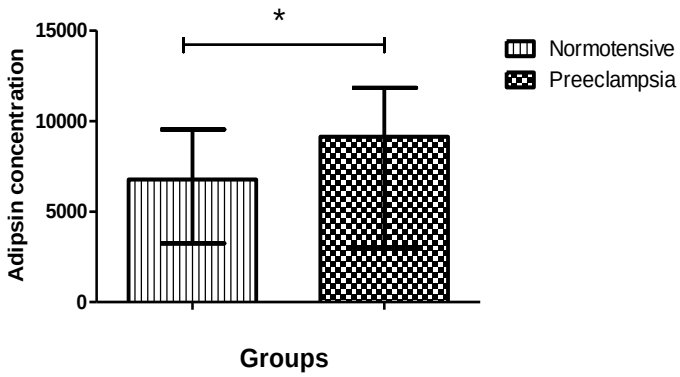
Across all groups: There was a significant difference in adipsin concentration between HIV negative normotensive (median=3607 pg/mL, IQR=5947 pg/mL) *vs* HIV negative

preeclampsia (median=11972 pg/mL, IQR=3240 pg/mL; $p < 0.05$), as well as a difference between HIV negative preeclampsia (median=11972 pg/mL, IQR=3240 pg/mL) *vs* HIV positive preeclampsia (median=4849 pg/mL, IQR=9618 pg/mL; $p < 0.05$). No differences were found amongst the other groups of pregnant women (Kruskal-Wallis H=11.68, $p = 0.0086$; figure 1C).

1A



1B



1C

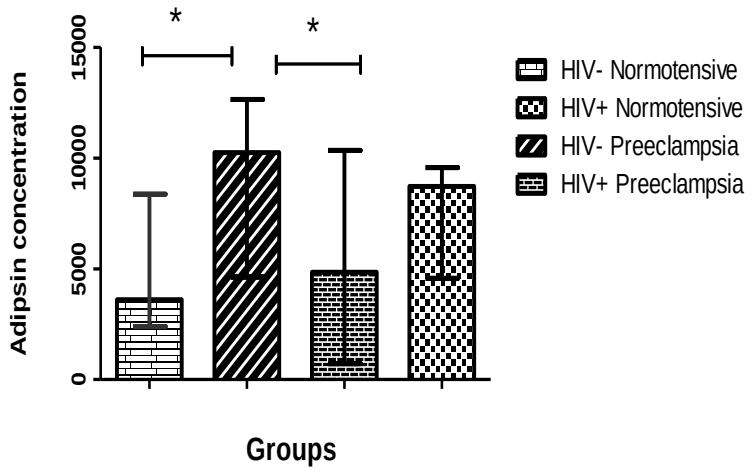


Figure 1: The Histogram illustrating serum concentration of adipsin (A) Preeclamptic vs Normotensive groups; (B) HIV positive vs HIV negative groups; (C) Across all groups. Data is represented by median and interquartile range. *p value < 0.05.

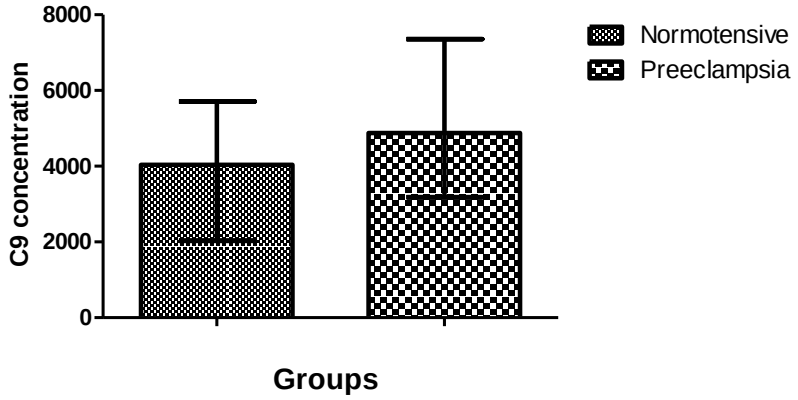
Serum concentrations of C9

Pregnancy type: C9 protein expression was not statistically different between the normotensive (median=4039 pg/mL, IQR=3683 pg/mL), and PE groups (median=4876 pg/mL, IQR=4169 pg/mL), regardless of HIV status (Mann-Whitney U=13.50, p =0.5365; figure 2A).

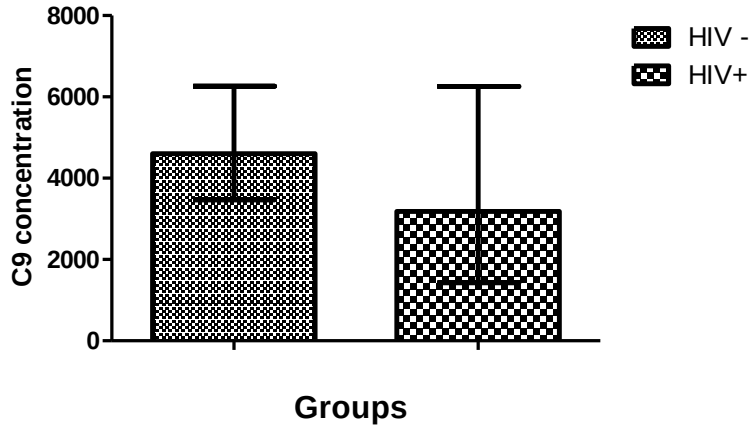
HIV status: No statistical significance in C9 expression was found between HIV positive groups (median=3180 pg/mL, IQR=4830 pg/mL) *vs* HIV negative groups (median=4610 pg/mL, IQR=2791 pg/mL), regardless of pregnancy type (Mann-Whitney U=13.50, p = 0.3166; figure 2B).

Across all groups: Similarly, no statistical significance was noted across all study groups (Kruskal-Wallis H=9.514, p= 0.0774; figure 2C).

2A



2B



2C

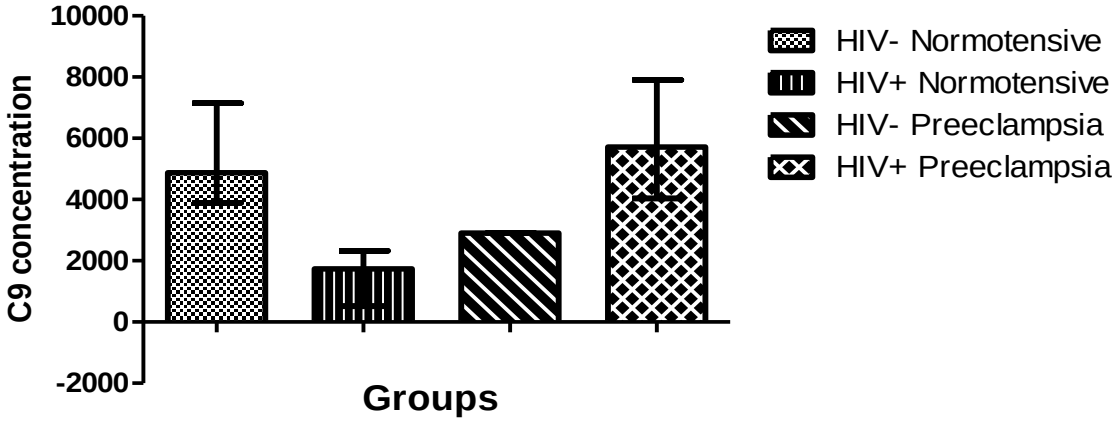


Figure 2: Histogram illustrating the serum concentration of C9; (A) Preeclamptic vs Normotensive groups; (B) HIV positive vs HIV negative groups; (C) Across all groups. Data is represented as median and interquartile range.

Discussion

The main finding of this novel study was the significant up-regulation of adipsin in the preeclamptic compared to the normotensive pregnant group, regardless of HIV status [figure 1A; $p = 0.0439$]. Poveda et al. (2016) reported similar findings in PE compared to normotensive pregnancy, albeit late in pregnancy (Poveda *et al.*, 2016). Notably, our study reflects adipsin concentration at term. Moreover, increased serum adipsin levels have been previously reported to correlate with an elevation of urinary adipsin in PE patients (Wang *et al.*, 2014; Wang *et al.*, 2016). Preeclampsia is characterized by glomerular endotheliosis (Gangaram *et al.*, 2005). The latter two studies suggest that urinary adipsin may be used as a predictor test value for diagnosing PE development.

Adipsin is a serine protease synthesized and released by adipose tissue; in our study, maternal body weight was similar between normotensive and preeclamptic groups. As mentioned earlier, adipsin forms C3 convertase, thereby increasing complement activation (Choy and Spiegelman, 1996). Off note, adipsin generates an acylation-stimulating protein (C3adesArg/ASP) (Morgan and Holmes, 2000) to stimulate triglyceride synthesis (Cianflone *et al.*, 2003). During pregnancy, triglyceride levels are a risk factor for the development of PE and preterm birth (Ghio *et al.*, 2011). Acylation-stimulating protein signalling also activates the P13K, protease kinase C, Akt and MAPK/ERK1/2 pathways, which are vital for trophoblast cell migration

This increase in adipsin levels may cause excessive complement activation, which may lead to pro-inflammatory and chemotactic anaphylatoxins being released, which have the potential to cause excessive inflammation in PE (Regal *et al.*, 2017). Several studies have correlated an increase in adipsin concentrations with body mass index (BMI) in PE compared to normotensive pregnant women; unfortunately, BMI was not available for our study population (Wang *et al*., 2014; Takeshita *et al*., 2010). An increase in BMI is associated with obesity and insulin resistance (Saad *et al.*, 2016). It is noteworthy that metabolic conditions such as obesity and diabetes results in chronic inflammation, a hallmark feature in PE (Rossheim *et al*., 2016). It is, therefore, plausible that the elevation in adipsin may be due to metabolic changes that occur in pregnancy and in PE development (Perez-Sepulveda *et al.*, 2014). In a recent study by He *et al*. (2020), a dysregulation of the alternative pathway in PE during the first trimester was observed, but this returned to normal pregnancy levels in the second trimester; adipsin in our study reflected term pregnancy.

Our study also found that serum adipsin concentrations were not influenced by HIV status, regardless of pregnancy type. The similarity in adipsin between the groups may be attributed to antiretroviral drug usage, a standard of care practice for HIV infection in South Africa. The effects of HAART are those of re-establishing the immune system and therefore, immune response (Landi *et al*., 2014). It is plausible that a compromised immune system due to HIV infection triggers a compensatory response of adipsin, to increase complement activation. This change in adipsin concentrations is most likely due to the immunological changes of the complement system, brought about by HIV infection (Yu, 2010), in tandem with HAART administration. Also, protease inhibitors impede triglyceride accumulation via modulation of ASP production (Soliman *et al*., 2009).

This novel study also demonstrates no significant difference in serum C9 concentration between normotensive and PE, irrespective of HIV status. In contrast, several other studies report higher C9 concentrations in PE compared to healthy pregnancies (Burwick *et al*., 2020; Derzsy *et al*., 2010). It is plausible that these results are due to excessive complement activation, as a result of the hyper-inflammatory state caused by PE. C3(H_2O) is cleaved by C9 to generate C3 convertase. This cleavage is slower without properdin, a positive regulator of AP convertase (Hourcade, 2006)

This study reports similar C9 concentrations based on HIV status. C9 is involved in the formation of the membrane attack complex, a part of our innate immune response that leads to the killing of foreign pathogens. The similar levels may be attributed to antiretroviral therapy in our study (Dudkina *et al*., 2016). The administration of HAART plays a conflicting role in these results, as it is known to reconstitute the immune system (Landi *et al*., 2014) whilst other studies indicate the opposite (Umar *et al.*, 2020). Also, HIV escapes complement attack by exploiting complement regulators such as CD55, and CD59 (Liu *et al*., 2014). Also, cancer cells reduce MAC concentration, is influenced by CD59 up-regulation, where it removes plasma membrane MAC via vesiculation (Shi *et al*., 2009). Moreover, patients with a deficiency of C9 protein are unable to form the MAC (Frank, 2000). Due to the lack of information on C9 in the duality of HIV-associated PE, further research is required.

One of the limitations of this study was the small sample size. Additionally, all the HIV-positive women were on HAART treatment, which may have confounded differences in analyte expression. The heterogeneity of the study population, which was not stratified into early and late-onset PE, may be seen as a limitation.

Conclusion

This study demonstrates a significant up-regulation of adipsin in PE compared to normotensive pregnancies, this finding correlates with the exaggerated inflammatory milieu of PE. Complement C9 protein was similar between pregnancy types. This similarity may emanate from properdin dysregulation or a genetic polymorphism. The concentration of adipsin and C9 proteins were not affected by HIV status due to the immune reconstitution effect of antiretroviral therapy. Furthermore, the up-regulation of adipsin in placental sites and urinary levels of PE in previous studies, in tandem with our findings, indicate the possibility of adipsin as a predictor value for PE development.

Declaration of interest

There are no conflicts of interest

Acknowledgements

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CHAPTER 3

3.1 Synthesis

3.1.1 Problem Identification

HIV infection remains one of the world's leading health concerns, with 38 million people living with and 1.7 million people newly infected with HIV at the end of 2019. Women and girls account for approximately 48% of all new HIV infections in 2019, and in Sub-Saharan Africa, women and girls account for 59% of all new infections. The prevalence of HIV infection in South Africa is 13.5% (SA STATS, 2019), of which 4.7 million are women in their reproductive age (UNAIDS, 2019). Approximately 30% of South African women who are pregnant are infected with HIV (Kalumba *et al*., 2013), with the province of KwaZulu-Natal having the highest prevalence. South Africa has the largest antiretroviral therapy (ART) rollout in the world (Nlooto and Naidoo, 2016). In contrast to HIV infection, ART reconstitutes the immune system (Kalumba *et al*., 2013).

In South Africa, HIV infection (31%) and hypertensive disorders of pregnancy (17%) account for the majority of maternal deaths (Moodley *et al*., 2018; Saving Mothers Report, 2017). Preeclampsia is a multisystemic disorder of pregnancy, accounting for 83% of HDP (Gathiram and Moodley, 2016). In normotensive pregnancies, adaptations of the innate and adaptive immune system are activated to ensure the survival of the fetus, whilst also protecting the mother (Pierik *et al*., 2019). During PE, however, there is deficient maternal tolerance or an altered immune response due to the increased release of monocytes and neutrophils, which results in excessive inflammation (Perez-Sepulveda *et al*., 2014).

The complement system is an integral part of the innate immune system and is comprised of various plasma proteins and cell membranes (Merle *et al*., 2015). Additionally, it is important in defence of the host by clearing injured tissue, immune complexes and acts as a mediator of the adaptive immune response (Lokki *et al*., 2014). During viral infection, the complement system is essential, as it is the first defence barrier to manage and maintain the spread of HIV. It also assists in the clearance and neutralisation of HIV (Posch *et al.*, 2011). Adipsin is a serine protease, that activates the alternative pathway of the complement system, therefore activating the body's natural defence against pathogens (Poveda *et al*., 2016). Complement C9 is a membrane protein, and the last of the complement components (Wagner and Frank, 2010). It is used in the hosts' defence against pathogens. C9 recruits other complement components (C5-C8) to form the membrane attack complex (MAC) (Dudkina *et al*., 2016), which causes several immunological changes including Apoptosis and the release of pro-inflammatory cytokines

(Vlaicu *et al*., 2013). These changes result in elevated complement activation, therefore excessive inflammation, as that in PE (Brewster *et al*., 2008). This study investigates the dysregulation of complement components, adipsin and C9 in HIV associated normotensive pregnant vs preeclamptic women, using a multiplex immunoassay technique.

3.1.2 Adipsin concentration in HIV associated pregnancy

The main finding of our study was that the concentration of adipsin was statistically different between the PE *vs* normotensive pregnant groups, irrespective of HIV status ($p = 0.0439$). Poveda et al. (2016) reported similar findings in PE compared to normotensive pregnancy, albeit late in pregnancy (Poveda *et al.*, 2016). Notably, our study reflects adipsin concentration at term. Moreover, increased serum adipsin levels have been previously reported to correlate with an elevation of urinary adipsin in PE patients (Wang *et al.*, 2014) (Wang *et al.*, 2016).

Adipsin is a protease produced by adipose tissue and activates the alternative pathway. In our study, the maternal weight did not differ ($p = 0.1196$) across the study groups. This similarity may be attributed to the immune-reconstitutive effect of ART's (Maharaj *et al.*, 2017). Nonetheless, it is plausible that the upregulation of adipsin in PE in our study may due to the exaggerated inflammatory state of PE. Also, adipsin produces an acylation-stimulating protein (C3adesArg/ASP) (Morgan and Holmes, 2000) that synthesis triglycerides (Cianflone *et al.*, 2003). Acylation-stimulating protein signals via the P13K, protease kinase C, Akt and MAPK/ERK1/2 pathways that mediate trophoblast cell invasiveness. Notably, triglyceride elevation is a risk factor for complications of pregnancy and intra-uterine growth retardation and small for gestational age infants. (Ghio *et al.*, 2011).

In our study, there was no significant difference in adipsin concentration between HIV-negative *vs* HIV-positive groups, irrespective of pregnancy type ($p = 0.6290$). This similarity may emanate from antiretroviral drug usage, a standard of care practice for HIV infection in South Africa. Notably, HAART re-constitutes the immune system and therefore, immune response (Landi, 2014). HIV infection triggers complement activation of adipsin (Yu, 2010), in conjunction with HAART administration. Furthermore, protease inhibitors regulate ASP secretion, thereby preventing triglyceride build-up (Soliman *et al*., 2009).

3.1.3 C9 concentration in HIV associated pregnancy

In our study, C9 protein concentration was not statistically different between the normotensive and PE groups, regardless of HIV status (p =0.5365). In contrast, several other studies report higher C9 concentrations in PE compared to healthy pregnancies (Burwick *et al*., 2020; Derzsy *et al*., 2010). Notably, these results are due to excessive complement activation, emanating from the hyper-inflammatory environment of PE. The cleavage of C3(H_2O) by C9 to produce C3 convertase. Properdin, a positive regulator of AP convertase, reduces this reaction (Hourcade, 2006). Complement C9 was found on the trophoblast membrane of PE patients, suggesting that the complement system and C9 may play a role during the vascular remodelling in PE (Romero *et al*., 2007). Additionally, urinary Adipsin levels were elevated in PE women, suggesting that Adipsin has the potential to be a biomarker for PE (Wang *et al*., 2016).

In our study, no statistical significance in C9 concentration was noted between HIV positive *vs* HIV negative groups, regardless of pregnancy type (p = 0.3166). C9 functions to create the membrane attack complex, which plays a role in the opsonization of foreign pathogens (HIV). The similar levels may be attributed to antiretroviral therapy in our study (Dudkina *et al*., 2016). Also, HIV escapes complement attack by exploiting complement regulators such as CD55, and CD59 (Liu F *et al*., 2014). A deficiency of the complement protein C9 results in the patient's inability to form MAC (Frank, 2000).

Similarly, no statistical significance of C9 was noted across all study groups (p= 0.0774) and may be attributed to ART, properdin dysregulation and genetic polymorphisms of complement components.

3.1.4 Limitations of the study

The limitations of this study include small sample size, the heterogeneity of the study population and the fact that all HIV-positive women received ART, which may have confounded analyte expression. Lastly, the PE group was not stratified by gestational age into early and late-onset subtypes.

3.1.5 Conclusion

This novel study demonstrates a significant up-regulation of adipsin in PE compared to normotensive pregnancies, regardless of HIV status. This finding correlates with the hyperinflammatory state of PE. Additionally, C9 was similar between normotensive pregnant and PE, irrespective of HIV status. This similarity is possibly due to properdin maladaptation,

or from a genetic variation. We also demonstrate that adipsin and C9 levels were not affected by HIV status due to the immune reconstitution effect of antiretroviral therapy. Furthermore, the up-regulation of adipsin in placental sites and urinary levels of PE in previous studies, in tandem with our findings, indicate the possibility that adipsin has a predictor value for PE development.

CHAPTER 4

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APPENDIX 1

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

04 June 2020

Prof T Naicker
Discipline of Optics and Imaging
School of Laboratory Medicine and Medical Sciences
naickera@ukzn.ac.za

Dear Prof Naicker

Title of Project: Exploring the pathogenesis HIV associate pre-eclampsia syndrome in a homogenous South African population group.
BREC Ref No.: BCA338/17

We wish to advise you that your letter received on 20 May 2020 to append the studies below to the above study has now been approved by a sub-committee of the Biomedical Research Ethics Committee

MMedSci	Rowen Govender	215023500	The role of complement component 4B (C4B) and complement factor I (CFI) in the duality of HIV infected preeclamptic women
MMedSci	Sumeshree Govender	21351694	The role of C5a and C2 protein in pre-eclampsia complicated by HIV infection.
MMedSci	Camille Naicker	214515577	The components C5 and Mannose- binding lectin (MBL) functionality in the complement system in relation to HIV and preeclampsia pregnant women in Durban, South Africa.
MMedSci	Mikyle David	216000603	The function of Adipsin and C9 protein in the complement system with relation to HIV-associated pre-eclampsia
MMedSci	Tashlin Abel	215013948	The regulation of SLK-1 and SFLT-4 and their involvement in Preeclamptic woman with HIV.
MMedSci	Omeshini Naiker	215028862	The role of angiostatin and PDGF in maintaining placental health in preeclamptic patients
MMedSci	Nqobile Mdlalose	216002159	The role of HER2 and HER 3 in HIV associated preeclampsia
MMedSci	Nitalia Naidoo	216013288	The role of osteopontin and neuropilin in HIV associated preeclampsia

The committee will be notified of the above approval at its next meeting to be held on 14 July 2020.

Yours sincerely

...

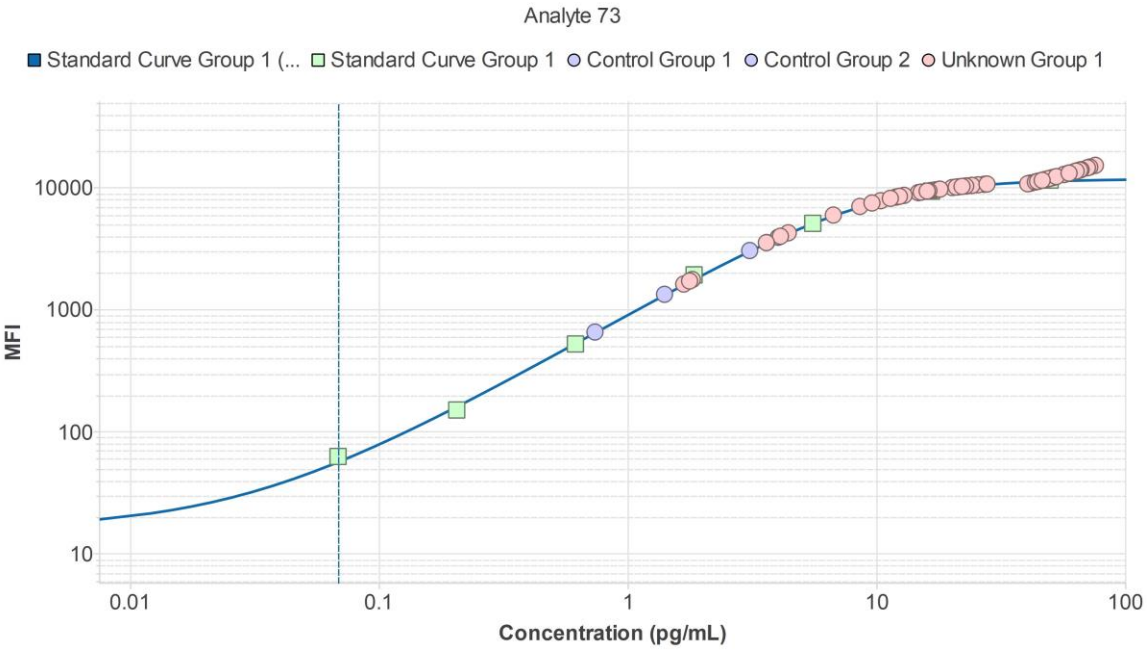
Ms A Marimuthu

(for) Prof D Wassenaar

Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

APPENDIX 2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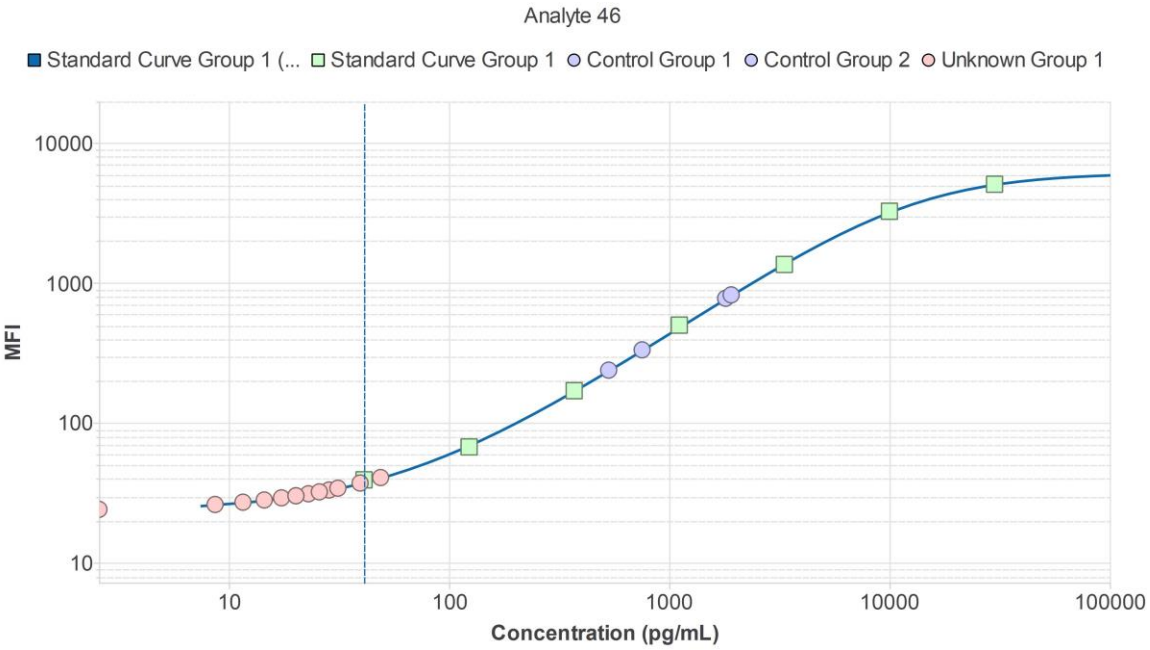
APPENDIX 3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