THE ROLE OF COMPLEMENT COMPONENTS C2 AND C5a 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 & Imaging Centre, Doris Duke Medical Research Institute, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa under the supervision of Professor T Naicker.

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DECLARATION

I, **Sumeshree Govender** declare that:

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

ii. This dissertation has not been submitted for any degree or examination at any other university.

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DEDICATION

First and foremost, I dedicate this thesis to God.

For the strength, guidance and spiritual support.

I also dedicate this work to my God-sent parents, Naughty, Indrani and sister, Deveshni.

Thank you for the unwavering love, guidance and encouragement that you have given me, cheering me when I was discouraged, helping me to succeed and instilling me with the confidence that I am capable of doing anything I put my mind to.

Thank you for everything.

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LIST OF ABBREVIATIONS

World Health Organization…….	WHO
Millennium Development Goals…….	MDG
Maternal Mortality Rate……..	MMR
Sustainable Development Goals……..	SDG
Hypertension Disorders of Pregnancy…….	HDP
Preeclampsia……...	PE
Hemolysis, elevated liver enzymes, low platelet count……...	HELLP
Human Immunodeficiency Virus……	HIV
South Africa……	SA
Highly Active Antiretroviral Therapy…….	HAART
Antiretroviral Therapy……	ART
Pro-inflammatory cytokine…….	Th1
Anti-inflammatory cytokine……...	Th2
Classical Pathway……...	CP
Alternative Pathway……	AP
Lectin Pathway……	LP
Mannose-binding lectin……...	MBL
Mannose-binding lectin serine protease 1……...	MASP-1
Mannose-binding lectin serine protease 2……...	MASP-2
Membrane Attack Complex……	MAC
Complement regulatory……...	Creg
Membrane cofactor protein…….	MCP
Decay-accelerating factor……	DAF
C1 esterase inhibitor……	C1NH

Vascular Endothelial Growth Factor……	VEGF
Placental Growth Factor……..……..	PIGF
Soluble Fms-like Tyrosine Kinase 1…….	sFlt-1
Soluble endoglin…….	sEng
Syncytiotrophoblast microvesicles…….	STBM
Dendritic cells…….	DC
Natural killer……...	NK
Complement component 2……..	C2
Complement component 5a…….	C5a
Polymorphonuclear neutrophils……..	PMN
Immunoglobulin M…….	IgM
Immunoglobulin G……..	IgG
Bacterial Lipopolysaccharides……..	LPS
Tumour Necrosis Factor- alpha……	TNF-α
Interleukin 6……	IL-6
Monocyte-derived macrophages…….	MDM
Systemic Lupus Erythematosus……...	SLE
Blood pressure…….	BP
Small interfering RNA……	siRNA
Complement component 5a receptor……...	C5aR
Desarginated Complement component 5a…….	$C5a^{desArg}$
Soluble Vascular Endothelial Growth Factor Receptor-1…….	sVEGFR-1

ABSTRACT

Background: In South Africa (SA), HIV infection and preeclampsia (PE) are the leading causes of maternal mortality and morbidity. In PE, immunologic maladaptation alters fetal tolerance. Our first line of defence against pathogens is controlled by the complement system, a central part of our innate immunity. In the complement cascade, complement component 5a (C5a) is a potent pro-inflammatory anaphylatoxin, whilst complement component 2 (C2) defends the onset of infections and immune complex removal. There is a dearth of information on these proteins in the synergy of HIV infection and PE development. In light of the high prevalence of HIV infection and PE in SA, the aim of this study was to determine the concentrations of C2 and C5a in HIV associated normotensive versus preeclamptic pregnancies.

Method: Post ethics approval, stored serum samples were obtained from 76 pregnant women and grouped according to pregnancy type, preeclamptic patients (n=38) and normotensive patients (n=38), this was further stratified by HIV status, normotensive HIV-positive (n=19), normotensive HIV-negative (n=19), preeclamptic HIV-positive (n=19), and preeclamptic HIV-negative (n=19). All HIV-infected patients received (Highly Active Antiretroviral Therapy) HAART. The expression of C5a and C2 was quantified using Bio-Plex multiplex immunoassay.

Results: Based on pregnancy type, a significant downregulation of C5a concentration was demonstrated in the preeclamptic *vs* normotensive pregnancies regardless of HIV status ($p = 0.0486$). There was no statistical significance in C5a concentration between the HIV-positive and HIV-negative groups, irrespective of pregnancy type ($p = 0.8002$). Furthermore, there was no significant difference in C2 levels between preeclamptic *vs* normotensive group, regardless of HIV status. Similarly, based on HIV status, no statistical significance regardless of pregnancy type was found ($p = 0.7469$).

Conclusion: This novel study demonstrates a significant decrease in the concentration of C5a in PE compared to normotensive pregnant women. This unexpected expression may be due to the rapid consumption of C5a in circulation, an altered affinity of C5a to its receptors or genetic polymorphisms. We also report similar C5a and C2 concentrations between HIV positive and HIV negative groups possibly reflecting the immune reconstitution effect of HAART. Complement dysregulation affects host innate defence in PE by exaggerating placental and fetal injury hence requires a large-scale study to evaluate individual proteins of the complement cascade in the synergy of HIV associated preeclampsia.

I-ABSTRACT

Umsuka: ENingizimu Afrika (SA), ukutheleleka ngesandulela ngculazi (HIV) kanye ne-preeclampsia (PE) yizimbangela ezihamba phambili zokushona kwabazithweleyo kanye nokugula kwabo. Kwi-PE ukungasebenzi kahle kwama sosha omzinba phecelezi i-*immunoligical maladaptation* kuguqula ukubekezelelana kwengane. Isiqalo sethu sokuvikela kwi-pathogen kulawulwe uhla olunconekayo, ingxenye yokuvikela ngaphakathi. Kukho lokuzivikela okuncomekayo, i-C5a yi-proinflammatory anaphylatoxin enamandla kakhulu, kanti iC2 ivikela ukutheleleka kokuqala Kanye nokususa okuyinkimbinkimbi yomzimba. Kukhona ukusweleka kwemininingwane yalawama-protheni yokuthi asebenza kanjan ekuthelelaneni kweHIV kanye nokuqala kwe PE. Ngokubeka sobala ukudlanga kokuthelelana nge HIV ne PE la NIngizimu Africa, injongo yaloluchungechunge lwaluwukuhlaziya ngokucophelela i-complment 2 (C2) kanye ne complement 5a (C5a) ukukhulelwa ngokujwayelekile kwabathelelene ngeHIV naba-preeclamptic.

Iziyathelo: Sekuphasisiwe, amasampula e-serum agciniwe athathwe kwabesifazane abakhulelwe abangamashumi ayisikhombisa nesithupha (76) futhi aqoqwa ngohlobo lokukhulelwa, iziguli ezi-preeclamptic (n = 38) kanye neziguli ezine gazi elishaya kahle (n = 38), loku kwaphinda kwabhekwa nange simo se-HIV, ezinegazi elishaya kahle zi HIV-positive (n = 19), ezinegazi elishaya kahle zi HIV-negative (n = 19), i-preeclamptic HIV-negative (n = 19) ne-preeclamptic HIV-positive (n=19). Zonke iziguli ezine-HIV zithole i-HAART. Ukuziveza kwe C5a ne C2 kwakulinganiswa ngokusetshenziswa kwe Bio-Plex multiplex immunoassay.

Imiphumela: Ngokuya ngohlobo lokukhulelwa, umehluko omkhulu ekuvezweni kokugxila kwe-C5a ukhonjisiwe phakathi kokukhulelwa kwe-preeclamptic vs kwa-negazi elishaya kahle ngale kwesimo se-HIV ($p = 0.0486$). Ngale kokubaluleka, ukugxila kwe-C5a kuphakanyisiwe ezigulini ezine gazi elishaya kahle uma kuqhathaniswa neqembu le-preeclamptic. Kwakungekho ukubaluleka okwatholakele phakathi kwamaqembu ane-HIV kanye nangenayo i-HIV, kungakhathalekile uhlobo lokukhulelwa ($p = 0.8002$). Masiqhubeka, bekungekho mehluko ophawulekayo obonwe emazingeni e-C2 phakathi kweqembu le-preeclamptic nelaba negazi elishaya kahle, nangale kokutholakala kwe-HIV. Masibheka mayelana nokwesimo seHIV, abukho ubufakazi obubalulekile phakathi kwalo lolubile uhlobo lokukhulelwa olwatholakala ($p=0.7469$).

Ukusonga: Lolu cwaningo lwenoveli lukhombisa ukwehla okukhulu ekugxileni kwe-C5a ku-PE uma kuqhathaniswa nabesifazane abakhulelwe abanegazi elishaya kahle. Le nkulumo engalindelekile ingabangelwa ukusetshenziswa okusheshayo kwe-C5a ekujikelezeni kwegazi, ukuhlangana okushintshiwe kwe-C5a kwi-zamukeli zayo noma ama-polymorphisms ezakhi zofuzo. Siphinde sibike ukugxila okufanayo kwe-C5a no-C2 phakathi kwamaqembu ane-HIV kanye

nangenayo i-HIV okungenzeka kukhombisa umphumela wokwakhiwa kabusha kwamasosha omzimba kwenziwa yimphumela yokusebenzisa i-HAART. Ukuhlukunyezwa kwama-complimenti kuthinta ukuzivikela okungokwemvelo kwe-PE ngokwandisa ukulimala kwe-placenta kanye nokukhulelwa kwengane ngakho-ke kudinga ucwaningo olukhulu lokuhlola amaprotheni ngamanye we-complement Cascade ekuhlanganisweni kwe-preeclampsia ehambisana ne-HIV.

CHAPTER 1

BACKGROUND AND LITERATURE

1.1 Maternal Mortality and Hypertension: A Global Crisis

Maternal mortality is a crucial public health issue in low- and middle-income countries (Girum and Wasie, 2017). According to the World Health Organization (WHO), approximately 810 maternal deaths/day occur globally, emanating from complicated pregnancies and childbirth (WHO, 2019). The adoption of the Millennium Development Goals (MDG) has led to a decline in maternal mortality by 44% (United Nations, 2019); however, many countries were unsuccessful in attaining the desired 75% reduction of their maternal mortality ratio (MMR) between 1990 and 2015 (Wijesinghe *et al.*, 2019; United Nations, 2019). Subsequently, the Sustainable Development Goals (SDG) 2016-2030 was developed to reduce the MMR to less than 70 maternal deaths per 100 000 live births (UNAIDS, 2019). Sub-Saharan Africa alone accounts for roughly two-thirds (196 000), whilst Southern Asia accounts for nearly one-fifth (58 000) of maternal deaths (Osungbade and Ige, 2011; WHO, 2019).

The prevalence of hypertensive disorders of pregnancy (HDP) such as preeclampsia (PE) in developing countries ranges from 1.8% to 16.7% (Osungbade and Ige, 2011). Globally, HDP disorders such as the HELLP (hemolysis, elevated liver enzymes, low platelet count) syndrome, eclampsia, and PE contribute to an estimated 30 000 deaths per year (Collier and Martin, 2018). In South Africa (SA), Human Immunodeficiency Virus (HIV) infection and HDP are the main causes of maternal deaths (Saving Mothers Report, 2017).

1.2 Preeclampsia: A Hypertensive Pregnancy Disorder

1.2.1 Clinical Features of Preeclampsia

Preeclampsia is a pregnancy-specific disorder occurring after 20 weeks of gestation. It is characterized by a new-onset high blood pressure of ≥140/90 mmHg (Brown et al., 2018), accompanied by one or more of the following conditions, during or after 20 weeks' gestation: proteinuria and/or evidence of multi-organ dysfunction (hematological complications, acute kidney injury and neurological complications) (Brown *et al.*, 2018). Fetal complications include intrauterine growth restriction, placental abruption, and perinatal death (Silasi *et al.*, 2010).

1.2.2 Epidemiology of Preeclampsia

Globally, PE is one of the leading causes of maternal mortality, accounting for 3-8% of maternal deaths (Nathan *et al.*, 2018). The WHO reports the incidence of PE to be seven-fold greater in low- and middle-income countries compared to high-income countries (WHO, 2019). In low-income countries such as SA, the incidence of maternal deaths due to PE development is 14.8% (Saving Mothers Report, 2017). In SA, the prevalence of PE is 17% (NCCEMD, 2018).

1.2.3 Aetiology of Preeclampsia

The exact aetiology of PE is unknown; however, immunological, genetic, and environmental factors contribute to the pathogenesis of PE (Thakoordeen *et al.*, 2018). Preeclampsia is considered a two-stage disorder. Stage 1 of PE (preclinical) occurs due to placental dysfunction as a result of shallow trophoblast invasion and limited physiological transformation of spiral arteries early in pregnancy (16-20 weeks) (Young *et al.*, 2010). The consequential outcome is reduced blood supply with resultant placental ischaemia, which pre-empts the release of placental factors into the maternal circulation thereby causing maternal inflammatory and oxidative stress (Redman, 1991). When stage 2 of PE (clinical) occurs, anti-angiogenic factors and other mediators that initiate systemic inflammation, oxidative stress, and endothelial cell (EC) dysfunction are released into circulation (Figure 1.1). This results in the induction of the classic clinical manifestation of hypertension, proteinuria, and other associated complications (Regal *et al.*, 2017).

The two main subtypes of PE are early-onset preeclampsia (EOPE) and late-onset preeclampsia (LOPE), the classification is based on the time of onset of the disease (Staff *et al*., 2013). A vital comparable difference between the two subtypes is the fetal growth restriction. During EOPE there is an elevated fetal growth restriction compared to LOPE (Huppertz, 2008). Moreover, the clinical signs of EOPE appear at <33 weeks, whereas in LOPE it appears in ≥34 weeks of gestation (Staff *et al*., 2013). According to Kovo *et al.*, (2012) more than 80% of preeclamptic pregnancies are of the LOPE subtype (Kovo *et al*., 2012).

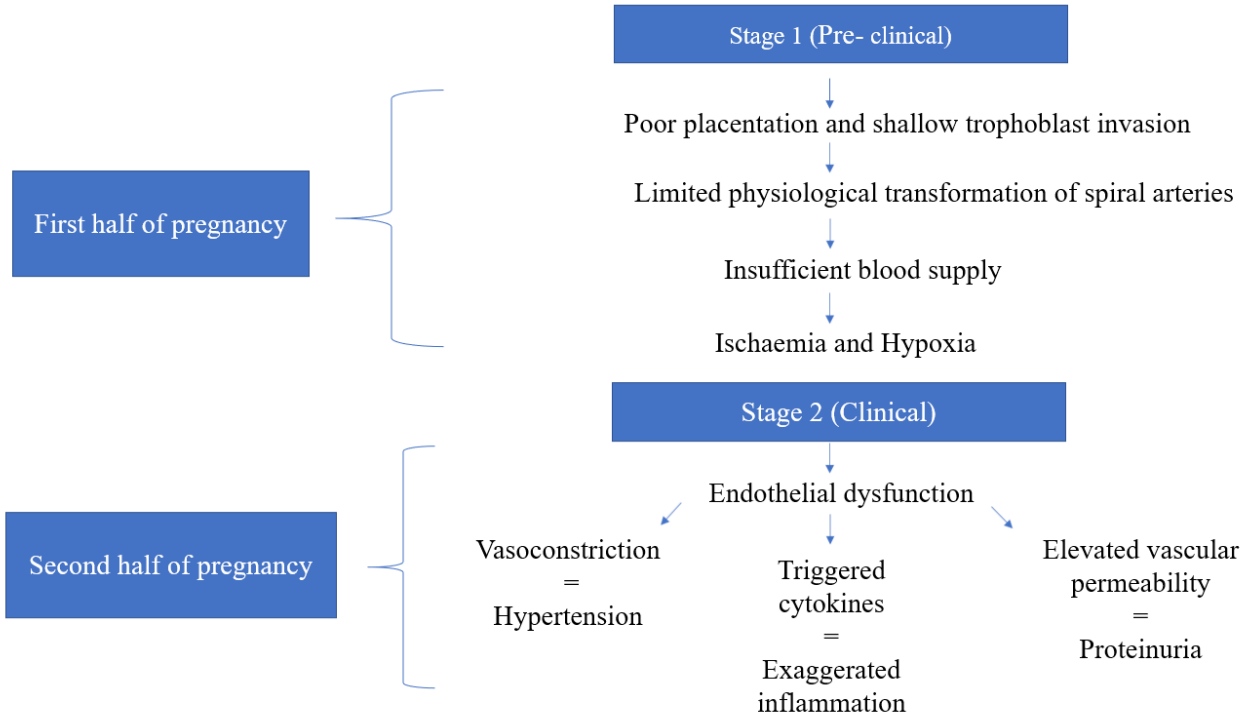


Figure 1.1. Development and progression of Preeclampsia: a two-stage placental disorder. (Adapted from Staff, 2019).

1.3 Human Immunodeficiency Virus Infection (HIV): A Global Pandemic

HIV infection is an immune invasive retrovirus infection that is responsible for the deterioration of cellular immunity. It leads to intensified susceptibility to foreign pathogens and opportunistic infections (Okoye and Picker, 2013; Maartens *et al.*, 2014). Globally, 37.9 million people are HIV infected (UNAIDS, 2019). SA is considered the epicentre of the pandemic with 7.9 million of its population (13%) living with HIV infection (Stats SA, 2020). In 2017, the prevalence of HIV infection in the province of KwaZulu-Natal (KZN, SA) was 27%. In SA, One-fifth of young females of childbearing age are HIV infected (Stats SA, 2020). The overall HIV antenatal prevalence is 30.7% with the highest prevalence (41.1%) occurring in KZN (National Antenatal Sentinel HIV Survey, South Africa, 2019). In light of the high prevalence of PE (12%) and HIV infection (41%) in KwaZulu-Natal, the duality of HIV infection superimposed on a preeclamptic pregnancy is high, hence warrants urgent investigation.

1.3.1 HIV Infection and Pregnancy

There is compelling evidence that demonstrates that HIV-1 exploits and destabilizes angiogenesis and lymphangiogenesis via its envelope glycoprotein (gp120), trans-activator of transcription (Tat), and its matrix protein (p17) (Zhang *et al.*, 2012; Caccuri *et al.*, 2014; Basta *et al.*, 2015). The accessory protein Tat evades host response due to its similar arginine- and lysine-rich sequence to vascular endothelial growth factor (VEGF), a strong angiogenic growth factor (Zhou *et al.*, 2013). Tat promotes endothelial adhesion via an elevated αvβ3 and α5β1 integrin expression (Zhou *et al.*, 2013). Tat also utilizes p17, the matrix protein to activate the protein kinase Akt and extracellular signal-regulated kinase (ERK) transduction pathways (Zhang *et al.*, 2012; Caccuri *et al.*, 2014). Therefore, Tat promotes a high inflammatory reaction in HIV-infected preeclamptic women (Abbas and Herbein, 2013). Notably, HIV-1 infection enhances PE prevalence via key immunomodulating circulating cytokines that are interlinked with HIV-associated immune activation (Pillay *et al.*, 2020).

A study was done by Paladugu *et al.*, (2003), noted that Tat protein weakened endothelium-dependent vasorelaxation and endothelial nitric oxide synthase expression and regulation in endothelial cells of porcine coronary arteries (Paladugu *et al.*, 2003). Furthermore, the study associated the long-term effect of Tat in PE patients with coronary artery disease. Additionally, Tat protein was shown to induce the expression of ICAM-1 and VCAM-1, indicating that HIV-1 infection mechanisms contribute to accelerates atherosclerosis and endothelial injury (Dhawan *et al.*, 1997; Liu *et al.*, 2005). These findings suggest that the defective angiogenesis in PE may emanate from the effect of d Tat homology with VEGF.

Nonetheless, in contrast to the heightened immune state observed in PE, there is substantial downregulation of immune response in HIV infection (Stoner *et al.*, 2016; Sebitloane and Moodley, 2017). The risk of PE development is lower in the presence of HIV infection; however, many studies show that highly active antiretroviral therapy (HAART), affects the prevalence of PE development receiving. The elevation of PE development emanates from the restoration of the immune response in patients receiving antiretroviral therapy (ARTs) (Sebitloane and Moodley, 2017; Naicker *et al.*, 2019; Saums *et al.*, 2019). A more recent study, however, reports no alteration in the risk of PE development between treated and untreated HIV-infected pregnant women (Saums *et al.*, 2019). Of note, some studies oppose the concept that HIV infection has protective characteristics against HDP development (Frank *et al.*, 2004).

1.4 Immunologic Maladaptation of Preeclamptic Pregnancies and HIV Infected Pregnant Women Receiving HAART

Currently, data on the synergy of HIV infection and PE development are contradictory. Immunologic maladaptation is one of the pathogenic developments of preeclamptic pregnancies (Khan *et al.*, 2016). Notably, the fetus is an allograft that carries paternal antigens, foreign to the mother's immune system (Alrahmani and Willrich, 2018). During normal pregnancy, the innate and adaptive immune system undergoes specific adaptations to enable the survival of the fetal allograft and to protect the mother and fetus from pathogens (Silasi *et al.*, 2010). In PE, these maternal immune specific responses are altered and the mother develops inadequate tolerance to the fetus (Hsu and Nanan, 2014).

As mentioned earlier, HIV infection suppresses the immune response. The development of PE has halted the neutralization of immune hyperactivity during HIV infection (Govender *et al.*, 2013; Hall *et al.*, 2014). However, the introduction of Highly Active Antiretroviral Therapy (HAART), a standard of care for all South African HIV patients, results in an increased incidence of PE development in HIV-infected women (Phoswa *et al.*, 2019). HAART exacerbates the exaggerated immune response of PE, therefore re-establishing immunocompetence (Kalumba *et al.*, 2013).

HAART administration downregulates pro-inflammatory cytokines and improves endothelial function thereby decreasing PE development. However, ART drugs such as protease inhibitors are potent anti-angiogenic factors that alter HIV-1 aspartyl protease, thereby promoting immune restoration. Anti-retroviral therapy promotes oxidative stress (Chai *et al.*, 2005), hence causes endothelial dysfunction (Zhong *et al.*, 2002; Fiala *et al.*, 2004). Moreover, a down-regulation of nitric oxide exacerbates oxidative stress and endothelial dysfunction during ART. A similar microenvironment occurs during PE development (Aouache *et al.*, 2018).

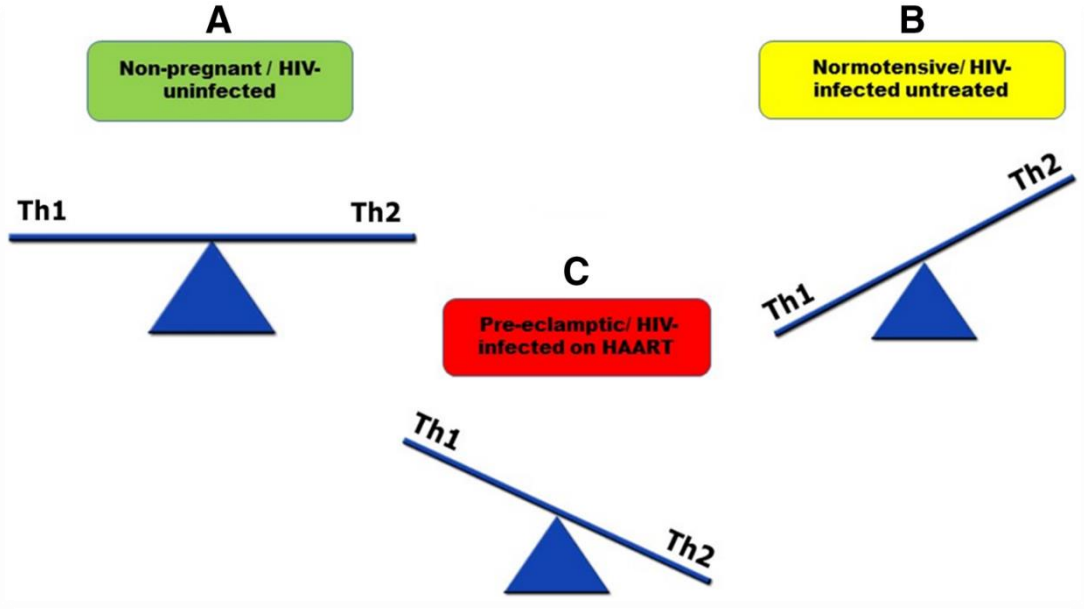


Figure 1.2. Schematic diagram showing the regulation of pro-inflammatory cytokine (Th1) and anti-inflammatory cytokine (Th2) in **A** non-pregnant or HIV-uninfected, **B** normotensive or infected untreated, and **C** preeclamptic or HIV-infected on HAART. **A** Shows a Th1 and Th2 distribution balance. **B** Shows more Th2 released as opposed to Th1, therefore, causing an imbalance, increasing HIV infection in untreated women. **C** Shows Th2 levels are lower than Th1. Th1 response is induced by HAART, following PE development. (Adapted from Machado *et al*., 2014 and Naicker *et al.*, 2019).

In a normal pregnancy, a shift of the maternal response from the Th1 to Th2 immune response is caused by the presence of the placenta (Figure 1.2), this is crucial for the semi-allogenic fetus to be tolerated by the mother (Machado *et al.*, 2014; Naicker *et al.*, 2019). Once accomplished, an immune-tolerant environment is favoured (Laresgoiti-Servitje *et al.*, 2010; Alrahmani and Willrich, 2018). In preeclamptic pregnancies, however, maternal immune regulation is further altered and the Th2 shift does not occur, therefore elevated Th1 immune response prevails (Hu *et al.*, 2007). This is usually attributed to an ischemic placenta that is unable to cause the Th1 to Th2 shift (Olusi *et al.*, 2000; Naicker et al., 2019). In HIV-infected pregnant women treated with HAART, there is a high expression of Th1 cytokines compared to HIV-uninfected pregnant or non-pregnant women (Alonso *et al.*, 2000). Th1 immune response is induced in infected pregnancies treated with HAART and reduced in HIV-uninfected pregnant and non-pregnant individuals (Alonso *et al.*, 2000). HAART treatment, reconstitutes the immune response, predisposing HIV-infected women to PE development by inducing the Th1 immune response in HIV-infected pregnant women (Maharaj *et al.*, 2017).

1.5 The Complement System

In humans, the first line of defence against pathogens is controlled by the complement system, a central part of our innate immunity (Merle *et al.*, 2015). Surface complement regulatory (Creg) proteins are expressed on normal cells, this prevents self-damage caused by an activated complement by regulating the complement activation (Lillegard *et al.*, 2013).

There are 3 pathways (Figure 1.3), the alternative pathway (AP), the lectin pathway (LP), and the classical pathway (CP) which contribute to the activation of the complement system (Khan *et al.*, 2016). Each of these pathways leads to a common terminal path that causes lysis of pathogens, elicits inflammation, and clears immune complexes (Merle *et al.*, 2015).

a) The CP (Figure 1.3) is activated by antibody binding to cell surfaces which exposes a C1q binding site (Regal *et al.*, 2017). Once C1q is bound to the antibody's Immunoglobulin G (IgG) and Immunoglobulin M (IgM)] Fc portion, C1r and C1s are activated. Upon activation, C4 and complement component 2 (C2) are cleaved from C1s, and as a result, C4bC2a is formed which is commonly known as C3 convertase (Sarma and Ward, 2011).

b) The LP (Figure 1.3) is activated when ficolin or mannose-binding lectin (MBL) binds to carbohydrate moieties located on the surface of pathogens (Noris and Remuzzi, 2013; Killick *et al.*, 2018). This binding activates serine proteases *viz*., mannan-binding lectin serine protease 1 (MASP-1) and mannan-binding lectin serine protease 2 (MASP-2), which cleave C4 and C2 to also form the C3 convertase, C4bC2a (Wallis, 2007).

c) As opposed to the CP and LP, the AP is constantly stimulated at low levels. In healthy individuals, this activation serves as a surveillance system (Merle *et al.*, 2015). Carbohydrates, proteins, and lipids on pathogens trigger activation of the AP (Figure 1.3) (Qu *et al.*, 2009). C3 is spontaneously hydrolyzed at low levels to form C3(H_2O), resulting in the binding of Factor B, and is thereafter cleaved by Factor D to form C3 convertase, C3bBb (Kemper *et al.*, 2009). In the presence of plasma protein properdin, C3 is stabilized. This protein can activate the AP through association with AP C3 convertase or pathogenic antigens (Sarma and Ward, 2011).

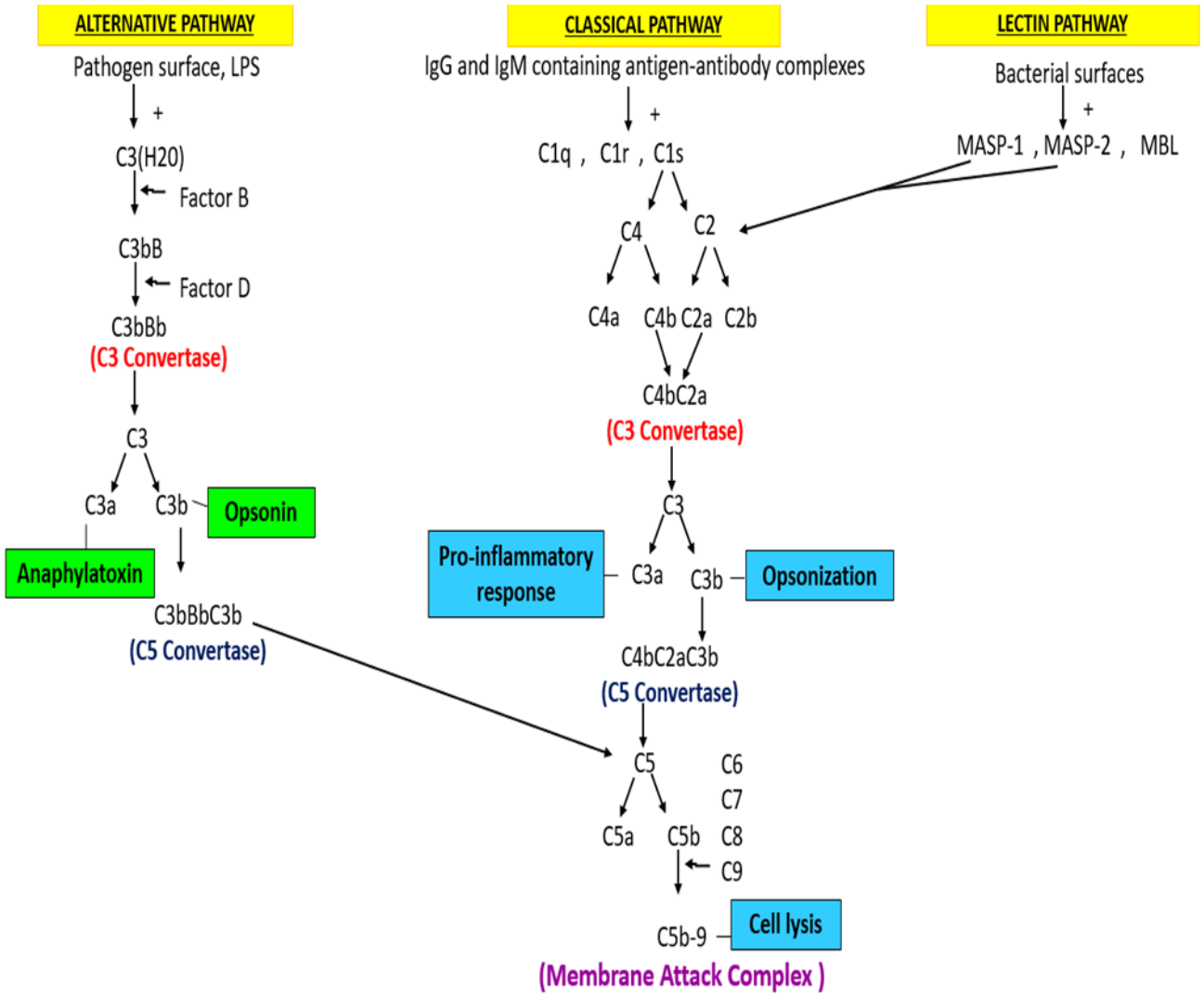


Figure 1.3. Schematic diagram showing the activation and regulation of the complement cascade. Complement activation occurs via 3 pathways, CP, AP, and LP. The CP is activated by antibody binding to cell surfaces which exposes a C1q binding site, the LP is activated when ficolin or MBL binds to carbohydrate moieties found on pathogen surfaces and the AP is activated when C3 spontaneously hydrolyzed at low levels to form C3(H_2O). All 3 pathways form a C3 convertase, cleaving C3a and C3b, resulting in membrane attack complex (MAC) (cell lysis), inflammation, and opsonization. (Adapted from Sarma and Ward, 2011).

C3 convertase (Figure 1.3) is cleaved upon catalyzation of the 3 pathways into functional fragments C3a and C3b. The enzyme C3 convertase is the central component during pathogen infection in the complement system (Sarma and Ward, 2011). C3a is known to be an inflammation mediator whereas C3b is an opsonin that attaches to immune complexes, pathogens, and apoptotic cells which initiate phagocytosis (Merle *et al.*, 2015).

Thereafter, C5 convertase is formed by the interaction of C3 convertase and C3b. C5 convertase is further cleaved into complement component 5a (C5a) and C5b. C3a and C5a are powerful anaphylatoxins that attract and activate leukocytes (Regal *et al.*, 2017). Vasodilation, as well as an acceleration in vascular permeability, are controlled by C3a and C5a (Noris and Remuzzi, 2013; Regal *et al.*, 2017). The membrane attack complex (MAC) is formed when C5b interacts with complement components C6, C7, C8, and C9, and to form a membrane pore (Figure 1.3). This initiates membrane rupture and as a result cell lysis is implemented by the activity of C5b-9 (Wills-Karp, 2007; McDonald *et al.*, 2015; Alrahmani and Willrich, 2018).

1.6 The Complement System in Normal Pregnancy

During pregnancy, the fetus is protected from harm due to complement activation by Creg proteins that halts activation (Richani *et al.*, 2005). Excessive complement activation is inhibited in a successful pregnancy. Within maternal tissue, invasion of extravillous trophoblast cells into maternal tissues are faced with complement activating antibodies hence they are protected from the maternal complement system (Regal *et al.*, 2017). A site that requires protection from complement activation is the syncytiotrophoblast, as this is the placental surface that is exposed to maternal blood (Rampersad *et al.*, 2008; Ito *et al.*, 2015; Pillay *et al.*, 2019).

Complement activation is controlled by 3 regulatory proteins, CD59 a glycophosphatidylinositol (GPI)-anchored protein, membrane cofactor protein (MCP), and decay-accelerating factor (DAF) which are found on the trophoblast cell membrane (Liszewski *et al.*, 1996). DAF halts C3 convertase formation and increases decaying of preformed C3 convertase (Figure 1.4). MCP cleaves C3b and C4b into their active forms whilst CD59 functions downstream to inhibit the formation of MAC (Francis *et al.*, 2006; Pacheco *et al.*, 2011; Denny *et al.*, 2013). Nonetheless, it is important to note that whilst complement activation is crucial for host defence, there is a delicate balance between complement regulation versus activation. Pregnancy disorders such as PE occurs when there is a dysregulation in complement regulation (Khan *et al.*, 2016).

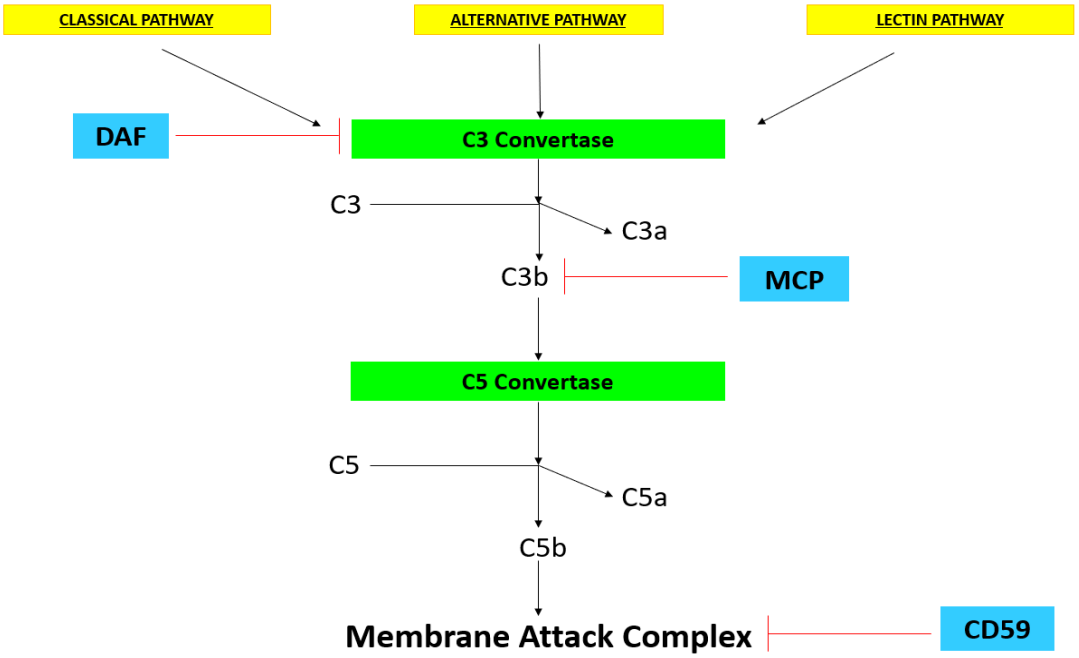


Figure 1.4. Schematic diagram showing the overview of the main effectors and regulators of the complement system. Complement is activated by the classical pathway, the lectin pathway, and the alternative pathway. DAF, MCP and CD59 are key complement regulators found on human placental tissue responsible for preventing inappropriate activation of complement. (Adapted from Denny *et al.*, 2013).

A study by Johnson and Gustavii, (1987), showed an increase of specific complement proteins *viz*., C2, C4, C3, C5, C6, and Factors B and H concentrations whereas C1q and C1r remained unaltered during normal pregnancy in a cohort of 72 women, (Johnson and Gustavii, 1987). Furthermore, a study by Derzsy *et al.*, (2010) reported that serum C1 esterase inhibitor (C1INH) concentration that controls the activation of the initial component of the CP was reported to be low (Ogston *et al.*, 1981; Halbmayer *et al.*, 1991; Cohen *et al.*, 1992; Derzsy *et al.*, 2010) or unaltered (Mellembakken *et al.*, 2001) during normal pregnancy [0.21 (0.19–0.23) g/l] and in preeclamptic pregnancies [(0.19 (0.17–0.22) g/l); p <0.05] (Derzsy *et al.*, 2010).

The activation of the complement cascade is a compensatory mechanism for the decline in adaptive immunity that occurs in normal pregnancy hence it serves to protect the host (and fetus) from microorganisms and other potential pathogens (Richani *et al.*, 2005). This effect is mediated by C1 esterase inhibitor (C1INH) regulates activation which may activate the complement cascade and therefore serve to elevate components C3a, C4a, and C5a in maternal plasma (Ogston *et al.*, 1981; Halbmayer *et al.*, 1991; Cohen *et al.*, 1992). Richani *et al.*, (2005), reported an upregulation of

plasma C3a, C4a, and C5a concentrations in normal pregnant (n=134) versus non-pregnant (n=40) women.

Moreover, C2 polymorphisms and its deficiency are associated with chronic inflammatory conditions such as systemic lupus erythematosus (SLE) (Agnello, 1978; Macedo and Isaac, 2016). The clinical manifestations of PE mimic that of SLE. In contrast to previous studies, a review by Lintner *et al.*, (2016), stated that SLE patients commonly show evidence of complement consumption leading to low serum levels of C4 and C3. The distinctive pattern of inactive SLE patients is that both C4 and C3 are reduced concurrently. C2 is split into two by-products, C2a and C2b. C2a (larger fragment) joins to C4b to produce C3-cleaving enzyme, C4b2a. Similar to C4, C2 plays an important role in producing the biological activity of C3 and thereafter the terminal components C5 through C9 (Lintner *et al.*, 2016). Furthermore a review by Pickering and Walport, 2000 stated that homozygous hereditary deficiency of each of the classical pathway components (C1q, C1r, C1s, C4, and C2) is related to a heightened susceptibility to SLE (Pickering and Walport, 2000).

1.7 Pathogenesis of Preeclampsia

1.7.1 Angiogenic Imbalance in the Pathogenesis of Preeclampsia

During normal pregnancy, physiological alteration of maternal spiral arteries facilitates adequate blood supply to the fetus (Silasi *et al.*, 2010). In PE, however, deficient cytotrophoblast invasion and a lack of remodeling of the spiral arteries occur within the myometrium. This leads to a reduction in the luminal diameter of blood vessels (Figure. 1.5) which causes an insufficient blood supply to meet the oxygen and nutritional demands of the fetus (Young *et al.*, 2010; Naicker *et al.*, 2019).

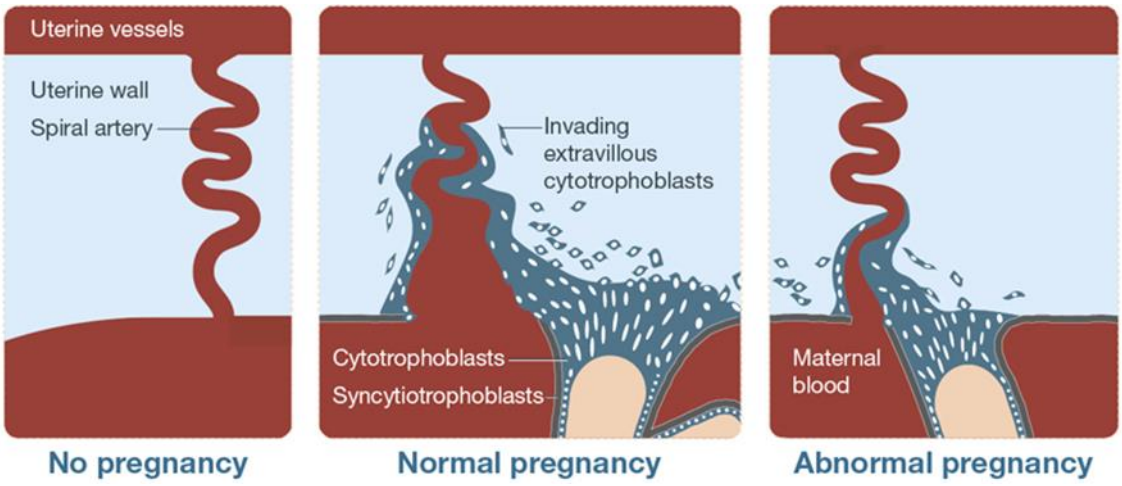


Figure 1.5. Diagram showing placentation in non-pregnant women, during normal pregnancy and abnormal placentation in problematic pregnancies**. (**Adapted from The Eunice Kennedy Shriver National Institute of Child Health and Human Development, 2017).

An imbalance of innate angiogenic factors plays a key role in the pathogenesis of PE (Maynard *et al.*, 2003). In normal pregnancies, vascular endothelial growth factor (VEGF), a pro-angiogenic factor maintains endothelial stability (Figure 1.6). On the other hand, placental growth factor (PIGF) stimulates angiogenesis under conditions of ischemia and inflammation (Carmeliet *et al.*, 2001; Autiero *et al.*, 2003). PIGF and VEGF inhibition in pregnant rats mediate a PE-like syndrome (Maynard *et al.*, 2003). This shows that PIGF and VEGF blockade would be crucial in the pathogenesis of soluble fms-like tyrosine kinase 1 (sFlt-1) induced endothelial dysfunction (Lam *et al.*, 2005).

In PE excess amounts of anti-angiogenic factors such as sFlt-1, soluble endoglin (sEng), and other inflammatory mediators are released by the ischaemic placenta. This causes a widespread endothelial dysfunction that results in hypertension, proteinuria, and other systemic manifestations of PE (Maynard *et al.*, 2003; Venkatesha *et al.*, 2006). This escalation of sFlt-1 levels is associated with a concurrent decline in pro-angiogenic factors *i*.e., VEGF, and PIGF (Figure 1.6) (Maynard *et al.*, 2003; Ahmad and Ahmed, 2004; Lam *et al.*, 2005; Venkatesha *et al.*, 2006).

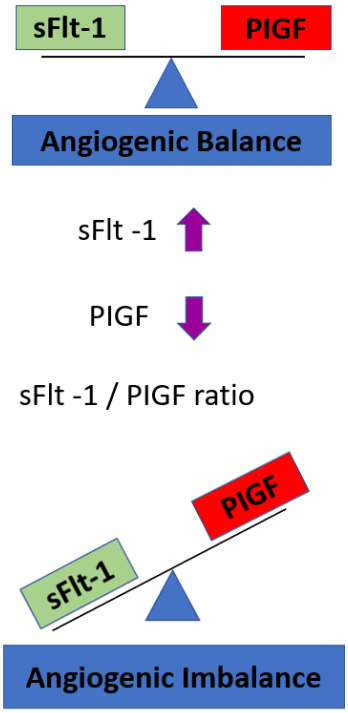


Figure 1.6. During normal pregnancies, sFlt-1 and PIGF establish physiological angiogenic balance. Numerous factors and mediators impact trophoblast invasion and placentation. Excessive production and release of sFlt-1 occur during preeclampsia and as a result, there is an upregulation of sFlt-1/PIGF ratio (angiogenic imbalance). (Adapted from Sitepu and Rachmadsyah, 2019).

Furthermore, sEng is a cell surface receptor for transforming growth factor-beta (TGF-β) that is found to be increased in PE (Figure 1.7 & 1.8) (Levine *et al.*, 2006). In pregnant rats, sEng escalates vascular damage carried out by sFlt-1, which induces a severe preeclamptic-like syndrome with features of the HELLP syndrome (Venkatesha *et al.*, 2006).

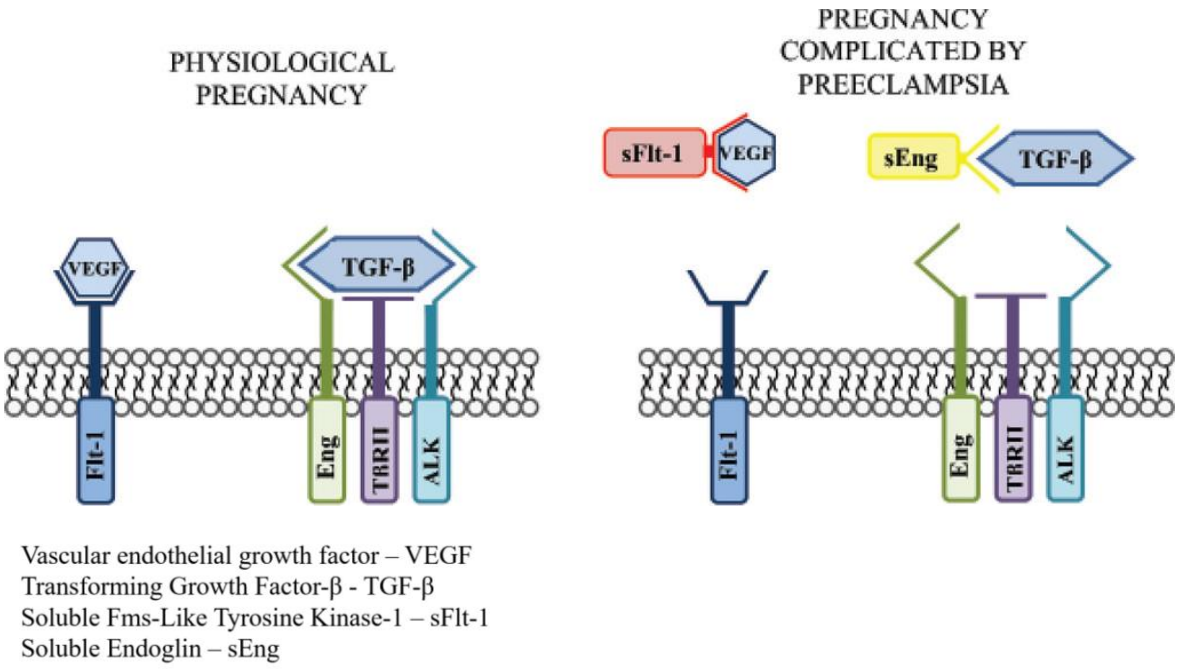
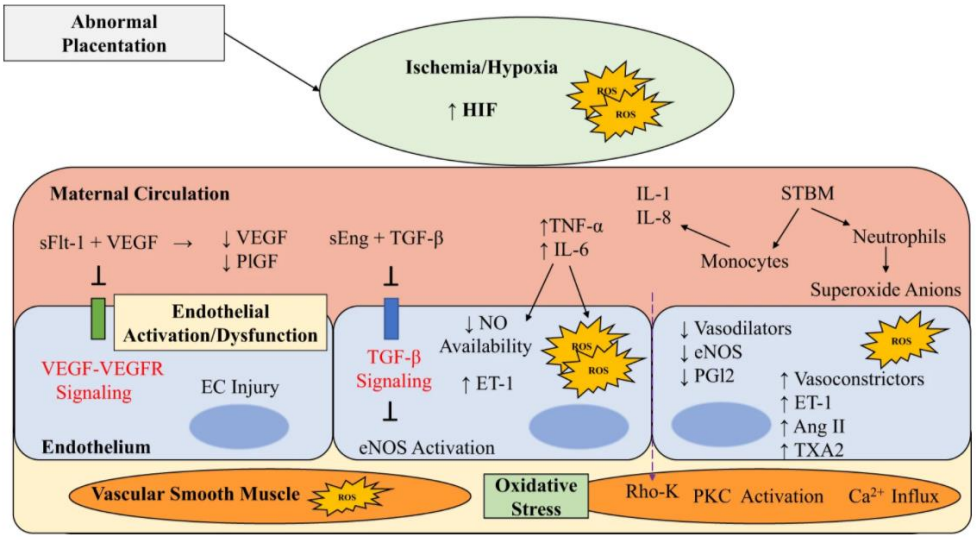


Figure 1.7. Angiogenic factors in the pathogenesis of Preeclampsia (Adapted from Rudic *et al.*, 2019)

sFlt-1 antagonises VEGF and PIGF by binding to them in circulation and preventing the interaction with their endogenous receptors (Kendall and Thomas, 1993). Maynard *et al*., (2003) demonstrated that exogenous sFlt-1 given to pregnant or non-pregnant rats produces a syndrome similar to that of PE (Figure 1.7). sFlt-1 is secreted by syncytiotrophoblasts into the maternal circulation as syncytiotrophoblast microvesicles (STBM) (Nagamatsu *et al.*, 2004). The expression of STBM's is elevated in PE (Knight *et al.*, 1998). In fact, in normal pregnancy STBM's are anti-angiogenic (Sunderland *et al.*, 1981), proinflammatory (Khalfoun *et al.*, 1986; Chua *et al.*, 1991), and procoagulant (Mukkala *et al.*, 1989), however, its function is dysregulated in PE (Sunderland *et al.*, 1981; Mukkala *et al.*, 1989).

Systemic inflammation together with hypoxia stimulates the release of excessive amounts of sFlt-1 in PE (Redman and Sargent, 2009). Endothelial injury observed in PE is caused by oxidative stress and nitrosative stress. This is derived from the imbalance between pro-oxidants and anti-oxidants (Paladugu *et al.*, 2003). Due to this stress, and upregulation in reactive oxygen species, possible weakened accessibility of anti-oxidant mechanisms and reactive nitrogen species production is triggered (Aouache *et al.*, 2018). STMB recruitment of monocytes and neutrophils to injured endothelial sites prompts the release of proinflammatory cytokines, namely tumour necrosis factor-alpha (TNF-α), interleukin (IL)-1, IL-6 and IL-8 from the ischemic placenta (Sani *et al.*, 2019). Apart from the downregulation of the bioavailability of nitric oxide and prostaglandin I2 (PGI2), an increase of endothelin-1, a potent vasoconstrictor is also produced by inflammatory cytokines.

Imbalance of endothelial vasodilators (NO and PGI2) and vasoconstrictors [Angiotensin II (Ang II), ET-1, and thromboxane A2 (TXA2)] during endothelial cell injury causes vascular smooth muscle contraction (Figure 1.8). Sustained vascular resistance and the hypertensive hallmark of endothelial injury observed in PE is the result of a decline in calcium ion efflux from smooth muscle cells through protein kinase C and Rho-kinase activation, due to vasoconstrictors (Maynard *et al.*, 2003; Touyz *et al.*, 2018).



Angiotensin II - (Ang II),
Endothelial nitric oxide synthase - (eNOS),
Endothelin-1 – (ET-1),
Hypoxia-inducible factor (HIF),
Interleukin-1, 6, and 8 (IL-1, IL-6, and IL-8),
Nitric oxide (NO),
Prostaglandin (PGI2),
Protein kinase C (PKC),
Placental growth factor (PlGF),
Reactive oxygen species (ROS),
Soluble endoglin (sEng),
Soluble fms-like tyrosine kinase-1 (sFlt-1),
Syncytiotrophoblast microparticles (STBMs),
Transforming growth factor-β (TGF-β),
Tumor necrosis factor-α (TNF-α),
Thromboxane A2 (TXA2),
Vascular endothelial growth factor (VEGF),
VEGF receptor (VEGFR)

Figure 1.8. Abnormal placentation in Preeclampsia. (Adapted from Sani *et al.*, 2019 and Naidoo *et al.*, 2021).

1.7.2 HIV Therapy in Endothelial Dysfunction

Regardless of an HIV-infected patient's $CD4^+$ count or progressive stage, the WHO has recommended that all HIV-infected individuals receive ART (WHO, 2020). However, HAART administration has been associated with the activation of severe PE development (WHO, 2020).

In fact, Lopinavir/Ritonavir-based (LPV) preferred and Alternative Second-Line Regimens in HIV-infected patients are believed to affect uterine decidualization and spiral artery remodeling in both *in vitro and in vivo* models (Kala *et al.*, 2020)**.** Upon LPV exposure of primary decidual cell cultures, an observed decreased expression VEGF, PlGF, angiopoietin-2, granulocyte-macrophage colony-stimulating factor, interferon-gamma and matrix metalloproteinase (MMP) 9 was shown (Kala *et al.*, 2020). Additionally, a downregulated expression of the transcription factor, signal transducer, and activator of transcription 3 (STAT3) occurs due to uterine natural kill cell reduction and deficient trophoblast invasion (Fitzgerald *et al.*, 2008). These findings result in endothelial dysfunction in PE and related adverse neonatal outcome. Nuclear factor-kappa (NF-κB) transcription factors that downregulate matrix metalloproteinase and VEGF expression thereby promoting angiogenesis dysregulation and PE development are impaired by HAART (Sgadari *et al.*, 2002). During PE, amplified expression of Flt-1 and sFlt-1 was detected within trophoblast cells, regardless of HIV status, suggesting the occurrence of autocrine signaling in trophoblast invasion and differentiation. This initiates abnormal placentation with endothelial cell dysfunction in PE (Govender *et al.*, 2014). Furthermore, a decline in PIGF and elevated sFlt-1 concentrations, in preeclamptic women compared to normal pregnant women occur prior to HAART exposure (Powis *et al.*, 2013).

PlGF and viral load were significantly associated to PE development in a multivariate analysis (Powis *et al.*, 2013). Elevated sFlt-1 and sEng concentrations correlated with preeclamptic women regardless of HIV infection (Govender *et al.*, 2013). In this study, a decrease in PIGF concentration was observed in HIV-negative women with PE in comparison to normotensive women. Nevertheless, HIV infection decreased PIGF concentration in normotensive pregnant women opposed to their HIV-negative counterparts ($p = 0.02$), thus prompting preeclamptic development in women (Govender *et al.*, 2013).

In contrast, a study done by Maharaj *et al.*, (2017) which demonstrated HIV-associated PE women revealed that HIV/HAART is related to significant decline of IL-2, TNF-α and IL-6, with substantial downregulation in IL-2 and TNF-α in preeclamptic women (Maharaj *et al.*, 2017).

1.7.3 Aberrant Complement Regulation in Preeclamptic Pregnancy

Impairment to the surface of intact host cells may be caused by complement dysregulation (Cho, 2015). Additionally, complement regulator deficiency can unsuccessfully tag modified self-cells, therefore causing interference with the removal of injured or modified self-cells, and hence is associated with the pathophysiology of numerous autoimmune diseases (Cho, 2015). Notably, co-opting host regulators found on some pathogens, allows them to evade the complement system, therefore resulting in infection (Cho, 2015). It is important that the complement system preserves the most appropriate balance between activation on pathogens and modified self-cells, and inhibition on intact host cells.

Notably, in PE the increase in maternal systematic inflammatory response involves both innate and adaptive immune systems (Redman *et al.*, 1999; Saito *et al.*, 2007). According to animal models, this activation results in complement deposition and fetoplacental unit destruction, with consequential fetal loss (Derzsy *et al.*, 2010). The incompatible relationship between placental cells and the maternal immune system may also participate in the dysregulation of complement activation. This can contribute to the pathogenesis of PE (Huppertz, 2018).

It is proposed that the dysregulation of the complement activation is the central component of PE pathogenesis (Fakhouri, 2016; Sabau *et al.*, 2016; Alrahmani and Willrich, 2018). Lynch et al., (2010) observed that during early pregnancy (10 - 20 weeks) there is a dysregulation of the AP activation fragment Bb in those women who develop PE. The presence of complement fragment Bb in early pregnancy predisposes one to the risk of PE development. Fragment Bb is a marker that is necessary for the activation of the alternative complement pathway, and functions as a protease to cleave additional C3 molecules that are crucial for the complement cascade (Soto et al., 2010).

Furthermore, the highest levels of Bb occur during early gestation before the onset of preeclamptic development, thus implying that there is an early activation of complement activity (Lynch *et al.*, 2008; Lynch *et al.*, 2011). The AP plays an essential role in protecting the developing fetus and placenta (Soto et al., 2010).

In contrast, in the second half of pregnancy (>24 weeks), an elevation of maternal blood Bb level occurs in PE compared to normotensive women of African–American ethnicity (Velickovic et al., 2015). Moreover, an increase in Bb levels from both maternal and umbilical venous blood in severe PE compared to a normotensive pregnancy has been noted (Soto et al., 2010; Hoffman *et al.*, 2014;

Sones *et al.*, 2018). These results thus implicate racial/ethnic 19 differences as well as disease type, and maternal/ fetal blood as confounding factors in the dysregulation of the complement cascade.

Also, an up-regulation of the AP occurs in women with PE and the HELLP syndrome (Alrahmani and Willrich, 2018). Both C5a and C5b-9 proteins have been reported to be elevated in severe preeclamptic compared to healthy pregnant women, suggesting that activation of the terminal pathway is a critical feature for this severe disease (Burwick *et al.*, 2013). The activation of the terminal pathway in PE is supported by a human case in which Eculizumab, an inhibitory monoclonal antibody against C5 is used successfully as a temporizing treatment for severe PE and HELLP syndrome (Burwick and Feinberg, 2013; Lokki *et al.*, 2017).

In a study by Qing *et al.*, (2011), the DBA/2-mated CBA/J mouse model was used to demonstrate the link between excessive complement activation and PE development (Qing *et al.*, 2011). The DBA/2-mated CBA/J mouse shares many pathological features with human PE including oxidative stress, elevated anti-angiogenic factors, placental dysfunction, and activation of coagulation pathways (Qing *et al.*, 2011). On day 5 of pregnancy, C3 inhibitor was administrated to DBA/2-mated CBA/J mice, it prevented oxidative stress, placental dysfunction, proteinuria, and renal pathology, features consistent with preeclamptic development. An upstream component of the complement cascade, C1q, is associated with the prevention of abnormal placentation and PE development. C1q is spread in the human decidual stroma and is vital in trophoblast migration and spiral artery remodeling, contributing to placental development (Bulla *et al.*, 2008; Agostinis *et al.*, 2010). Singh *et al.*, (2011) reported that C1q deficiency in mice exhibits proteinuria and hypertension i.e., characteristics of PE; emphasizing the significance of C1q in normal pregnancy (Singh *et al.*, 2011). These findings are constant with the high incidence of pregnancy complications such as systemic lupus erythematosus (SLE), malaria, or antiphospholipid antibody syndrome (APLAS) in women with acquired or genetic deficiency of classical pathway components (Salmon et al., 2011).

1.7.4 The Complement System and Angiogenic Imbalance in the Pathogenesis of Preeclampsia

The complement cascade mediates the release of anti-angiogenic factors which cause excessive complement activation in pregnancy (Denny *et al.*, 2013). In animal studies, VEGF decreases complement deposition, therefore, leading to poor placentation (Sones *et al.*, 2018). Moreover, trophoblast cells are known to produce complement components C3, C4, and C1q (Bulla *et al.*, 2012).

The increase in sFlt-1 levels in PE (Levine *et al.*, 2004), is induced by complement activation at sub-lethal levels on syncytialized human trophoblast cells (Banadakoppa *et al.*, 2018). sFlt-1 is released

upon the trigger of local generation C5a from infiltrating inflammatory cells. sFlt-1 weakens trophoblast proliferation, decreases placental blood flow, and prompts ischemia which initiates the increased production of placental sFlt-1 (Girardi *et al.*, 2006).

1.8 Complement System in HIV Infection

1.8.1 Activation

The complement system aids in the protection of the host against HIV infection, whilst on the other hand, it may also enhance HIV infectivity (Yu et al., 2010). There are two mechanisms of complement activation in HIV infection *viz.*, antibody-independent and antibody-dependent activation. During antibody-independent activation, studies have shown that the CP is directly activated by HIV, without the presence of virus-specific antibodies (Sölder *et al.*, 1989; Boyer *et al.*, 1991; Ebenbichler *et al.*, 1991; Spear *et al.*, 1991).

Also, HIV-1 viral envelope protein gp160 induces complement activation (Sölder *et al.*, 1989). The activation site is localized to the transmembrane protein gp41, which binds C1q, thereafter activating the reconstituted C1 complex (Ebenbichler *et al.*, 1991). C1q binds to gp41 reaction site localized on the globular head (Thielens *et al.*, 1993), resulting in the activation of the C1 complex (Figure. 1.9). Activation of subsequent components triggers C3 activation (Sölder *et al.*, 1989) and opsonization of the virus with surface-bound C3 fragments (Marschang *et al.*, 1993).

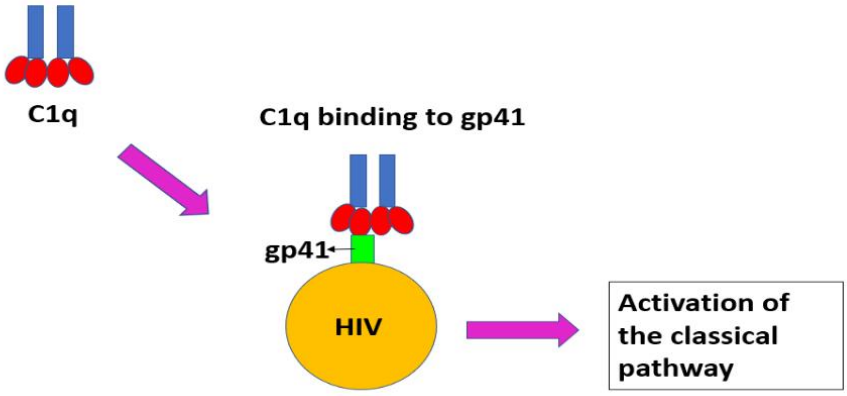


Figure 1.9. Complement activation by HIV, adhesion of C1q, and transmembrane protein gp41 leading to the CP being activated. (Adapted from Marschang *et al.*, 1994).

Furthermore, the binding of carbohydrate side chains of HIV envelope protein gp120 with MBL triggers the activation of the LP (Thielens *et al.*, 2002), increasing phagocytic uptake and inhibits viral entry into susceptible cells (Eisen *et al.*, 2008; Liu *et al.*, 2014). Studies have shown that MBL deficiencies are associated with enhanced susceptibility to HIV infection and disease progression (Tan *et al.*, 2009; Sheng *et al.*, 2010; Li *et al.*, 2013).

In antibody-dependent activation, the CP is further activated by HIV-specific antibodies (Stoiber *et al.*, 2001). Irrespective of a strong antibody response, only a fraction of antibodies produces neutralizing activity, which is insufficient to prevent the initiation of HIV infection (Huber and Trkola, 2007).

1.8.2 Complement Enhances HIV-1 Infectivity

Despite complement system activation during HIV-1 infection, HIV-1 in circulation is able to evade complement-mediated lysis. Hence the role of the complement system and antibody immunity is vital during HIV-1 virion clearance, and to prevent the spread and maintenance of the virus in the infected host. The deposition of complement activation products, C3 fragment and C5a into HIV-1 virions facilitates its interaction with monocytes/macrophages and dendritic cells (DC) that express complement receptors CR3 and CR4 (Bajtay *et al.*, 2004; Pruenster *et al.*, 2005; Stoiber *et al.*, 2008). Furthermore, the binding of C3 fragments to the gp160 complex and enhanced infection of C3 receptor-bearing target cells is a result of HIV complement activation. CR1 and CR2 independently contribute to the penetration of the opsonized virus into complement receptor-expressing T-cells (Delibrias *et al.*, 1993). According to recent studies by Lund *et al.*, (1995), the interactions of CD4-gp120 and C3d-CR2 also enhance viral adhesion to target cells, a step important for viral entry (Lund *et al.*, 1995).

Additionally, host cell Creg proteins such as; CD59 and CD55 (Figure 1.10) may also be incorporated into the HIV-1 viral envelope (Yu *et al.*, 2010). These regulatory proteins are acquired by the virus from the host cell in the budding process (Hu et al., 2010). Moreover, Hu *et al.*, (2010), demonstrated that the inhibition of human CD59 activity enhances complement-mediated virolysis of HIV-1 (Hu *et al.*, 2010). However, the complement regulator factor H binds to HIV-1 providing additional protection against complement attack (Yu *et al.*, 2010; Liu *et al.*, 2014).

Serum Factor H is also an important cofactor for the generation of C3d-opsonized infectious HIV-1 reservoirs on follicular DC's and B cells in HIV-infected individuals (Bánki *et al.*, 2006). Dendritic cells (DCs) are the first cell type to come into contact with HIV-1; they enhance viral dissemination to activated CD4 T cells and spread to lymph nodes (Granelli-Piperno *et al.*, 1999). B-cells are not

readily infected by HIV, however similar to follicular DC's, they may serve as extracellular reservoirs for HIV-1. B cells can circulate in peripheral blood and migrates through tissues where they may interact with and transfer the virus to T cells (Moir *et al.*, 2000). The direct interaction between B and T lymphocytes and direct binding of the opsonized virus to receptors on B cells is critical for HIV-1 amplification (Jakubik *et al.*, 1999; Jakubik *et al.*, 2000; Döpper *et al.*, 2003)

The deposition of complement fragments (*eg*: C3 and C5a) present on the surface of HIV-1 virions mediates the interaction between HIV-1 and cells expressing complement receptors namely, macrophages, dendritic cells and non-immune cells (erythrocytes) (Tjomsland *et al.*, 2013). As a consequence, trans-infection of CD4+ T cells is heightened (Hu et al., 2010). Apart from DC's being the first type of cell to interact with HIV-1, they also intensify the viral spread to newly triggered CD4+ T cells. MHC class-1 exhibition of HIV-derived antigens by DCs is stimulated by complement opsonization of HIV-1 particles (Tjomsland et al., 2013).

Furthermore, the adhesion of anti-HIV antibodies to complement-opsonized HIV-1 virions facilitates HIV-1 interaction with erythrocytes. HIV-1 attaches to erythrocytes in a complement/CR1-dependent manner and this interaction plays an important role in the progression of the primary infection (Horakova *et al.*, 2004).

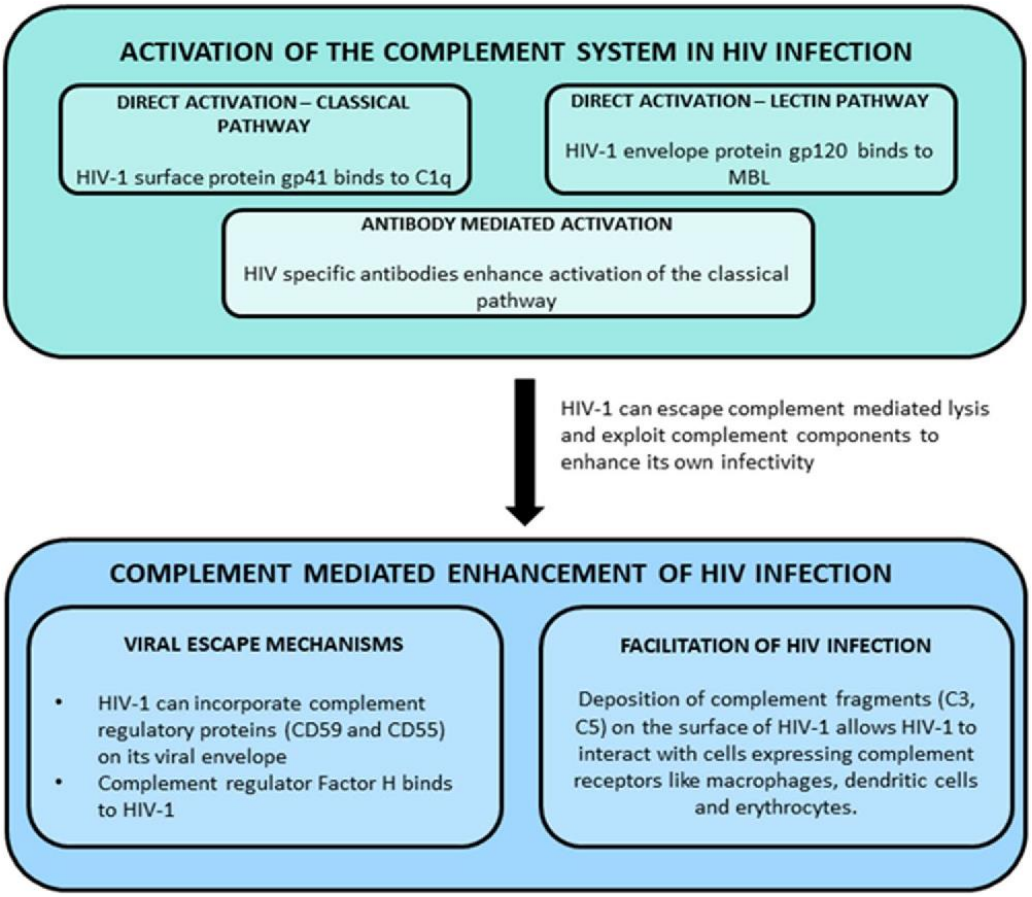


Figure 1.10. Schematic diagram showing the activation and enhancement of the complement system in HIV infection. The CP and LP activation are the results of direct activation of the complement system by HIV infection via gp41 and gp120 binding. HIV-specific antibodies can further activate the complement system. CD59 and CD55 proteins incorporation on viral envelope cause HIV-1 to escape complement-mediated lysis. Complement deposition present on the HIV-1 surface facilitates the virus and cells expressing complement receptor interaction. (Adapted from Pillay *et al*., 2019).

1.9 Complement System in HIV Infected and Preeclampsia

Due to the high prevalence of HIV infection and PE in SA, it is important to understand the association between both conditions (Frank *et al.*, 2004). Previous studies have reported that pregnant women with HIV infection have a lower risk of developing PE (Wimalasundera *et al.*, 2002; Conde-Agudelo *et al.*, 2008; Kalumba *et al.*, 2013; Landi *et al.*, 2014; Machado *et al.*, 2014; Sansone *et al.*, 2016). HIV-1 infection neutralises the exaggerated immune response in PE thereby preventing preeclamptic development (Frank *et al.*, 2004). However, the use of HAART reconstitutes immune status thereby increasing the risk of PE development.

These complement regulatory (Creg) proteins are inhibitors of the complement cascade. Khan *et al*., (2016), reported an increase in Creg proteins namely, CD35 and CD55 levels on neutrophils using flow cytometry in PE (n=50) compared to normotensive pregnant (n=50) women, irrespective of their HIV status (Khan *et al.*, 2016). CD55 upregulation is seen as a protective mechanism against excessive complement activation in PE (Khan *et al.*, 2016).

The role of the complement system in HIV infection is multifaceted. It can aid in the protection of the host against HIV infection, on the other hand, it can also enhance HIV infectivity (Yu et al., 2010). However, an upregulation of CD55 may lead to enhanced HIV-1 infection (Yu *et al.*, 2010). The upregulation of these regulatory proteins has been implicated as an adaptive phenomenon in response to elevated complement-mediated cell lysis which occurs in HIV infection which is further aggravated by preeclamptic complement activation (Khan *et al.*, 2016).

In contrast, an *in-vitro* transcriptome profiling, ELISA and flow cytometry study by Ellegard *et al*., (2018) observed complement-opsonized HIV-1-modulated DC response and their cross-talk with natural killer (NK) cells. This inhibits killing and promotes the increase of factors associated with immune suppression (PD-1, TIM3, LAG-3) and susceptibility to infection (TCM, CD38, CXCR3, CCR4) on CD4 T cells (Ellegård *et al.*, 2018). Therefore, it is plausible to assume HIV infection in combination with the complement system may also weaken the heightened inflammatory state in PE.

1.10 Complement Component 5a

1.10.1 Structure and Function

Human C5a is an 11 kDa, 74 amino acid glycoproteins released from the alpha-chain of C5 by the C5 convertase enzyme (Manthey *et al.*, 2009). This powerful anaphylatoxin prompts oxidative burst in neutrophils, stimulates the production of oxygen radical species, chemo-attracts granulocytes, reduces apoptosis, and enhances phagocytosis in normal pregnancies (Mollnes *et al.*, 2002). Cleavage of C5 into C5a and C5b fragments are brought about by activated phagocytic cells. Polymorphonuclear neutrophils (PMN) myeloperoxidase generated oxidant and kallikrein activity is responsible for C5 cleavage and thereafter the production of C5a (Vogt, 1996).

Anaphylatoxins may also be produced by neutrophil elastase enzymatic activity and macrophage serine protease (Huber-Lang *et al.*, 2002). C5a is an important part of the innate immune response and evidence suggests that it may also play a role in adaptive immunity (Köhl, 2006). C5a is not

necessarily an initiating factor, however excessive and uncontrolled production occurs in many inflammatory diseases (Guo and Ward, 2005).

1.10.2 C5a Receptors

The transduction of C5a occurs via its attachment to two receptors, C5aR1 (CD88) and C5aR2 (C5L2) with a seven-transmembrane (7TM) helical structure (Klos *et al.*, 2013). In humans, C5aR1 and R2 share about 35% homology at the primary sequence level (Ohno et l., 2000). C5aR1 occurs on cell surfaces whilst C5aR2 occurs intracellularly (Klos *et al.*, 2013). Both receptors also bind to C5a-des-Arg, a naturally occurring cleavage product of C5a (Klos *et al.*, 2013). Unlike C5aR2, C5aR1 is a G-protein coupled receptor (GPCR) both receptors efficiently recruit β arrestins (βarrs) (Figure 1.11; Pandey et al., 2020). In contrast to C5aR1, C5a activation does not result in ERK1/2 phosphorylation and MAPK activation downstream of C5aR2 (Pandey *et al.*, 2020).

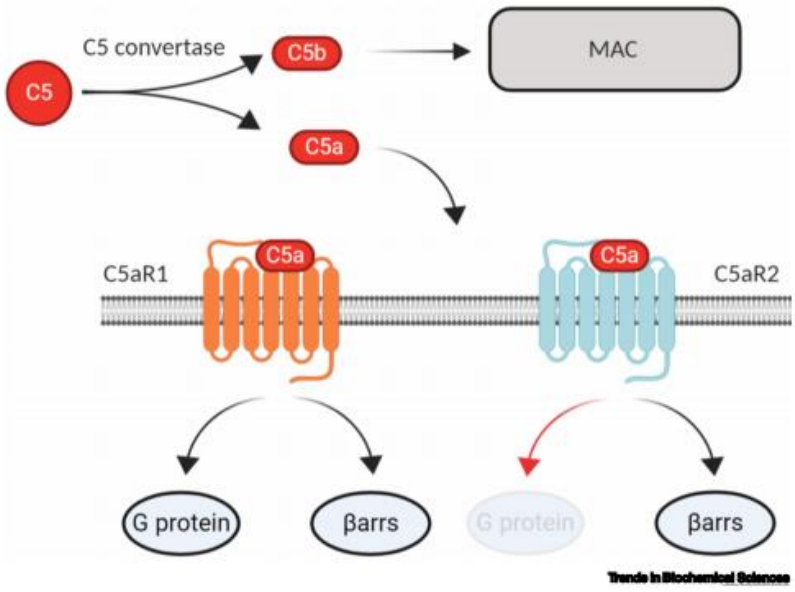


Figure 1.11. Schematic representation of C5aR1 and C5aR2. C5a convertase cleaves C5 into C5a and C5b. C5a activates C5aR1 and C5aR2. C5aR1 is a G-protein-coupled receptor and binds to β arrestins (βarrs) whilst C5aR2 does not have any G-protein coupling. (Adapted from Pandey *et al.*, 2020).

1.10.3 C5a Levels in Preeclampsia and HIV Infection

C5aR1 forms a heterodimer with a chemokine receptor CCR5, the major receptor for viral entry. A reduction of C5aR1 reduces HIV infection (Moreno-Fernandez *et al.*, 2016). Low CCR5 and high chemokine levels correlate with slow HIV-1 progression (Reynes *et al.*, 2000).

The potent pro-inflammatory anaphylatoxin C5a has been demonstrated in both normal and complicated pregnancies (Denny *et al.*, 2013). Richani *et al.*, (2005) demonstrated an elevation of C3a, C4a, and C5a in maternal plasma during normal pregnancy with C5a levels being [12.4 ng/ml (1.2 – 87.1); $p < 0.0001$] compared to non-pregnant women [4.1ng/ml (0.9 – 13.1); $p < 0.0001$] (Richani *et al.*, 2005). Also, both C3a and C5a concentrations are higher in preeclamptic patients compared to normal pregnant women (Haeger *et al.*, 1992; Soto *et al.*, 2010), where it induces inflammation, recruits DC's and macrophages for amplifying of HIV-1 spread (Liu *et al.*, 2014). In a normal pregnancy, there is a low or unchanged concentration of C1INH which regulates initiation of the CP (Mellembakken *et al.*, 2001). This decrease may elevate C3a, C4a, and C5a levels in maternal plasma (Richani *et al.*, 2005). The increase of C5a concentration is associated with the elevation of white blood cell count, monocytes, and initiation of granulocytes during pregnancy (Richani *et al.*, 2005).

C5a correlate significantly with Abdominal Aortic Aneurysm's, where values greater than 101 ng/ml had an odds ratio of 11 (95% CI 1.1-114.1) compared with values below 70ng/ml (Zagrapan *et al.*, 2020). Moreover, in patients chronically infected with hepatitis B virus, complement C5a is an indicator of fibrosis (95% CI; 0.976, 0.999) (Deng *et al.*, 2017).

One mechanism by which C5a may exert its harmful effects is by inducing the release of the potent anti-angiogenic factor, soluble vascular endothelial growth factor receptor-1 (sVEGFR-1), also known as sFlt-1 (Girardi *et al.*, 2011). sFlt-1 antagonises pro-angiogenic VEGF and PlGF, which ensures successful pregnancy. Circulating levels of sFlt-1 are elevated both in women with recurrent miscarriage and in murine models of pregnancy loss (Girardi *et al.*, 2006). Numerous studies show strong evidence of elevations in circulating levels of sFlt-1, with correspondingly reduced levels of PIGF and VEGF in women who develop PE or fetal injury in recurrent pregnancy loss (Figure 1.12) (Levine *et al.*, 2004; Rana *et al.*, 2012; Denny *et al.*, 2013).

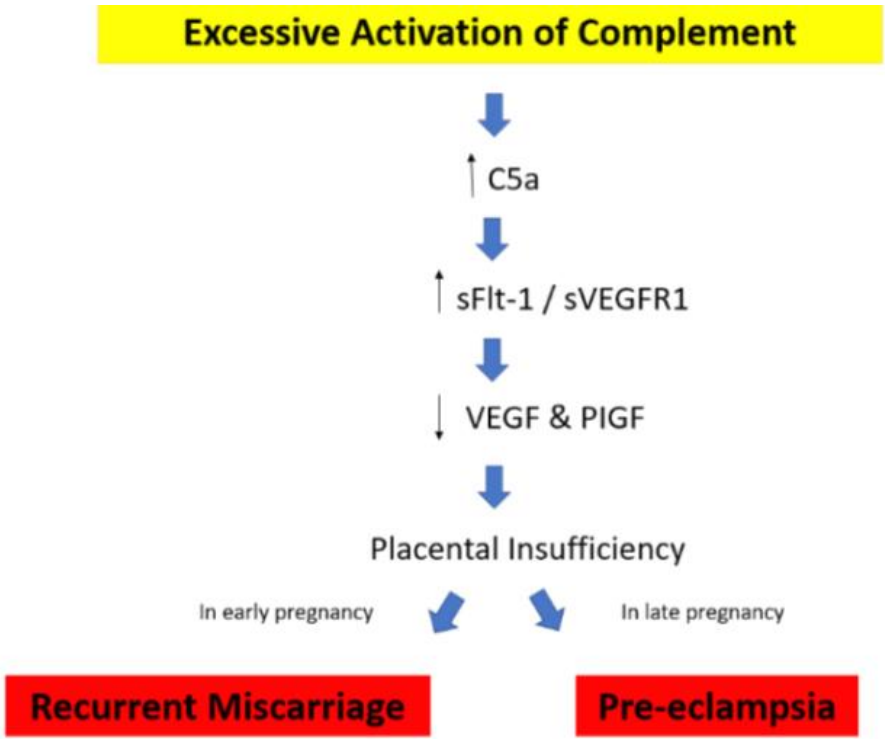


Figure 1.12. Schematic diagram showing the role of C5a in complicated pregnancies. Excessive activation of complement with an elevation of C5a during pregnancy results in poor pregnancy outcomes. C5a promotes induction of the release of soluble VEGF receptor-1 (sVEGFR-1)/ sFlt-1. Decreased availability of VEGF and PlGF leads to placental insufficiency, which in early pregnancy may lead to recurrent miscarriage and in late pregnancy, to PE development. **(**Adapted from Denny *et al*., 2013).

C5a is produced during acute and chronic infection with bacteria or intracellular pathogens and in autoimmune disorders (Hugli, 1990). Bacterial lipopolysaccharides (LPS) and C5a act interdependently in the induction of pro-inflammatory cytokine release Tumour Necrosis Factor-alpha (TNF-a), IL-1, and Interleukin 6 (IL-6) by monocytes and macrophages (Cavaillon *et al.*, 1990; Montz *et al.*, 1991). Therefore, the generation of complement-derived anaphylatoxins during local inflammation at mucosal surfaces of the genital tract may increase the efficiency of sexual transmission during the earliest phases of HIV infection (Bentwich *et al.*, 1995).

The increase of pro-inflammatory cytokines TNF-α and IL-6 have been reported in the presence of C5a, both liable for promoting HIV-1 infection and regulation (Kacani *et al.*, 2001; Popko *et al.*, 2010). The augmentation of HIV infection induced by C5a is reversed by the inhibition of complement component 5a receptor (C5aR) (Kacani *et al.*, 2001). Therefore, the chronic

inflammatory state in HIV infection and PE could be further aggravated by excessive complement activation.

C5a was also demonstrated to be a potent stimulatory factory *in vitro* studies where it increased the susceptibility of monocyte-derived macrophages (MDM) to HIV infection (Kacani *et al.*, 2001). The stimulatory effect of C5a correlated with secretion of endogenous TNF-α and IL-6, cytokines that are known to up-regulate HIV replication in an autocrine/paracrine manner (Poli *et al.*, 1990; Tadmori *et al.*, 1991; Weissman *et al.*, 1994). A 2-day treatment of MDM with C5a before viral pulse, 40-produced a 40-fold enhancement of HIV infectivity (Kacani *et al.*, 2001).

1.11 Complement Component 2

1.11.1 Structure and Function

Complement component gene, C2 is found on HLA class III, a part of the short arm of chromosome 6. Serum C2 is a precursor protein, formed as a result of the activation of C1 into C2b and C2a fragments. C2 shares sequence homology with serine proteinases, but has a catalytic chain with an extended N-terminus that has a 60-amino-acid-residue repeat structure (Reid and Porter, 1981; Bentley, 1986). C2 is similar to the primary structure of Factor B.

C2a forms C3 convertase (C4b2a) along with C4b (Walport, 2001). C2 is a key component of the CP and LP, defending the onset of microbial infections and immune complex removal (Lintner *et al.*, 2016). MBL or ficolin's together with MASP-1 adhere to carbohydrate molecules. This activates MASP-2, cleaving C2, and C4, resulting in a C3 convertase that is similar to that of the CP (Wallis *et al.*, 2007).

1.11.2 C2 Signal Transduction

Human complement receptor type 2 (CR2; CD21) is a surface-associated glycoprotein that binds to a variety of endogenous ligands, including the complement component C3 fragments iC3b, C3dg and C3d, the IgE receptor CD23, and the type I cytokine, interferon-alpha. CR2 links the innate complement-mediated immune response to pathogens and foreign antigens with the adaptive immune response by binding to C3d that is covalently attached to targets, and which results in a cell signaling phenomenon that lowers the threshold for B cell activation (Paul Hannan, 2016).

A 28-bp deletion, located on exon 6 at the 3' terminal end is observed in more than 95% of C2 deficient patients. This identical molecular genetic defect results in premature termination of transcription (Johnson *et al.*, 1992). A conserved MHC haplotype comprising HLA-B18, C2*Q0,

Bf*S, C4A*4, C4B*2, and Dr*2 is linked with the deletion (Winkelstein and Sullivan, 2010). Amongst Caucasians, C2 deficiencies are furthermost predominant amongst the genetically determined complete complement deficiencies. Those of European descent who are C2 deficient have a gene frequency of between 0.05 and 0.007. Resulting in a 1:10 000 prevalence of homozygotes (Rohrer *et al.*, 2019).

1.11.3 C2 Levels in Preeclampsia and HIV Infection

There is a lack of data on the exact role of C2 in pregnancy, however, a study by Johnson and Gustavii, (1987) showed an increase in the concentration of complement proteins (C2, C4, C3, C5, C6, and Factors B and H) in maternal blood of normal pregnant women compared to non-pregnant women (Johnson and Gustavii, 1987). In contrast, C2 polymorphisms and deficiencies have also been linked to many chronic inflammatory conditions: age-related macular degeneration (Gold *et al.*, 2006; Richardson *et al.*, 2009) and systemic lupus erythematosus (SLE) (Agnello, 1978). In PE, advanced maternal age is a risk factor (Whitelaw *et al.*, 2014). It is possible that functional differences of C2 variants each differentially influence the level of complement activation (Morris Jr *et al.*, 2009). In C2 deficiency, activation of the LP and CP is impaired. In these patients, there are increased levels of circulating immune complexes predisposing them to autoimmune diseases. SLE has a similar exacerbated immune microenvironment like PE, where a 10% penetrance of C2 deficiency occurs (Lintner *et al.*, 2016). Moreover, in pregnant women with SLE, there is complement-mediated injury, predisposing them to a higher risk of PE development, placental insufficiency, fetal growth restriction, and miscarriage (Teirilä *et al.*, 2019).

Nonetheless, there is a paucity of information regarding C2 in HIV infection. HIV may be inactivated through the combination of complement activation and antibody activation (Spear *et al.*, 1990). The requirement for antibody response, together with the observation that C2-deficient serum plus antibody does not release reverse transcriptase, indicates that HIV neutralization occurs by the CP. This mechanism of neutralization is similar to that shown for many other enveloped viruses (Hirsch, 1982).

Huson *et al.*, (2015) reported increased C3 and C1q-C4 levels in asymptomatic patients with HIV infection compared to healthy controls. However, MBL deficiency does not influence complement activation, suggesting HIV infection activates the complement system primarily via the CP (Huson *et al.*, 2015).

There is a paucity of data on the role of the complement system in HIV associated PE. Urgent research evidence on complement activation will aid in unraveling the aetiology of PE thereby enabling better clinical management strategies for PE. Therefore, the aim of this study is to compare

the immune-expression of complement components C2 and C5a, in the duality of HIV associated PE.

1.12 Hypothesis of study

- Complement components C2 and C5a levels will remain dysregulated between normotensive and preeclamptic pregnant women, regardless of their HIV status
- Complement components C2 and C5a levels will remain dysregulated between HIV infected and uninfected pregnant women, regardless of pregnancy type (normotensive and preeclamptic)

1.13 Aim of study

- To assess whether HIV infection affects the expression of complement components C2 and C5a levels in preeclamptic pregnancy**.**

1.14 Specific objectives

- To assess whether pregnancy type (normotensive versus preeclamptic pregnant women) regardless of their HIV status affect complement components C2 and C5a levels using Bio-Plex Multiplex Immunoassay
- To assess whether HIV status (HIV infected versus HIV uninfected) regardless of their pregnancy type affect complement components C2 and C5a levels using Bio-Plex Multiplex Immunoassay
- To assess whether complement components C2 and C5a levels are dysregulated across the study groups using Bio-Plex Multiplex Immunoassay
- To assess whether demographic features of the study groups affect complement components C2 and C5a expression in HIV-infected preeclamptic pregnancies.

CHAPTER 2

Citation:

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THE ROLE OF COMPLEMENT COMPONENTS C2 AND C5a IN HIV ASSOCIATED PREECLAMPSIA

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Abstract

Objective: To evaluate the expression of complement components C2 and C5a in HIV associated preeclampsia.

Materials and Methods: The sample population (n=76) was divided by pregnancy type into preeclamptic (n=38) and normotensive pregnant (n=38) groups; these were further stratified by patient's HIV status (HIV-positive and HIV-negative). Bio-Plex multiplex immunoassay method was used to quantify serum concentration of complement components C5a and C2.

Results: The concentration of C2 was not statistically different between preeclamptic and normotensive pregnant women, irrespective of HIV status as well as by HIV status regardless of pregnancy type. However, based on pregnancy type (preeclamptic *vs* normotensive), the expression of C5a was statistically different (p = 0.05); been down-regulated in preeclampsia compared to normotensive women, irrespective of HIV status. Both C2 and C5a concentrations did not differ across all study groups.

Conclusion: This novel study reports a loss of regulation of complement activation as shown by the down-regulation of C5a in preeclamptic compared to normotensive pregnant women, regardless of HIV status. Complement dysregulation affects the host innate defence, and as a consequence, intensifies placental and fetal injury. Moreover, HIV status did not influence the expression of both C5a and C2, irrespective of pregnancy type, this may be attributed to Highly Active Antiretroviral Therapy (HAART).

Keywords: C2, C5a, HIV, Preeclampsia

Introduction

Despite intensive research, maternal mortality remains a global health concern particularly in low- and middle-income countries [1]. Human Immunodeficiency Virus (HIV) infection, haemorrhage, and hypertensive disorders in pregnancy (HDP) are the main factors contributing to maternal mortality in South Africa (SA) [2]. Preeclampsia (PE) is a common and potentially fatal HDP, affecting 3-8% of all pregnancies worldwide [3]. It is diagnosed by a new-onset high blood pressure of ≥140/90 mmHg with/without proteinuria (> 300 mg/d) and/or evidence of maternal organ dysfunction at or after 20 weeks of gestation [4]. PE is a two-stage disorder. During stage 1, abnormal placentation involves inadequate trophoblast invasion with resultant deficient myometrial spiral artery remodeling that leads to an ischemia/ hypoxic micro-environment [5]. The maternal syndrome of PE is a consequence of ischaemia which leads to widespread endothelial damage, multi-organ involvement, and the clinical signs and symptoms of PE or stage 2 of this pregnancy disorder [6].

During normal pregnancy, the adaptations of the innate and adaptive immune system ensure the survival of the fetus [7]. However, during PE, immune hyper-reactivity results in maternal intolerance of the fetus [8]. In contrast, in HIV-infected individuals, the immune response is suppressed. Thus, in HIV associated PE, a neutralisation of immune response may occur [9, 10]. The use of Highly Active Antiretroviral Therapy (HAART) causes reconstitution of the immune system [11], thereby predisposing HIV-infected women to the development of PE [12].

The complement cascade is a fundamental part of the innate immune system (Figure 2.1). Extensive simulation of the complement system contributes to the pathogenesis of PE [13-15], hence its inhibition facilitates a successful pregnancy [16]. The complement system is activated via one of three pathways *viz*., classical, lectin, or alternative [17].

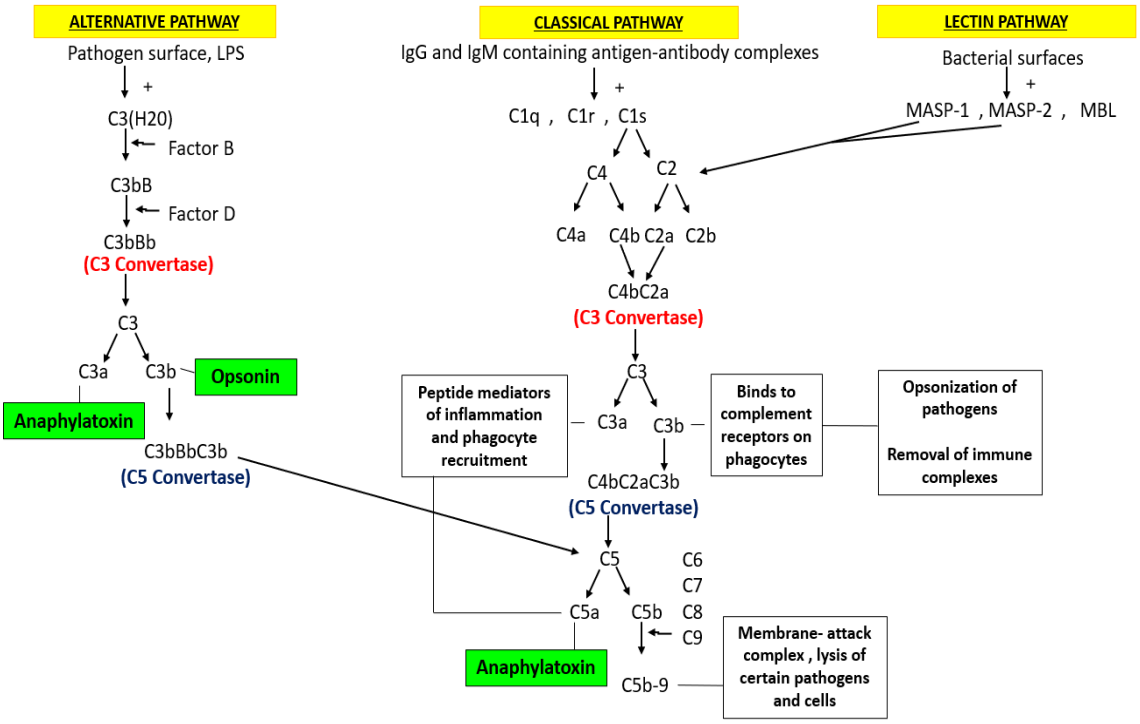


Figure 2.1. Schematic diagram showing an overview of the normal complement cascade and its main components. The complement cascade can be initiated via three pathways, classical, alternative, and lectin. The classical pathway (CP) is initiated by immune complexes interacting with C1q, the lectin pathway is triggered when mannose-binding lectin (MBL) fixes to carbohydrate moieties located on pathogen surfaces. The CP and lectin pathway (LP) generate the same C3 convertase, C4bC2a, and the alternative pathway (AP) is activated when C3 spontaneously hydrolyzed at low levels to form C3(H_2O) later on forming C3 convertase, C3bBb. All 3 pathways form a C3 convertase, cleaving C3a and C3b, resulting in membrane attack complex (cell lysis), inflammation, and opsonization. Adapted from Sarma and Ward, Janeway Jr *et al.* and De Vriese *et al.* [18-20].

All 3 pathways merge at a central point, the production of C3 convertase [21]. The cleavage of C3 convertase leads to a central terminal path that mediates host defence via the opsonization of pathogens, eliciting inflammation, lysis of pathogen cells, and/or clearing of immune complexes [15, 17, 22-24]. However, the role of the complement system in HIV infection is complex, as it may protect the host from HIV infection and/or enhance the infectivity of HIV [25].

Anaphylatoxins are potent inflammatory mediators targeting a vast spectrum of immune and non-immune cells [26]. Complement component 5a (C5a) is a powerful anaphylatoxin [15, 18, 27]. C5a mediates the release of potent anti-angiogenic factors, soluble vascular endothelial growth factor receptor-1 (sFlt-1) and soluble endoglin (sEng) [28]. The concomitant decline in pro-angiogenic growth factors including placental growth factor (PIGF) and vascular endothelial growth factor (VEGF) is crucial for placental development [28, 29]. Additionally, increased C5a elevates pro-

inflammatory cytokines such as Tumour Necrosis Factor-alpha (TNF-α) and Interleukin 6 (IL-6) both known to promote HIV infection [27, 30].

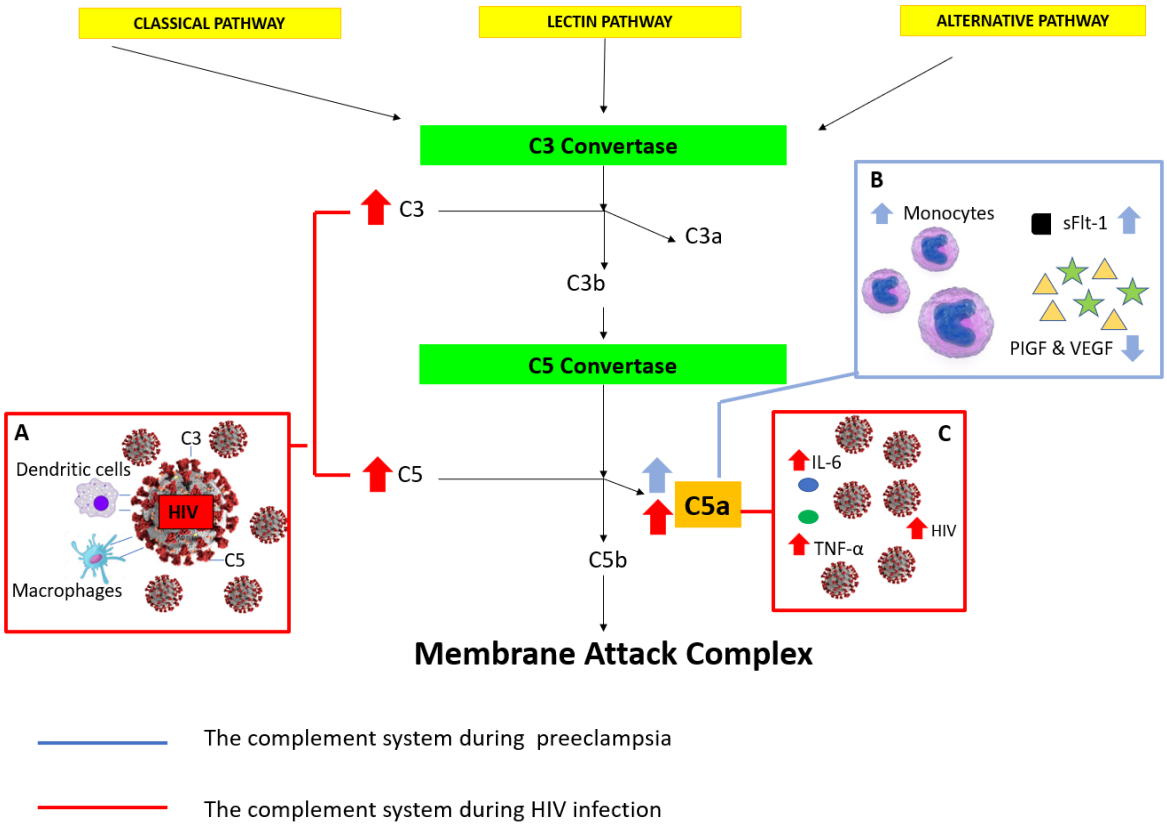


Figure 2.2. Schematic diagram showing the role of complement component C5a in the complement pathway of HIV associated preeclamptic women in **A:** Enhancement of C3 and C5 on the surface of HIV-1 enables the interaction of cells expressing complement receptors such as macrophages and dendritic cells (DC) with HIV-1 thereafter facilitating in HIV infection, **B:** Increased levels of C5a is associated with an increased number of monocytes. The escalation of C5a releases sFlt-1 resulting in the decrease of PIGF and VEGF, therefore, leading to preeclamptic development. **C:** C5a in excessive amounts is liable for the elevated production of pro-inflammatory cytokines, IL-6, and TNF-α and as a result, the promotion of HIV-1 infection and regulation. Adapted from Conroy *et al.*, Soederholm *et al.*, and Teirilä *et al.* [29, 31, 32].

Complement component 2 (C2) is a precursor protein and is a critical factor in both the CP and LP [33, 34]. C2 is cleaved to form C3 convertase, C4bC2a [6, 18]. The exact role of C2 in pregnancy and during HIV infection is sparse. Nonetheless, existing research shows that C2 concentrations are high in pregnancy [16]. Moreover, complement components C1q – C4 are increased in asymptomatic HIV-infected patients compared to healthy controls [35]. In light of the paucity of information

available on the regulation of complement components in HIV associated PE, this study aimed to evaluate the concentration of serum C5a and C2 using the Bio-plex multiplex immunoassay.

Methods and Materials:

Ethical Approval

This prospective study utilized retrospectively collected serum samples for which institutional ethics class approval (BCA 338/17) was obtained. Informed patient consent, hospital manager's approval and the regulatory authority consent were obtained in the primary study for use of the samples in subsequent studies. All patient identities were replaced with codes. Reports on the study protected confidentiality and all participants remained anonymous.

Sample Size

Post consultation with an institutional biostatistician, sample size was calculated. To detect a moderate effect size of 0.66 between two groups normotensive and preeclamptic women or HIV positive and HIV negative assuming equal groups (n=38 per group), a sample size of 76 pregnant women was required. To compare four groups, normotensive (HIV+ *vs* HIV-) and preeclamptic (HIV+ *vs* HIV-), a sample size of 19 in each group was needed to detect a large effect size of 0.95. All calculations are with 80% power and 95% probability and were done using G*Power statistical software.

Study population

A study population (n=76) was recruited from a large regional hospital, consisting of 38 normotensive and 38 preeclamptic women. Both groups are further stratified by HIV status into HIV-positive preeclamptic (n=19), HIV-negative preeclamptic (n=19), HIV-positive normotensive pregnancy (n=19) and HIV-negative normotensive pregnant women (n=19).

Inclusion Criteria: this study group consisted of primigravid and multigravida participants, diagnosed with PE (≥ 140/90 mmHg and/or the presence of a single incidence of proteinuria) [4], and participants with a normotensive pregnancy serving as the control group. All HIV-positive women received antiretroviral therapy (ART).

Exclusion Criteria: women with polycystic ovarian syndrome, intrauterine death, cardiac disease, chorioamnionitis, unknown HIV status, eclampsia, sickle cell disease, active asthma that requires medication during the gestational period, abruption placentae, chronic renal disease, patients who

have been declined from participation, systematic lupus erythematosus, pre-existing seizure disorders, and thyroid disease were not included in the study group.

Sample type

Maternal blood samples were collected and centrifuged at 3000 g for 10 minutes at 20°C. Serum was aliquoted and stored at -80°C until required.

Bio-Plex Multiplex (Bio-Rad) Immunoassay

A cytometric mechanism with a bead-based flow constructed the assay, where multiplex analyses are permitted. Using the manufacturer's instructions, Human Complement Magnetic Bead Panel (Millipore by Sigma – Aldrich, catalogue number: HCMP1MAG-19K), Bio-Plex®MAGPIX™ (Bio Rad Laboratories, Inc., USA) was utilized, using serum samples. Blank captured antibody with magnetic beads, C5a and C2 samples, antigen samples (1:4 dilution), and standards (serial dilution) were incubated. A triple wash eliminated any unbound substances. Prior to the incubation of the assay plate, a biotinylated detection antibody was added. Once the incubation period was over. A triple wash using wash buffer was once again performed to ensure the removal of unbound biotinylated detection antibodies. Thereafter, into each well, 1x streptavidin-phycoerythrin (SA-PE) was added. The plate was thereafter incubated for 10 min at 850 ± 50rmp in a dark room. The assay plate was washed 3 times with wash buffer and re-suspended in assay buffer for 30 seconds at 850 ± 50rmp. Lastly, a Bio-Plex®MAGPIX™ Multiplex Reader (Bio Rad Laboratories, Inc., USA) was used to read the assay plate

Statistical Analysis

Data were statistically analysed utilizing GraphPad Prism 5.00 for Windows (GraphPad Software, San Diego California USA). The Kolmogorov Smirnov normality test was used to check for parametric or non-parametric distribution. Non-parametric data are represented as median and interquartile range. Statistical significance according to pregnancy type (preeclamptic *vs* normotensive) and HIV status (negative *vs* positive) was determined using a Mann-Whitney's *U* test. The Dunn's Multiple Comparison *post hoc* test and the Kruskal-Wallis test determined statistical significance across all groups. A *p*-value of <0.05 was considered to be statistically significant. Spearman's Rank Correlation Coefficient (*r*) was calculated to determine the relation between clinical/ demographic data versus C2 and C5a concentrations across the study population (-1 and 1). The outcome results were elucidated based on the degree of association as strong (0.7–1), moderate (0.5– 0.7), or low (< 0.5) after taking significant correlation values into consideration.

Results

Patient Demographics and Clinical Characteristics

Patient demographics and clinical characteristics (Table 1) were non-parametrically distributed hence, are represented as median and interquartile range (IQR). Statistically significant differences were reported for maternal age ($p = 0.03$), parity ($p = 0.01$), gravidity ($p = 0.04$), gestational age ($p = 0.001$), systolic ($p < 0.0001$) and diastolic blood pressures ($p < 0.0001$) across the study groups. However, there was no significant difference reported for maternal weight ($p = 0.11$), across all study groups.

Table 1. Patient demographics across study groups

Total Sample Population (N=76)	**Normotensive HIV Negative (n=19)**	**Normotensive HIV Positive (n=19)**	**Preeclamptic HIV Negative (n=19)**	**Preeclamptic HIV Positive (n=19)**	***p*-value**
Weight (kg)	74.00(22)	81.00(28)	90.00(46)	79.50(35)	0.1063 (ns)
Gestational Age (weeks)	27.00 (9)	25.00(14)	24.00(10)	23.00(10)	0.0008***
Parity	1.00(1)	2.00(1)	1.00(2)	2.00(1)	0.0085 *
Systolic BP (mmHg)	109.00(20)	112.00(16)	146.00(14)	147.00(20)	<0.0001 ***
Diastolic BP (mmHg)	65.00(13)	72.00(14)	92.00(9)	97.00(13)	<0.0001 ***
Maternal Age (years)	25.00(9)	31.00(11)	29.00(16)	34.00(14.50)	0.0304*
Gravidity	2.00(2)	3.00(2)	2.00(2)	3.00(2)	0.0425 *

Results are represented as the median (IQR)

ns = non-significant,

* $p < 0.05$

*** $p < 0.001$

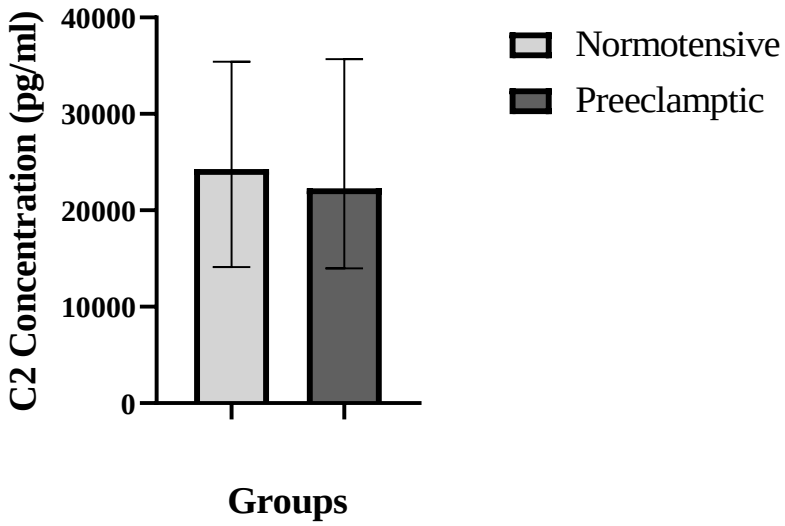
Serum concentrations of complement component C2

Pregnancy type - C2 concentration was non-significantly different between the normotensive pregnant (median = 24285 pg/ml, IQR = 21319 pg/ml) *vs* the preeclamptic (median = 22287 pg/ml, IQR = 21701 pg/ml) groups, regardless of HIV status (Mann-Whitney U = 654; $p = 0.4837$; Figure 2.3A).

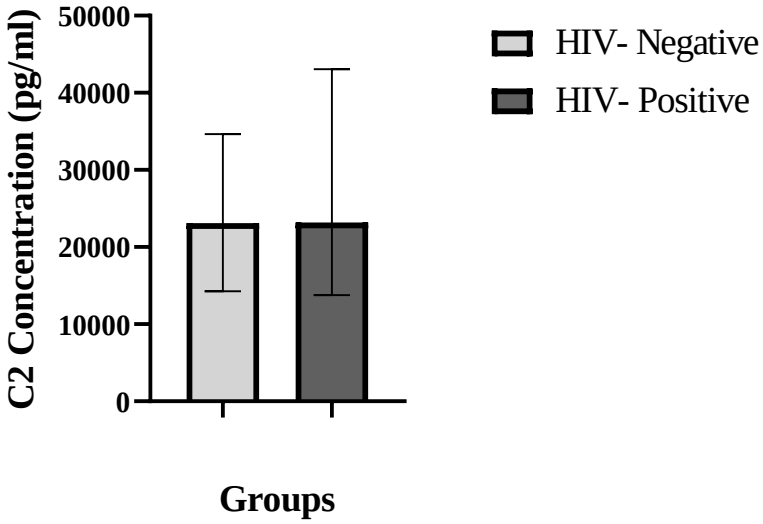
HIV status – The concentration of C2 level between the HIV-positive (median = 23180 pg/ml, IQR = 29300 pg/ml) *vs* HIV-negative (median = 23118 pg/ml, IQR = 20373 pg/ml) groups showed no statistical significance, irrespective of pregnancy type, (Mann-Whitney U = 690.5; $p = 0.7469$; Figure 2.3B).

Across all groups – The concentration of C2 was similar across all groups (Kruskal-Wallis H =1.098; $p = 0.7776$; Figure 2.3C; Table 2).

3A


C2 Concentration (pg/ml)
40000
30000
20000
10000
0
Groups
Normotensive
Preeclamptic

3B


C2 Concentration (pg/ml)
50000
40000
30000
20000
10000
0
Groups
HIV- Negative
HIV- Positive

3C

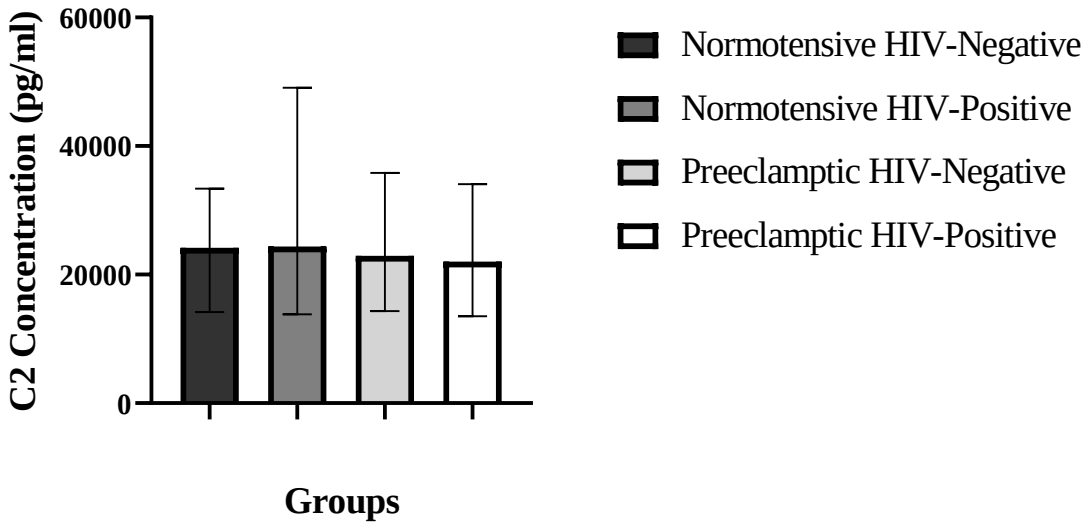


Figure 2.3 (A-C). C2 concentration are depicted in: (A) Normotensive *vs* Preeclamptic groups, (B) HIV infected *vs* HIV uninfected groups and, (C) across all groups.

Serum concentrations of complement component C5a

Pregnancy type - The serum concentration in the normotensive (median = 6562 pg/ml, IQR = 5302 pg/ml) was significantly higher than that of the preeclamptic (median = 4745 pg/ml, IQR = 3279 pg/ml) groups, regardless of HIV status (Mann-Whitney U = 532; $p = 0.0486$; Figure 2.4A).

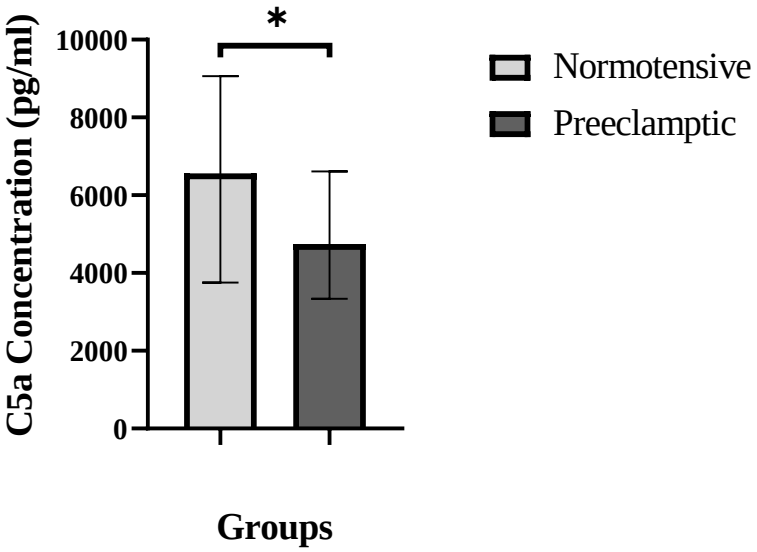
HIV status - Serum C5a level was similar between the HIV-positive (median = 5383 pg/ml, IQR = 4202 pg/ml) *vs* HIV-negative (median = 5548 pg/ml, IQR = 4726 pg/ml) groups, irrespective of pregnancy type, (Mann-Whitney U = 697; $p = 0.8002$; Figure 2.4B).

Across all groups – The concentration of C5a did not differ across all groups (Kruskal-Wallis H =4.352; $p = 0.2259$; Figure 2.4C; Table 2).

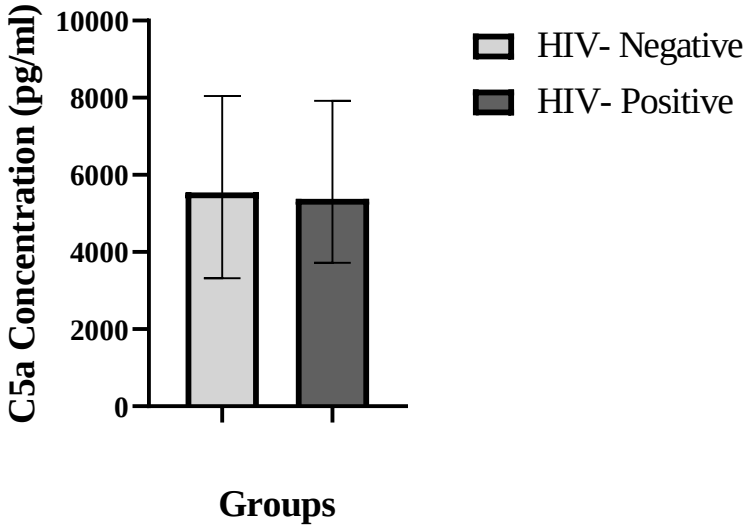
Normotensive HIV status – No statistical significance was shown between the normotensive HIV-negative group (median = 5965 pg/ml, IQR = 6010 pg/ml) *vs* normotensive HIV-positive group (median = 7160 pg/ml, IQR = 4958 pg/ml). (Mann-Whitney U = 162; $p = 0.6032$; Figure 2.4D).

Preeclamptic HIV status – C5a expression was non-significantly different between the preeclamptic HIV-negative group (median = 5044 pg/ml, IQR = 3093 pg/ml) *vs* preeclamptic HIV-positive group (median = 4447 pg/ml, IQR = 3642 pg/ml); (Mann-Whitney U = 159; $p = 0.5441$; Figure 2.4E).

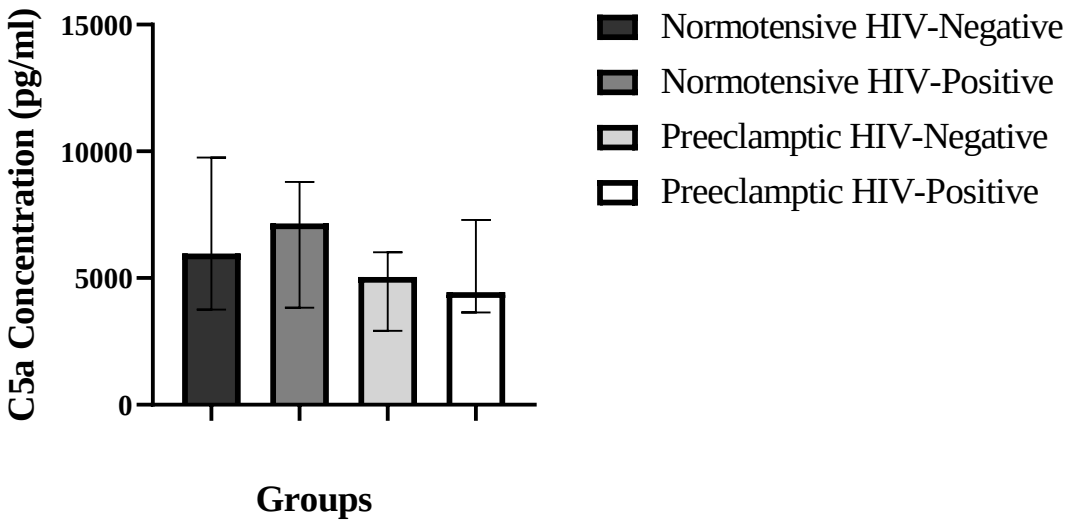
4A


*
10000
8000
6000
4000
2000
0
C5a Concentration (pg/ml)
Groups
Normotensive
Preeclamptic

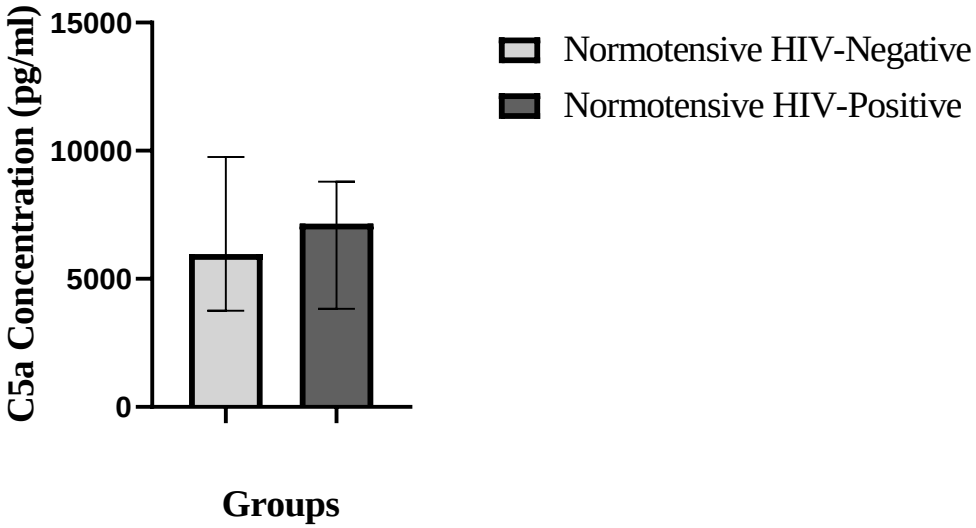
4B


10000
8000
6000
4000
2000
0
C5a Concentration (pg/ml)
Groups
HIV- Negative
HIV- Positive

4C


C5a Concentration (pg/ml)
15000
10000
5000
0
Groups
Normotensive HIV-Negative
Normotensive HIV-Positive
Preeclamptic HIV-Negative
Preeclamptic HIV-Positive

4D


C5a Concentration (pg/ml)
15000
10000
5000
0
Groups
Normotensive HIV-Negative
Normotensive HIV-Positive

4E

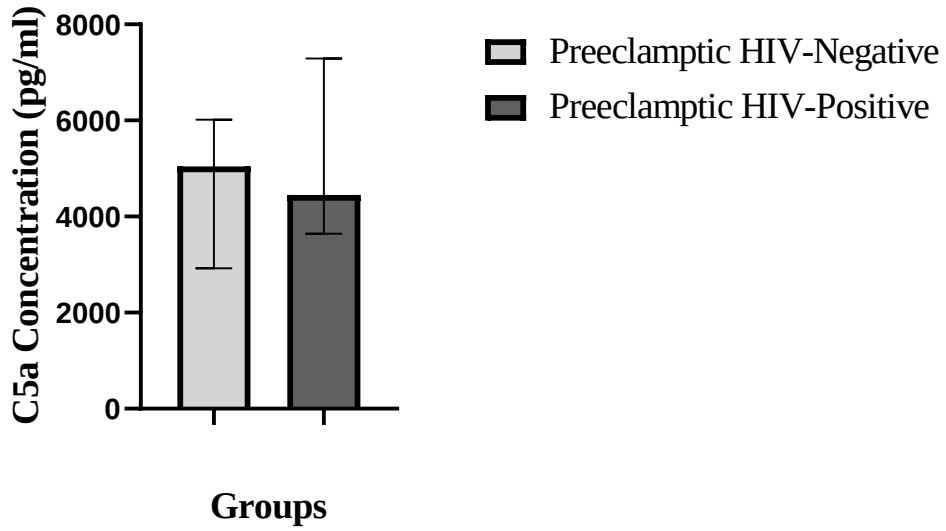


Figure 2.4 (A-E). C5a concentration are depicted in: (A) Preeclamptic *vs* Normotensive groups, (B) HIV infected *vs* HIV uninfected groups, (C) across all groups. Serum concentration of C5a are significantly different between preeclamptic and normotensive groups, $p = 0.0486$. Serum concentration of C5a have no significant difference between HIV-positive and HIV-negative groups $p = 0.8002$, as well as across all groups $p = 0.2259$ (D) Normotensive HIV-negative *vs* Normotensive HIV-positive group and, (E) Preeclamptic HIV-negative *vs* Preeclamptic HIV-positive group.

Table 2. Serum concentration (pg/ml) of complement analytes across all study groups

Total Sample Population (N=76)	**Normotensive**		**Preeclamptic**		***p*-value**
	HIV Negative (n=19)	**HIV Positive** (n=19)	**HIV Negative** (n=19)	**HIV Positive** (n=19)	
C2 (pg/ml)	24185 (19167)	24386 (35236)	22929 (21506)	22047 (20535)	0.7776 (ns)
C5a (pg/ml)	5965 (6010)	7160 (4958)	5044 (3093)	4447 (3642)	0.2259 (ns)

Results are represented as median (interquartile range)

ns = non-significant

Table 3. Spearman Rank Correlation Co-Efficient (*r*) and its level of significance (*p*) for Serum concentration (pg/ml) of C2 and C5a.

	C2 (pg/ml)	**C5a (pg/ml)**	***r* – value**	***p* – value**
Normotensive	24285 (21319)	6562 (5302)	0.08856	0.5970 (ns)
Preeclampsia	22287 (21701)	4745 (3279)	0.1534	0.3578 (ns)
HIV Negative	22929 (20473)	5548 (4726)	0.2274	0.1759 (ns)
HIV Positive	23180 (29300)	5383 (4202)	0.1089	0.5151 (ns)

Results are represented as median (interquartile range)

ns = non-significant

* $p < 0.05$

*** $p < 0.001$

Spearman's Rank Correlation Co-Efficient Analysis

Correlation between maternal demographics and serum C2 concentration:

Diastolic blood pressure correlates with C2 concentration in normotensive HIV-negative participants [r = - 0.463 ($p < 0.05$)] and in preeclamptic HIV-negative participants [r = - 0.483 ($p < 0.05$)]. A negative correlation co-efficient demonstrated a relationship between maternal age and C2 [r = - 0.482 ($p < 0.05$)] in preeclamptic HIV-positive patients. Table 4 displays the Spearman Rank Correlation Co-efficient for all other statistical non-significant maternal clinical/demographic data and C2 concentrations.

Correlation between maternal demographics and serum C5a concentration:

There was a significant correlation between gestational age and C5a concentration [r = -0.523 ($p < 0.05$)] in normotensive HIV-positive patients. Additionally, a positive correlation co-efficient r= 0.615 ($p < 0.001$) was noted between diastolic BP and C5a concentration preeclamptic HIV positive group, likewise a positive correlation was observed between systolic blood pressure and C5a concentration r = 0.483 ($p < 0.05$) in preeclamptic HIV-negative patients.

Effect of C2 and C5a on each other:

There was no statistically significant correlation of C2 on C5a concentration and vice versa (Table 3).

Table 4. Spearman's correlation coefficient (*r*) and its level of significance (*p*) for Serum concentration (pg/ml) of C2.

	Weight (kg)		**Gestational Age (weeks)**		**Parity**		**Systolic BP (mmHg)**		**Diastolic BP (mmHg)**		**Maternal Age (years)**		**Gravidity**	
	r	***p***	***r***	***p***	***r***	***p***	***r***	***p***	***r***	***p***	***r***	***p***	***r***	***p***
Normotensive HIV-Positive	0.133	0.644	-0.057	0.816	0.320	0.182	0.021	0.932	0.002	0.994	0.440	0.059	0.384	0.104
Normotensive HIV-Negative	0.090	0.715	0.209	0.390	0.001	0.997	-0.342	0.152	-0.463	0.046 *	0.314	0.191	-0.065	0.791
Preeclamptic HIV-Positive	0.049	0.842	-0.122	0.619	-0.011	0.965	-0.125	0.611	-0.051	0.836	-0.482	0.045 *	-0.011	0.964
Preeclamptic HIV-Negative	0.187	0.444	0.150	0.541	0.400	0.090	-0.054	0.827	-0.483	0.036 *	0.157	0.522	0.440	0.060

* $p < 0.05$

*** $p < 0.001$

Table 5. Spearman's correlation coefficient (*r*) and its level of significance (*p*) for Serum concentration (pg/ml) of C5a.

	Weight (kg)		**Gestational Age (weeks)**		**Parity**		**Systolic BP (mmHg)**		**Diastolic BP (mmHg)**		**Maternal Age (years)**		**Gravidity**	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Normotensive HIV-Positive	0.442	0.058	-0.523	0.022 *	0.163	0.505	-0.182	0.455	0.023	0.926	0.165	0.498	0.125	0.610
Normotensive HIV-Negative	0.206	0.397	0.318	0.185	0.341	0.154	-0.104	0.670	-0.447	0.055	0.362	0.127	-0.346	0.147
Preeclamptic HIV-Positive	-0.016	0.949	-0.027	0.912	-0.050	0.839	0.276	0.253	0.615	0.005 ***	-0.260	0.297	0.047	0.849
Preeclamptic HIV-Negative	-0.159	0.516	0.291	0.227	-0.134	0.584	0.483	0.036 *	-0.344	0.149	-0.155	0.526	-0.048	0.847

* $p < 0.05$

*** $p < 0.001$

Discussion

Our study demonstrates that a statistically significant down-regulation of serum C5a concentration in preeclamptic compared to normotensive pregnancies, irrespective of HIV status. This unexpected down-regulation of C5a in PE may be attributed to C5a being consumed faster than production, resulting in its rapid removal from circulation [36]. Alternatively, there may be a dysregulation of its receptor (C5aR) mediated affinity that facilitates signal transduction. Moreover, the knockdown of C5aR with small interfering RNA (siRNA) salvages endothelial cell migration and vessel formation [37].

Normal pregnant women show mild systemic inflammation in response to the semi-allogenic fetus whilst PE there is excessive maternal inflammation [38]. Notably, the anaphylatoxin C5a represents fragments of activated complement proteins that are the main mediators of an inflammatory response. In contrast to our finding, previous studies have reported that both the mother and fetus are exposed to significantly higher levels of this pro-inflammatory anaphylatoxin in PE [39,40].

A study conducted by Burwick *et al*. (2013) demonstrated that plasma concentrations of C5a and C5b-9 are exaggerated in PE, this results in the excretion of excess C3a, C5a, and C5b-9 in urine [36]. Richani *et al*. (2005) showed that plasma C5a concentration is elevated in normotensive pregnancies compared to non-pregnant women [12400 pg/ml (1200 – 87100 pg/ml) versus 4100 pg/ml (900 – 13100 pg/ml) respectively] [16]. In contrast, plasma C5a levels are elevated in PE compared to normotensive pregnancies [(8200 pg/ml ± 1300 pg/ml) versus (4500 pg/ml ± 500] [40]. These results differ from our study in that C5a concentration in PE was significantly lower compared to normotensive pregnancies [4746 (3368) pg/ml versus 6563 (5484) pg/ml; p= 0.2259]. The downregulation of C5a in our study may be due to sample type, as we utilized serum; the small sample size, or due to the synergy of HIV infection and PE. Furthermore, renal clearance of these circulating complement proteins occurs, due to normal plasma clearance mechanisms being overwhelmed [41]. This suggests that complement dysregulation in ongoing disease arises mainly at the level of C5 [36] and maybe a possible explanation for the downregulation of C5a during gestation as observed in PE in our study.

Notably, C5a may exert its damaging effect by inducing the release of the potent anti-angiogenic factor, soluble vascular endothelial growth factor receptor-1 (sVEGFR-1), also known as sFlt-1 [28]. It is widely accepted that PE is associated with a rise of this scavenger receptor, sFlt-1. This elevation together with a concurrent decline in VEGF and PlGF inhibits their beneficial effects on endothelial cell migration, tube formation and integrity [28]. Moreover, Govender *et al*. (2013) demonstrated

elevated serum sFlt1 and sEng in PE, regardless of HIV infection supporting an offset of the immune hyperactivity in PE [9]. sEng weakens TGF-β1 receptor transduction and prevents activation of the endothelial nitric oxide synthase 3 (eNOS) action, thereby promoting the development of hypertension. However, our study found downregulation of C5a hence a decline of sFlt-1 in PE. This confounding result may be attributed to the small sample size in our study. Additionally, our contradictory results likely emanate from drug usage in PE. In a study by Burwick and Feinberg (2013), Eculizumab a monoclonal antibody inhibitor of C5 decreases the production of complement components C5a and C5b-9 and their downstream effects [42,43].

Worldwide, C5 deficiency has been correlated with genetic defects. It is possible that a polymorphism of the C5 gene may be involved in C5a dysregulation, in fact, a complement C5 gene, c.754G>A:p.A252T mutation has been demonstrated in the Western Cape, South Africa in Black African patients infected with meningococcal disease [44-46]. A single nucleotide polymorphism in maternal C5, C5a and fetal CD55 and CD59 may constitute independent risk factors for PE development. It would be interesting to examine the C5a gene in Black African women of isiZulu origin in South Africa.

Based on HIV status, our study reports statistically similar C5a levels between HIV-negative compared to HIV-positive women, regardless of pregnancy type. It is widely accepted that this pattern recognition component is activated during HIV infection because C5a serves to attract dendritic cells and macrophages to sites of HIV entry [31]. Of note, C5aR1 is critical for viral entry [47]. In fact, the targeted reduction of C5aR1 expression reduces HIV infection by 50% because C5aR1 acts as an enhancer of CCR5-mediated HIV entry into macrophages. It is therefore plausible that a dysregulation of C5, C5aR1 concentration, or ART usage may have contributed to the similar C5a concentrations in both groups.

Nonetheless, C5a has a small amphipathic configuration with an antiviral action [48,49] that has been shown to block herpes simplex virus 1 (HSV-1), HSV-2, hepatitis C, and HIV-1 by disrupting the integrity of viral membranes [50-52]. Also, HIV induces the cleavage of C5 to generate the anaphylatoxin C5a, which attracts immature dendritic cells (DC) to promote viral amplification and dissemination [31]. A plausible explanation for the similarity between HIV-negative and HIV-positive groups in our study may be the inadequate binding of C5a to complement component 5a receptor (C5aR), mediated by antiretroviral therapy [31].

More specifically, the removal of C-terminal arginyl residues from C5a by the enzyme carboxypeptidase N converts it into a proinflammatory form called desArg C5a [53]. Desarginated C5a ($C5a^{desArg}$) is a potent stimulatory factor that primes monocyte-derived macrophages for HIV entry. Furthermore, kinetic analyses of HIV replication have shown that exposure to C5a leads to the acceleration of HIV infection in macrophages [27].

Moreover, C5a acts interdependently in the induction of pro-inflammatory cytokine release of Tumour Necrosis Factor-alpha (TNF-α), IL-1, and Interleukin 6 (IL-6) by monocytes and macrophages [54,55]. The increase of pro-inflammatory cytokines TNF-α and IL-6 have been reported in the presence of C5a, both liable for promoting HIV-1 infection and regulation [27,30]. Maharaj *et al*. (2017) reported a decrease of IL-2 and TNF-α concentrations in PE and a lower IFN-γ and IL-6 concentrations in HIV-infected preeclamptics receiving HAART relative to uninfected preeclamptic women. HIV infection together with HAART lowers the production of inflammatory cytokines during pregnancy in both successful and preeclamptic pregnancies [56]. It is plausible to hypothesize that C5a production has a directly proportional effect on the release of cytokine TNF-α and IL-6. Also, since these cytokines are reduced in HIV-infected patients, one may deduce that this may be attributed to a lower C5a level.

In addition, we demonstrate a down-regulatory trend (non-statistical significance) of C2 concentration in PE compared to normotensive pregnant women, irrespective of HIV status. This observation in PE is consistent with previous reports where C2 polymorphisms and its deficiency is linked to chronic inflammatory conditions such as SLE [57,58]. The clinical manifestations of PE mimic that of SLE. Systemic lupus erymatosus has a similar exacerbated immune microenvironment like PE, where a 10% penetrance of C2 deficiency occurs [34]. Moreover, in pregnant women with SLE, there is complement-mediated injury, predisposing them to a greater risk of PE development, placental insufficiency, miscarriage and fetal growth restriction [32].

It is widely accepted that the complement cascade is activated in PE via the LP and/or the CP [59]. Activation of these pathways are impaired in C2 deficient patient where during periods of active disease, serum complement activity is reduced emanating from a low expression of CP components (C1q, C2, C4) [60]. Also, C2 deficiency predominates in females [61]. Of note, hepatocytes synthesize 90% of plasma complement components [62]. Since liver enzyme abnormalities occur in approximately 10% of pregnant women with PE [63], it is plausible that the C2 and C5a downregulation in our study may be attributed to liver dysfunction in the PE cohort. In fact, in C5 deficient mice, the administration of murine C5 or C5a restores hepatocyte regeneration while obstruction of C5aR prevents hepatocyte proliferation [64].

Also, it is plausible to hypothesize that a domino effect takes place at the initiator part of the complement pathway prior to the generation of C3 convertase coupled with a progressively increased terminal complex formation [59]. In PE, activation of complement pathway by autoantibodies results in the production of C3b which sets in motion the powerful amplification loop of the AP [65]. C3b deposition, the release of C3a and downstream mediators, C5b-9 and C5a generate effector activity of complement function [65]. Moreover, complement component C1q plays a crucial role in trophoblast migration and spiral artery remodeling, contributing to placental development [66,67]. Notably, mice deficient in C1q are predisposed to PE development [28]. C1q deficient mice develop the characteristic features of human PE: hypertension, proteinuria, and glomerular damage [68]. From existing research, we know that the frequency of C1q, C4 deficiencies occur twice as often in preeclamptic patients compared with normal controls [69]. This may have a direct relation to C2 deficiencies observed as C2 is the split product of C1 and C4 [70].

Furthermore, a novel human complement regulatory receptor called C2 receptor inhibitor tri-spanning (CRIT), occurs on hemopoietic cells and on other tissues in the body. C2 binds to the N-terminal extracellular domain 1 of CRIT, it blocks C2 cleavage and prevents C3 convertase formation and the resultant complement-mediated inflammation [71]. It is possible that the non-statistically significant down-regulatory trend in our study may be due to the regulator CRIT and the subsequent inhibition of C3 convertase formation.

Based on HIV status regardless of pregnancy type, our study demonstrated an upregulated non-significant trend of serum C2. These results are similar to that of Mahajan *et al.* (2017) who reported *in vitro* studies of HIV-1 infected astrocytic cell lines and primary astrocytes showed significant upregulation of the complement factors C2 and C3 [72]. Huson *et al.* (2015) reported increased C3 and C1q-C4 levels in asymptomatic patients with HIV infection compared to healthy controls [35]. Even though an upregulated trend was observed in our study, the non-statistical significance found may be attributed to HAART therapy, which the standard treatment for all HIV-positive patients in South Africa (SA). We can deduce that there is a possibility that HAART lowers C2 levels to re-instate immune response [73] and therefore suggest further investigations on the effects of HAART on C2 expression.

Besides, HIV-1 increases mRNA levels of C2 in an astrocytic cell line U373 [74]. This regulation of C2 production may be attributed to HIV viral proteins Nef, Rev, and Tat on the complement promoter protein synthesis in the host cell. Furthermore, structural proteins such as gp120 and gp41 may also regulate HIV expression [75]. Also, the modulation of C2 production may be a secondary result of HIV-induced transcription factor NFκB, the main controlling factor in viral transcription.

In our study, we report a moderate correlation co-efficient between gestational age (r=-0.523), diastolic (r=0.615) and systolic blood pressure (r=0.483) with C5a concentration in normotensive HIV positive, preeclamptic HIV positive and preeclamptic HIV negative respectively. High blood pressure has been previously correlated with elevated C5a in humans [76]. Notably, the development of high blood pressure is also associated with the development of vascular damage [77,78]. In our study, gestational age was negatively correlated with C5a concentration [r = -0.523 (p < 0.05)] in normotensive HIV-positive group. Whilst normal pregnancy is associated with a generalized complement stimulation, gestational age does not correlate with the anaphylatoxin C5a [16].

We also report a moderate negative correlation between diastolic blood pressure and C2 concentration in the normotensive HIV-negative [r = - 0.463 (p < 0.05)] and preeclamptic HIV-negative [r = - 0.483 (p < 0.05)] participants. In hypertension, C3, C4 and C5 levels, all by-products of C2 correlate with development of hypertension [77]. C2 downregulation has been previously linked with Age-related Macular Degeneration and genetic polymorphisms in people of Indian ethnicity [79]. Maternal age is a predisposing factor to PE development [80]. In our study, there was no statistically significant correlation of the effect of C2 concentration on C5a and vice versa.

Limitations

Limitations of this study may have influenced analyte expression include the small sample size, all women who received ART such as HAART, and the duration of HAART.

Conclusion

This novel study reports a significant downregulation of C5a in PE. This observation suggests a loss of regulation of complement activation, dysregulation of signal transduction and/ or polymorphisms of C5a in women of African descent. We also demonstrate a non-significant distribution of C5a and C2 expression by HIV status, which may be attributed to HAART therapy. Notably, the dysregulation of complement activation will impact the host innate defence, enhancing placental and fetal injury.

Further studies should include a large cohort that investigates C2, C5a, C5a-desArg, C5aR1 and C5aR2 together with pro-inflammatory polypeptides in the development of PE. Single nucleotide polymorphisms investigations of these components are required to prevent premature delivery and enable better clinical management.

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Declaration of Interest

There are no conflicts of interest.

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CHAPTER 3

3.0 SYNTHESIS

Preeclampsia (PE) and Human Immunodeficiency Virus (HIV) infection are the leading causes of maternal morbidity and mortality in South Africa (SA). In the province of Kwa-Zulu-Natal, the prevalence of PE is 12% (Saving Mothers Report, 2017). Moreover, SA is considered the epicentre of the HIV epidemic with 13.0% of its population living with HIV infection (Stats SA, 2020). One-fifth of young females of childbearing age are HIV infected (Stats SA, 2019) hence the incidence of HIV infection comorbid with PE is high.

The opposing neutralised immune responses of HIV infection and PE are, however, are affected by antiretroviral therapy (ART) that serves to reconstitute the immune response (Pillay *et al.*, 2019). The exact pathophysiology of PE requires clarity (Thakoordeen *et al.*, 2018) however, immunologic maladaptation particularly of the complement system is one of the pathogenic developments of PE (Khan *et al.*, 2016). In light of the high prevalence of HIV infection and PE in SA; and the paucity of evidence on the immunological dysregulation of the complement system, the complement component 5a (C5a) and complement component 2 (C2) was evaluated in the synergy of HIV infection and PE using the Bio-Plex multiplex immunoassay procedure.

3.1 Pregnancy Type

C2: The main finding of our study demonstrated a statistical non-significant down-regulatory trend of C2 concentration in PE compared to normotensive pregnant women, regardless of HIV status. C2 is cleaved by C1 into C2a and C2b. C2a is an important component of C4b2a, the C3 cleaving enzyme of the classical pathway. Thus, C2 plays a vital role in generating the biological activity of C3 and C5 via C9 (Walport, 2001).

It is important to note that C2 deficient individuals have a genetic defect at 28-bp deletion at the 3' end of exon 6, this deletion causes a termination of transcription (Johnson *et al.*, 1992; Sullivan *et al.*, 1994). and commonly occurs in Caucasian's of European descent at a frequency of 0.05-0.007 (1:10000 homozygosity) This deficiency is the most common of the genetically determined complement deficiencies in Caucasians and the gene frequency of this deletion is between 0.05 and 0.007 in individuals of European descent, which translates into a prevalence of homozygotes of approximately 1 : 10 000.47,48 (Rohrer *et al.*, 2019). Moreover, this deletion is associated with a conserved MHC haplotype consisting of HLA-B18, C2*Q0, Bf*S, C4A*4, C4B*2, and Dr*2.45–47 (Winkelstein and Sullivan, 2010)

However, the non-significant C2 deficiency (down regulatory trend) observed in our study is novel as it occurred in isiZulu women of African descent. Further genetic studies are required to elucidate if this genetic defect occurs in an African population.

Also, hepatocytes are responsible for the biosynthesis of 90% of plasma complement components (Morgan and Gasque, 1997). Since liver enzyme abnormalities occur in approximately 10% of pregnant women with PE (Hammoud and Ibdah, 2014), it is plausible that the C2 deficiency in our study may be attributed to liver dysfunction in the PE cohort. Unfortunately, we do not have clinical data confirming the number of PE patients with liver dysfunction in our study.

The results of our study are also corroborated by other studies who reported that C2 deficiency is associated with an increased risk of developing immune disorders such as systemic lupus erythematosus (SLE) (Jönsson *et al.*, 2007; Truedsson *et al.*, 2007). Between 10 and 20% of individuals with C2 deficiency develop an upregulation of their immune response; notably PE represents an exaggerated inflammatory response (Buyon *et al.*, 1986). In addition, females with C2 deficiency are more likely to have an elevated immune response than their male counterparts (Jönsson *et al.*, 2007; Truedsson *et al.*, 2007). Notably, up to 30% of all lupus pregnancies are complicated by PE development (Erkan and Sammaritano, 2003; Bramham *et al.*, 2011; Schramm and Clowse, 2014).

During pregnancy, T-cells play an important role in modulating the maternal immune system as it adapts to a semi-allogeneic fetus (Santner-Nanan *et al.*, 2009). Fewer regulatory T-cells (Treg) and increased T helper-17 cell (Th17) activity have been found in women with PE (Wong *et al.*, 2008; Becker-Merok *et al.*, 2010; Tower *et al.*, 2013). Also, C2 deficient patients have a low titre of antinuclear antibody and antibodies to double-stranded DNA, whereas the presence of anti-Ro antibodies in C2 deficient SLE patients is greater than in non-C2 deficient patients (Provost *et al.*, 1983; Hauptmann *et al.*, 1988).

C5a: Our study also reports a significant downregulation of C5a concentration in PE compared to normotensive pregnant women, regardless of HIV status. This unexpected decrease of C5a observed in PE may be attributed to the strong affinity of C5a to its receptors; therefore, being cleared from circulation faster than production (Burwick *et al.*, 2013). Notably, both C3a and C5a bind to their respective receptors on placental trophoblast cells and as a result of cell damage, release vasoactive substances into circulation (Parrish *et al.*, 2010; LaMarca *et al.*, 2011; Wang *et al.*, 2012). This in turn induces excessive local placental inflammatory responses and promotes the onset of PE development.

C5 deficiency has been correlated with genetic defects worldwide. It is possible that a polymorphism of the C5 gene may be involved in C5a dysregulation, in fact, a complement C5 gene, c.754G>A:p.A252T mutation has been demonstrated in the Western Cape, South Africa in Black African patients infected with meningococcal disease (Aguilar-Ramirez *et al.*, 2009; López-Lera *et al.*, 2009; Schejbel *et al.*, 2013). A single nucleotide polymorphism in maternal C5, C5a and fetal CD55 and CD59 may constitute independent risk factors for PE development.

It is important to note that plasma C5a levels are elevated during normal pregnancies compared to non-pregnant women respectively [12400 (1200 – 87100) versus 4100 (900 – 13100) pg/ml] (Richani *et al.*, 2005). Also, maternal plasma C5a levels are elevated in the pathological state of preeclampsia compared to normotensive pregnancies [(8200 pg/ml ± 1300) versus (4500 pg/ml ± 500] (Denny *et al.*, 2013). C5a in our study was downregulated in preeclampsia compared to normotensive pregnancies [4746 (3368) pg/ml] versus [6563 (5484) pg/ml; p= 0.2259]; albeit non statistically non-significant in serum samples. Notably, the anaphylatoxin C5a represents fragments of activated complement proteins that are the main mediators of inflammatory response. Normal pregnant women show mild systemic inflammation in response to the semi-allogenic fetus whilst in PE there is excessive maternal inflammation (Chaouat *et al.*, 2013). The downregulation of C5a in our study may represent variation in the sample, as we utilized serum.

In contrast to our findings, Wang *et al.*, (2012), also demonstrated that the levels of C3a and C5a were significantly increased after the onset of PE. After the onset of PE, the complement system is activated due to local placental ischaemia and hypoxia, that produce large amounts of C3a and C5a, and the natural resultant cascade of the complement pathway accelerates the progression of PE development (Wang *et al*., 2012). Ye *et al*., (2016) also reported increased concentration of C3a in early-onset PE suggesting that complement activation does not progress beyond C3 activation, possibly because of step-specific regulators in the activation sequence. Complement activation in pregnancy is regulated by regulatory proteins such as CD46, CD55, and CD59 (Tedesco *et al.*, 1993; Ye *et al.*, 2016). In PE, expression of these regulators is reduced, leading to complement activation with resultant anaphylatoxin generation and a resulting exacerbation of the proinflammatory maternal-fetal milieu (Denny *et al.*, 2013).

It is also plausible to hypothesize that the down-regulation of C5a in our study may be caused by preeclamptic treatment. With evidence of complement activation, complement-targeted therapy is an intriguing prospect. Eculizumab is a medication approved by the US Federal Drug Administration for the treatment of PE (Krysiak *et al.*, 2015). It is a recombinant humanized IgG2/IgG4 kappa monoclonal antibody that selectively targets and inhibits the terminal portion of the complement

cascade, by binding to C5 thereafter inhibiting the downstream effects of C5a and C5b (Alrahmani and Willrich, 2018).

3.2 HIV status

C2: In our study, we report an up-regulated trend of C2 concentration (albeit non-significantly) in HIV-positive compared to HIV-negative women, irrespective of pregnancy type. These results are corroborated by the findings of Dierich *et al*., (1993), who using size exclusion chromatography showed that the complement system is activated via the alternate pathway when HIV-1 infected H9 cells are incubated in fresh human serum (Dierich *et al.,* 1993). They reported that C1q, rather than C1s directly binds to HIV-1. This suggests that HIV-1 binds to C1 via C1q, thereby inducing activation of C1, which will catalyse the activation of C4 and C2, and eventuating in the activation of C3 in the cascade (Dierich *et al.*, 1993).

In our study, the level of C2 was similar between the HIV-infected and HIV uninfected groups. This non-significant down-regulatory trend may be attributed to our small sample size and/or to the effect of ART specifically Highly Active Antiretroviral Therapy (HAART). HAART down-regulates C2 concentrations to re-establish immune response (Pillay *et al.*, 2019). Furthermore, C2 deficiency has been associated with increased susceptibility to blood-borne infections albeit pyogenic organisms (Jönsson *et al.*, 2005; Jönsson *et al.*, 2007).

C5a: In our study, we also report a statistically non-significant down-regulatory trend of C5a concentration between HIV infected compared to HIV uninfected women. Similar to C2, the statistical non-significance of C5a may also be attributed to ARV's, a standard of care practice for HIV infection in SA. Notably, C5a via its receptor (C5aR1) facilitates CCR5-mediated HIV entry into macrophages (Moreno-Fernandez *et al.*, 2016). Studies have also shown that complement opsonization facilitates viral infection of T and B cells, thymocytes and macrophage cultures (Bajtay *et al.*, 1998; Thielens *et al.*, 2002; Bánki *et al.*, 2005).

More specifically, the removal of C-terminal arginyl residues from C5a by the enzyme carboxypeptidase N converts it into desArg, a pro-inflammatory form (Zwirner *et al.*, 1998). Desarginated C5a ($C5a^{desArg}$) is a potent stimulatory factor that primes monocyte-derived macrophages for HIV entry. Furthermore, kinetic analyses of HIV replication have shown that exposure to C5a leads to the acceleration of HIV infection in macrophages (Kacani *et al.*, 2001). Furthermore, the increased secretion of pro-inflammatory cytokines correlates with higher susceptibility of macrophages to HIV infection after treatment with C5a and $C5a^{desArg}$ (Kacani *et al.*, 2001). PE is also associated with a heightened inflammatory response (Whitelaw *et al.*, 2014). Also, pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF- α) and interleukin 6 (IL-6)

have been shown to act in a positive feedback loop on HIV replication. TNF-α and IL-6 increase HIV replication and HIV infection of monocytic cells which in turn further increases the secretion of these cytokines, thereby promoting a chemotactic and proinflammatory microenvironment (Griffin *et al.,* 1989; Merrill *et al.,* 1990).

Moreover, a study by Maharaj *et al*., (2017) reported a decline in cytokine IL-2 and TNF-α levels with a concurrent decline of cytokines IFN-γ and IL-6 in HIV-positive compared to HIV-negative pre-eclamptic patients on receipt of HAART. It is plausible to assume that HIV infection combined with HAART downregulates the concentration of inflammatory cytokines in both uncomplicated and preeclamptic pregnancies (Maharaj *et al.*, 2017). We can assume that the release of cytokine TNF-α and IL-6 is dependent on the production of C5a, hence the low concentration of this complement component in our study.

In our study, there was no statistically significant correlation of C2 on C5a concentration and vice versa.

3.3 Across all groups

In the synergy of HIV infection and pregnancy, no statistical significance was noted for both C2 and C5a.

3.4 Correlation

The strength and the direction of the linear relationship between maternal demographics versus C2 levels were absent except for a moderate negative correlation between diastolic blood pressure and C2 in the normotensive HIV-negative [r = - 0.463 ($p < 0.05$)] and preeclamptic HIV-negative [r = - 0.483 ($p < 0.05$)] participants. This implies that C2 downregulation/deficiency correlates with an increase in blood pressure, supportive studies show that non-pregnancy-related hypertension is associated with high circulating levels of C3, C4 and C5 (Ruan and Gao, 2019).

In our study we also observed a negative correlation of C2 concentration [r = - 0.482 ($p < 0.05$)] with maternal age in preeclamptic HIV-positive patients. Whilst not in pregnancy a previous study investigated the association of C2 with age-related Macular Degeneration in an Indian cohort (Kaur *et al.*, 2010). The latter study illustrates that 3 single nucleotide polymorphisms in the C2 gene were associated with a reduced risk of Age-related Macular Degeneration (Kaur *et al.*, 2010). Age is a risk factor for PE development (Whitelaw *et al.*, 2014).

Moreover, our study reports a relationship between diastolic blood pressure and C5a [r= 0.615 (p < 0.001)] in preeclamptic HIV positive women, likewise between systolic blood pressure and C5a in preeclamptic HIV-negative patients [r = 0.483 (p < 0.05)]. Our findings are corroborated by Zhang et al. who reported a correlation between C5a and high blood pressure in humans (Zhang *et al.*, 2014). This activation of this anaphylatoxin promotes the progression of vascular injury and the development of hypertension (Ruan and Gao, 2019; Wenzel *et al.*, 2020). In our study, gestational age was negatively correlated with C5a concentration [r = -0.523 (p < 0.05)] in normotensive HIV-positive group. In contrast despite the generalized complement activation in normal pregnancy, the concentration of C5a was not affected by gestational age (Richani *et al.*, 2005).

3.5 Limitations

Limitations of this study is the small sample size and the fact that all HIV participants were treated with ART's, the duration of which was not known.

3.6 Conclusion

This novel study demonstrates a statistically significant downregulation of the anaphylatoxin C5a within the serum of preeclamptic compared to normotensive pregnant women, regardless of HIV status. C5a is a pro-inflammatory polypeptide generated in response to complement activation. The down-regulation in our study suggests a loss of regulation of complement activation in the hypoxic oxidative stressed microenvironment of PE. It may also reflect signal transduction inhibition. In this study, we also report no statistical significance of both C5a and C2 concentration between HIV-infected versus uninfected women, regardless of pregnancy type. This similarity may be attributed to HAART, duration of ARV usage and/or PE drug management. It is also plausible that mutation in genes and encoding C2 and C5a may be directly associated with PE development. Notably, host innate defence is impacted by the dysregulation of complement activation, this is further responsible for the augmentation of placental and fetal injury.

3.7 Future Direction

Further studies should include a large cohort that investigates C5a, C5a-desArg, C5aR1 and C5aR2 together with pro-inflammatory polypeptides in the development of PE. Large-scale prospective studies are required to establish the role of C5a and C2 in the synergy of HIV and PE, thereby preventing premature delivery and enabling better clinical management of the mother and neonate.

CHAPTER 4

4.0. REFERENCES

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APPENDIX

Appendix 1A – Ethics Approval

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

[redacted]

……
Ms A Marimuthu
(for) Prof D Wassenaar

Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee

Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

INSPIRING GREATNESS

Appendix 1B – Ethics Approval

Institutional class ethics approval (BCA338/17) was obtained from UKZN BREC prior to the initiation of this project. This enabled the student to utilize stored serum samples. The primary class approval authorizes the use of archived samples stored at the Optics and Imaging Centre; hence we had obtained institutional approval on behalf of the participants.

Appendix 2

Methods and Materials:

Ethical Approval

This prospective study utilized retrospectively collected serum samples for which institutional ethics class approval (BCA 338/17; Appendix 1) was obtained. Informed patient consent, hospital manager's approval and the regulatory authority consent was obtained in the primary study for use of the samples in subsequent studies project.

Sample Size

Post consultation with an institutional biostatistician (Ms. Fikile Nkwanyana), sample size was calculated. To detect a moderate effect size of 0.66 between two groups normotensive and preeclamptic women or HIV positive and HIV negative assuming equal groups (n=38 per group), a sample size of 76 pregnant women was required. To compare four groups, normotensive (HIV+ vs HIV-) and preeclamptic (HIV+ vs HIV-), a sample size of 19 in each group was needed to detect a large effect size of 0.95. All calculations are with 80% power and 95% probability and were done using G*Power statistical software.

Study population

A study population (n=76; Figure 4.1) was recruited from a large regional hospital, consisting of 38 normotensive and 38 preeclamptic women. Both groups are further stratified by HIV status into HIV-positive preeclamptic (n=19), HIV-negative preeclamptic (n=19), HIV-positive normotensive pregnancy (n=19) and HIV-negative normotensive pregnant women (n=19).

Inclusion Criteria: this study group consisted of primigravid and multigravida participants, diagnosed with PE (≥ 140/90 mmHg and/or the presence of a single incidence of proteinuria) [4], and participants with a normotensive pregnancy serving as the control group. All HIV-positive women received antiretroviral therapy (ART).

Exclusion Criteria: women with polycystic ovarian syndrome, intrauterine death, cardiac disease, chorioamnionitis, unknown HIV status, eclampsia, sickle cell disease, active asthma that requires medication during the gestational period, abruption placentae, chronic renal disease, patients who have been declined from participation, systematic lupus erythematosus, pre-existing seizure disorders, and thyroid disease were not included in the study group.

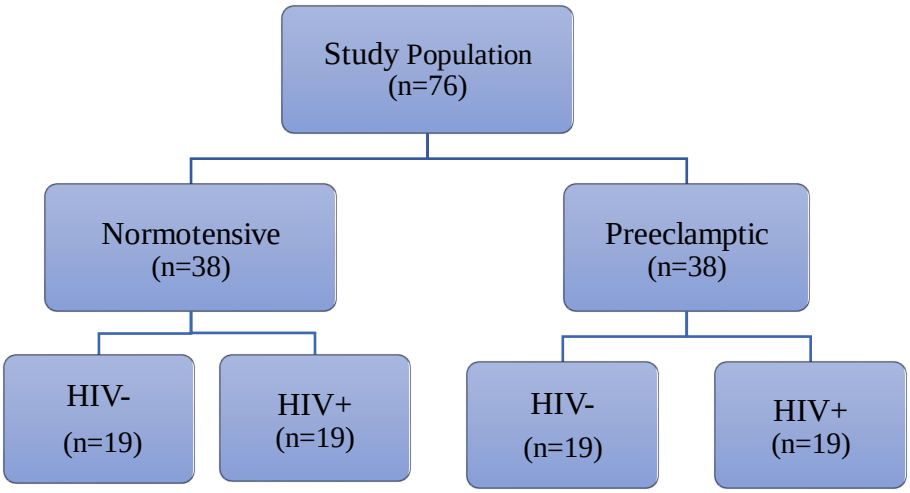


Figure 4.1: Schematic illustration of the study population

Data and Sample Collection Methods and Tools

Patient demographic data were obtained in the labour ward and antenatal clinic. Data was entered into data forms and transposed onto an excel spreadsheet. This data comprised of maternal age, gestational age, HIV status, CD4 count, HIV treatment, gravidity, parity, weight, height, blood pressure, proteinuria, obstetric and neonatal outcomes as well as neonatal and maternal complications. All patient identities were replaced with codes. Reports on the study protected confidentiality and all participants remained anonymous.

Sample type

Blood samples were previously collected in EDTA-coated vacutainer tubes by the research nurse during gestation and centrifuged at 3000 g for 10 minutes at 20°C. Serum was aliquoted and stored at -80°C until required.

Principles of the Multiplex Immunoassay

The Bio-Plex® multiplex immunoassay system enables the multiplexing of different analytes (C2 and C5a) within a single sample. This technique involves coloured bead sets created by the use of two fluorescent dyes at specific ratios. These beads are then conjugated with a reagent particular to a specific bioassay. The technology enables multiplex immunoassays in which one antibody to a specific analyte is attached to a set of beads with the same colour, and the second antibody to the

analyte is attached to a fluorescent reporter dye label. The use of different coloured beads enables the simultaneous multiplex detection of many other analytes in the same sample. (Figure 1).

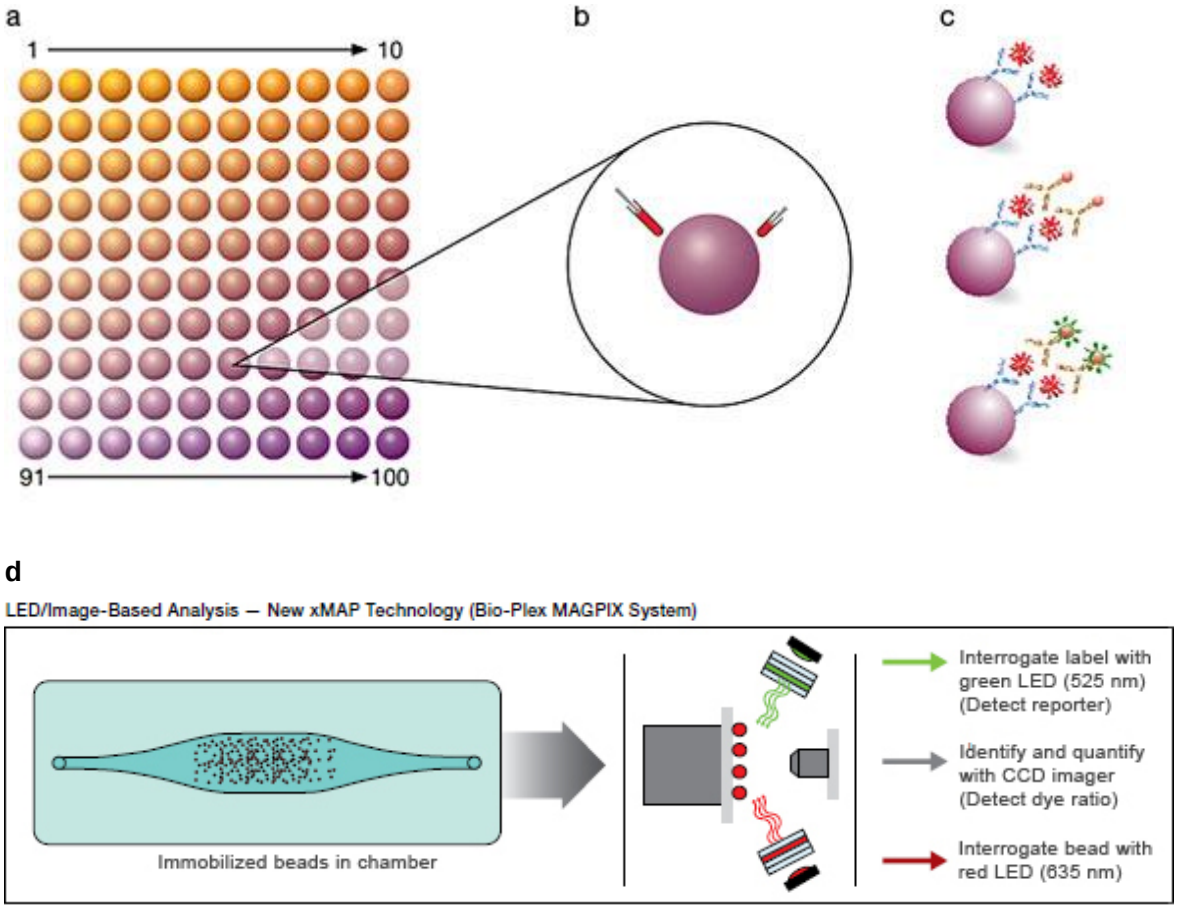


Figure 4.2. Multiplex immunoassay technology. Beads are coloured internally with two different fluorescent dyes (red and infrared). Different concentrations of red and infrared dyes are used to generate up to 100 distinct bead regions. Each bead region is conjugated to a specific target analyte (a) followed by binding with a biotinylated detection antibody (b), a reporter dye, streptavidin-conjugated phycoerythrin (c) and an analysis (d).

During data acquisition, the contents of each microplate well are drawn into the array reader, depending on the light-emitting diode (LED)/image-based analysis in the Bio-Plex® MAGPIX™ multiplex reader and magnetically immobilized. The software recorded the fluorescent signals simultaneously for each bead, translating the signals into data for each bead-based assay (Figure 4.2 d).

Bio-Plex Multiplex (Bio-Rad) Immunoassay

A MILLIPLEX MAP Human Complement Panel 1 - Immunology Multiplex Assay was performed according to the manufacturer's instructions (Millipore by Sigma-Aldrich, catalog no: HCMP1MAG-19K). Blank captured antibody with magnetic beads, C5a and C2 samples, antigen samples (1:4 dilution), and standards (serial dilution) were incubated. A triple wash eliminated any unbound substances. Prior to the incubation of the assay plate, a biotinylated detection antibody was added. Once the incubation period was over. A triple wash using wash buffer was once again performed to ensure the removal of unbound biotinylated detection antibodies. Thereafter, into each well, 1x streptavidin-phycoerythrin (SA-PE) was added. The plate was thereafter incubated for 10 min at 850 ± 50rmp in a dark room. The assay plate was washed 3 times with wash buffer and resuspended in assay buffer for 30 seconds at 850 ± 50rmp. Lastly, a Bio-Plex®MAGPIX™ Multiplex Reader (Bio Rad Laboratories, Inc., USA) was used to read the assay plate.

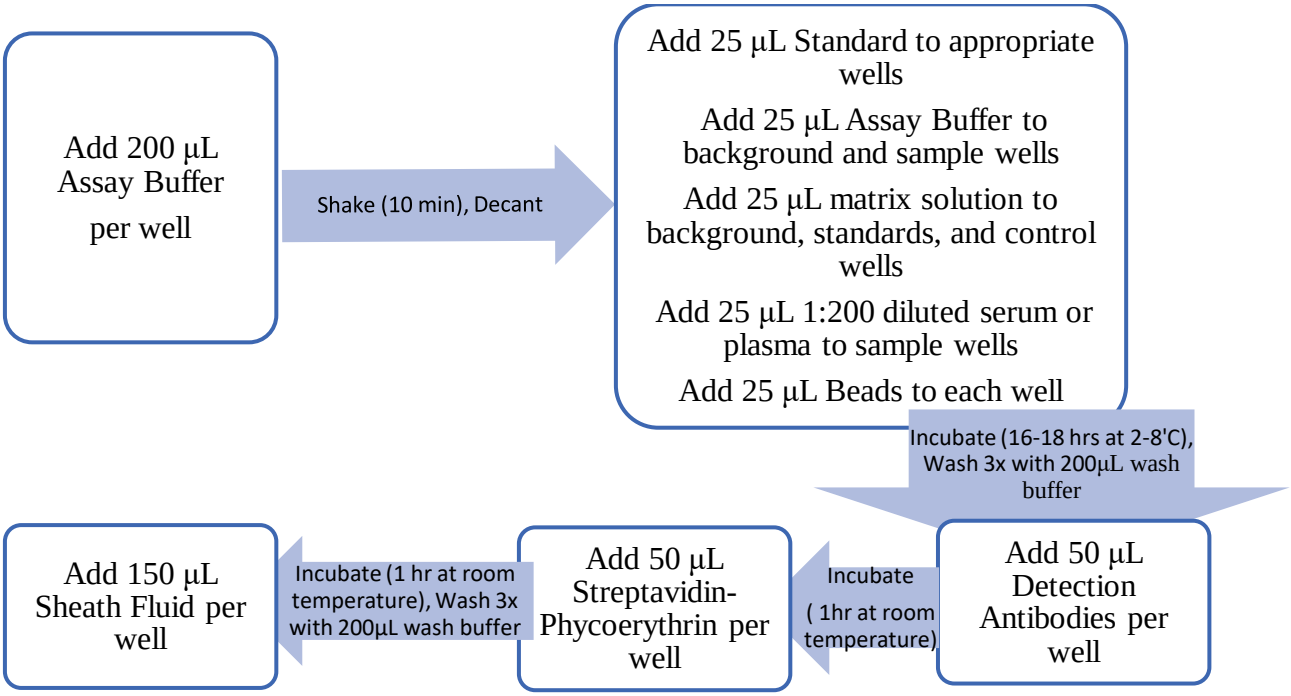


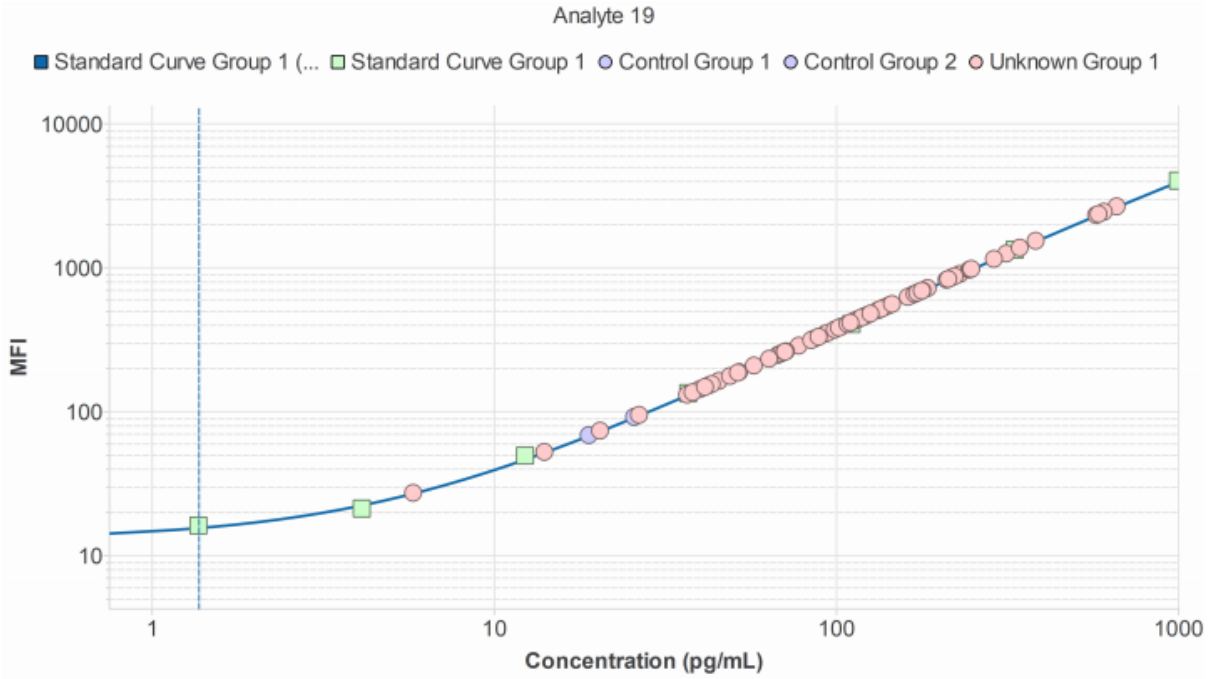
Figure 4.3. Bio-Plex Multiplex immunoassay procedure.

Statistical Analysis

Data were statistically analysed utilizing GraphPad Prism 5.00 for Windows (GraphPad Software, San Diego California USA). The Kolmogorov Smirnov normality test was used to check for parametric or non-parametric distribution. Non-parametric data are represented as median and interquartile range. Statistical significance according to pregnancy type (preeclamptic *vs*

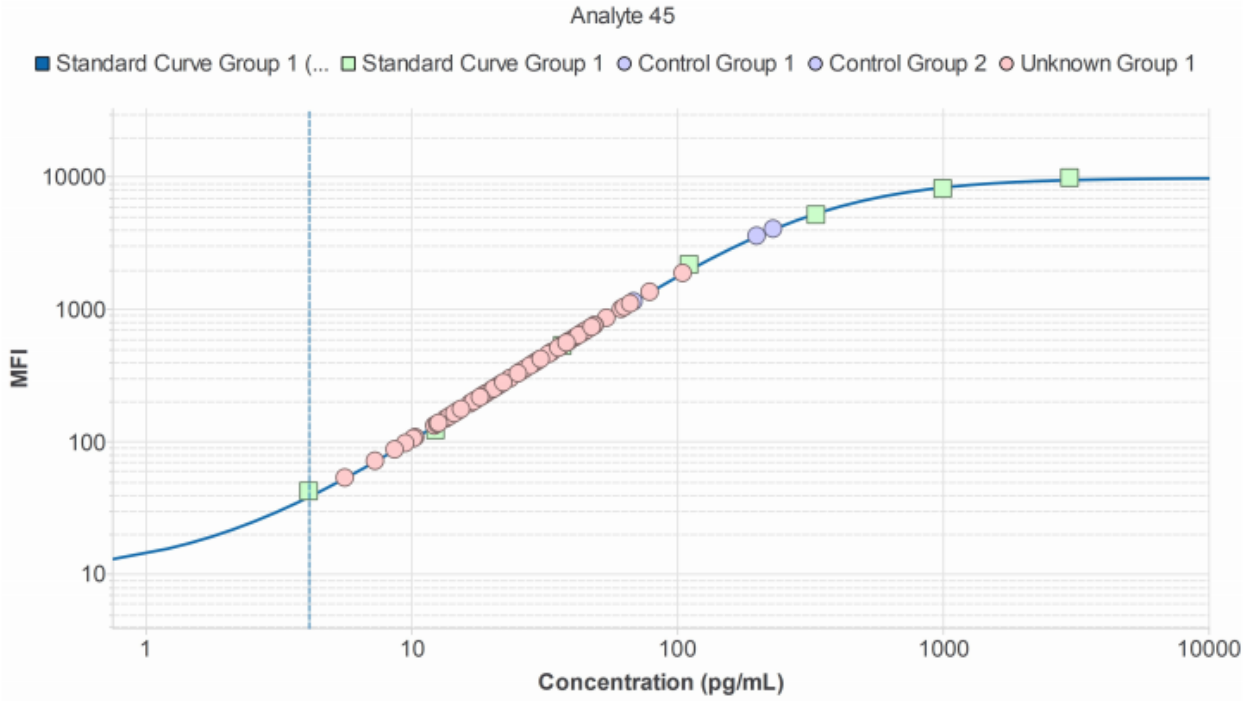
normotensive) and HIV status (negative *vs* positive) was determined using a Mann-Whitney's *U* test. The Dunn's Multiple Comparison *post hoc* test and the Kruskal-Wallis test determined statistical significance across all groups. A *p*-value of <0.05 was considered to be statistically significant. Spearman's Rank Correlation Coefficient (*r*) was calculated to determine the relation between clinical/ demographic data versus C2 and C5a concentrations across the study population (-1 and 1).

Appendix 3 – Standard curve C2



Fit	LLoQ	MDD	LoD	R Squared	Slope	Weighting	Equation
5PL	1.37	N/A	N/A	1.00	3.33	1/y^2	y = 12.96 + (269573.78 - 12.96)/((1 + (22356.29/x)^0.47))^2.54

Appendix 4 – Standard curve C5a



Fit	LLoQ	MDD	LoD	R Squared	Slope	Weighting	Equation
5PL	4.12	N/A	N/A	1.00	9.60	1/y^2	y = 10.13 + (9767.03 - 10.13)/((1 + (319.41/ x)^1.44))^0.93

Appendix 5 – Turn it in Receipt

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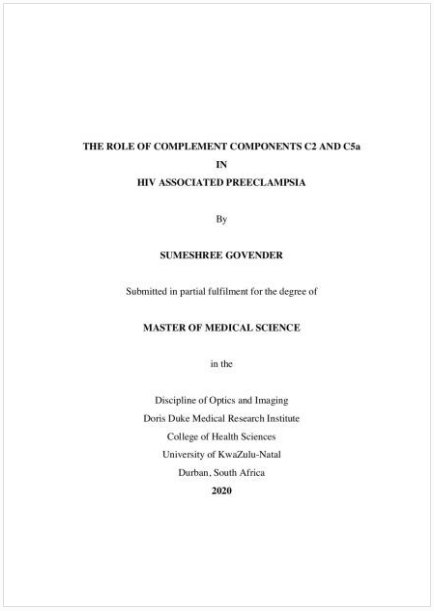


THE ROLE OF COMPLEMENT COMPONENTS C2 AND C5a
IN
HIV ASSOCIATED PREECLAMPSIA

By

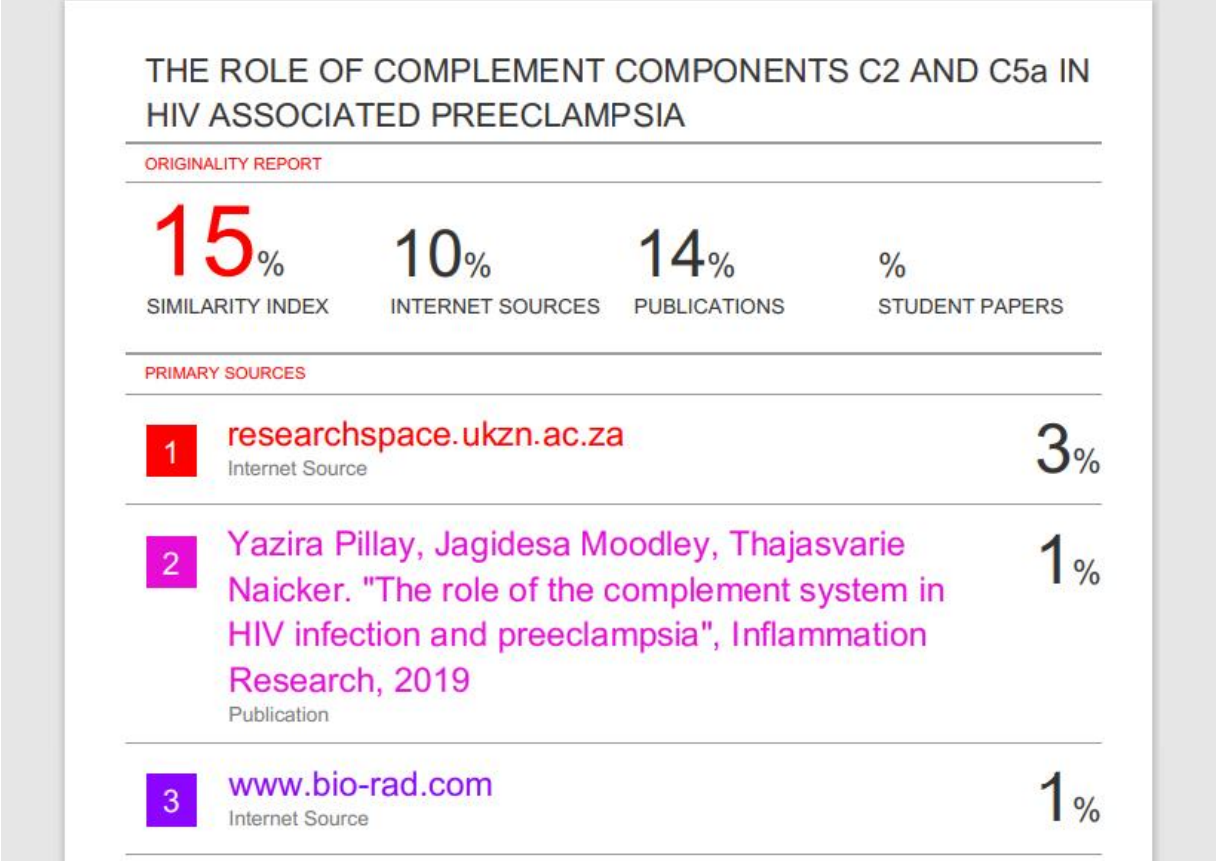
SUMESHREE GOVENDER

Submitted in partial fulfilment for the degree of

MASTER OF MEDICAL SCIENCE

in the

Discipline of Optics and Imaging
Doris Duke Medical Research Institute
College of Health Sciences
University of KwaZulu-Natal
Durban, South Africa
2020



THE ROLE OF COMPLEMENT COMPONENTS C2 AND C5a IN HIV ASSOCIATED PREECLAMPSIA

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