**THE ROLE OF C- REACTIVE PROTEIN AND SERUM AMYLOID A IN**

**HIV ASSOCIATED PRE-ECLAMPSIA**

By

**AKHONA SIKUTSHWA**

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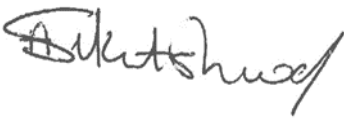
University of KwaZulu-Natal

Durban, South Africa

**2017**

**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 Thajasvarie Naicker.

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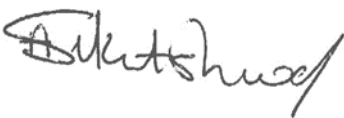       Akhona Sikutshwa                                             Professor Thajasvarie Naicker

   (Student number: 210522713)                                   (Supervisor)

# DECLARATION

I, **Akhona Sikutshwa** declare that:

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# DEDICATIONS

To God- None of this would've been possible without you! Thank you for this opportunity and giving me

the strength to go on

My beautiful Mother- Oh mama! What a phenomenal woman you are! You have been with me at every

moment of this journey. Your love and prayers have been the most amazing support for me. Each day you

continue to inspire me. I love you and with this, I hope I have made you proud.

My family and friends- I express my deepest love and appreciation for your incredible support,

motivation and for always believing in me. I love you

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# LIST OF ABBREVIATIONS

PE       -Pre-Eclampsia

HIV      -Human Immunodeficiency Virus

N-       -Normotensive HIV Negative

N+        -Normotensive HIV Positive

PE-      -Pre-eclamptic HIV Negative

CRP      -C-reactive Protein

SAA      -Serum Amyloid A

IL-6     -Interleukin 6

IL-8     -Interleukin 8

TNF-α  -Tumour necrosis factor –α

VEGF   -Vascular Endothelial Growth Factor

sFlt-1   -Soluble Fms–Like Tyrosine Kinase-1

PlGF    -Placental Growth Factor

sEng    -Soluble Endoglin

AIDS    -Acquired Immunodeficiency Syndrome

HAART -Highly Active Antiretroviral Therapy

pg/ml    -picogram per milliliter

mmHg  -millimeters mercury

BP       -blood pressure

# ABSTRACT

**Introduction**

Pre-eclampsia, an obstetric disorder specific to human pregnancy remains a major cause of maternal mortality and morbidity globally. The acute phase proteins, C-reactive protein (CRP) and serum amyloid A (SAA) and other known markers of inflammation have been implicated in the development of pre-eclampsia. However, a dose-response relationship between PE and inflammation remains to be elucidated. This study aims to assess CRP and SAA levels in HIV-associated pre-eclamptic pregnancies.

**Method**:

The study population (n = 76) was divided based on pregnancy type ie., normotensive (n = 38) and pre-eclamptic (n = 38) and further stratified by HIV status. CRP and SAA levels were quantified using a Bioplex immunoassay.

**Results**:

CRP was significantly decreased in pre-eclamptic compared to normotensive pregnancies, irrespective of HIV status $(p = 0.0073)$. Based on HIV status, there was an upregulation of CRP levels albeit non-significantly, regardless of pregnancy type $(p = 0.74)$. In contrast, SAA did not differ by pregnancy type, HIV status and across all study groups respectively $(p = 0.07; p = 0.44; p = 0.76)$. However, a slight increase of SAA levels albeit non-significantly was noted in pre-eclamptic versus normotensive pregnancy. Furthermore, although serum SAA levels did not differ based on HIV status and across all study groups, an increasing trend was noted with regards to HIV status *ie.*, in HIV- positive *vs* HIV -negative and PE-positive *vs* PE-negative pregnant groups.

**Conclusion**

This study demonstrates significant reduction of CRP and with a concomitant non-significant elevation of SAA in PE versus healthy pregnant women. Decreased CRP is suggestive of a neutralized immune response in HIV associated pre-eclampsia.

# CHAPTER ONE

# 1. INTRODUCTION

## 1.1 Maternal Mortality: A Global Crisis

Over the years maternal mortality rates have decreased, however, this still remains a major global concern. Every day women die from pregnancy related complications and during childbirth. The global estimate of women dying from complications arising during and after child birth and pregnancy is approximately 303 000 deaths (WHO, 2015). While most of them can be prevented, approximately 99% of all maternal deaths occur in developing countries (WHO, 2015). In developing countries, it is estimated that a woman faces a risk of about 33 times greater of dying from pregnancy-related complications in her lifetime, in comparison to another woman residing in a developed country (WHO, 2015). More than half of all maternal deaths occur in Sub-Saharan Africa, with about 546 maternal deaths per 100 000 live births per year (WHO, 2015). Of this, 138 deaths per 100 000 live births occur in South Africa which is a middle-low income country (WHO, 2015).

Obstetric haemorrhage is the major cause of maternal mortality contributing 27 % of all maternal deaths worldwide, whilst in South Africa it accounts for 16%, followed by hypertensive disorders (14%). Non-pregnancy related infections account for 34.7 % of all maternal deaths (Mothers, 2014).

## 1.2 Human Immunodeficiency Virus Infection (HIV): A Global Pandemic

The HIV epidemic remains a major health concern globally and continues to grow. In the year 2015, there was a global estimate of 2.1 million new HIV infections resulting to a total of 36.7 million people living with HIV (UNAIDS, 2016). Additionally, 1.1 million deaths occurred due to HIV-related illness in the same year (UNAIDS, 2016). Sub-Saharan Africa is dubbed as the epicenter of this global epidemic with approximately 7 million people living with HIV in South Africa alone (UNAIDS, 2016).

### 1.2.1   HIV and Women

HIV and pregnancy-related illnesses are the leading cause of maternal deaths worldwide. This remains a serious obstetric dilemma as it has devastating consequences on women of reproductive age (Mothers, 2014).Young women face a threefold risk of HIV infection and specifically in South Africa; the prevalence of HIV infection is greater in young women aged 15-24 years compared to their male counterparts (UNAIDS, 2016).

In South Africa, non-pregnancy related illnesses (mostly being HIV) account for 35.8% of all maternal deaths as well as a HIV prevalence rate of 37.7% in pregnant women in the KwaZulu-Natal province. Furthermore, women of reproductive age contribute a HIV incident rate of 18.8% in the same province (Saving Mothers Report, 2014, Department of Health SA, 2012).

### 1.3   Hypertension

Hypertensive disorders along with haemorrhage and infection are the cause of the majority of all maternal deaths and morbidity. Specifically, women younger than 20 years of age face a greater risk of death as a result of hypertension during pregnancy. In South Africa, 14.8% of maternal deaths is attributed to hypertension during pregnancy, with 83% these pregnancies complicated by pre-eclampsia (PE) (Thakoordeen, 2017). KwaZulu-Natal has a PE prevalence rate of 12% which makes this province an ideal location for conducting a study involving HIV and PE.

## 2.   PRE-ECLAMPSIA

### 2.1 Definition

Pre-eclampsia is a common disorder specific to human pregnancy which clinically presents with an elevated blood pressure (systolic blood pressure $\geq$ 140 mmHg and/or diastolic blood pressure $\geq$ 90 mmHg) and

proteinuria ($\geq$ 300 mg per 24 hours) after 20 weeks of gestation or during labour and/or postpartum (Gathiram and Moodley, 2016, Kalumba *et al.*, 2013)

Additionally, PE presents with many other clinical manifestations and serum markers for haemolysis, coagulation, oedema, liver and renal organ impairment (Engin-Üstün *et al.*, 2007, Gathiram and Moodley, 2016). Furthermore, if untreated, this multi-systemic syndrome can progress to eclampsia with presentation of severe symptoms including seizures and or cortical blindness following an asymptomatic period (Håpnes, 2014). This particularly poses a major threat to both the mother and baby as it may result in maternal and perinatal death (Håpnes, 2014).

Pre-eclampsia is characterized into two types namely; early onset pre-eclampsia (EOPE) and late onset pre-eclampsia (LOPE) in which clinical manifestation occurs at 33 weeks and both at and after 34 weeks' gestation respectively (Raymond and Peterson, 2011). Most of the pre-eclamptic patients are diagnosed with LOPE; however, the high maternal and neonatal mortality and morbidity rates are attributable to EOPE (Gathiram and Moodley, 2016).

Although the exact cause of PE remains unclear, it is postulated to be caused by an excessive maternal inflammatory response to pregnancy which is associated with reduced placental perfusion and an increased maternal inflammatory response which leads to widespread endothelial dysfunction (Jeyabalan, 2013, Kristensen *et al.,* 2009). The only known cure for this disorder is placental and fetal delivery; however, this may not always be the best of options at the time of diagnosis. Administration of treatment to pre-eclamptics is greatly dependent on the severity of the disorder and gestational age (Chaiworapongsa *et al.,* 2014a). Corticosteroids and anticonvulsants may be administered to mature the foetal lungs and the latter to prevent seizures in the event of possible eclampsia. Additionally, treatment may also include medication to lower blood pressure (Chaiworapongsa *et al.,* 2014a, Steegers *et al.,* 2010)

**2.2 Epidemiology**

Pre-eclampsia complicates approximately 3-7% of pregnancies globally and is the leading cause of maternal and perinatal deaths due to hypertensive disorders (Sansone et al., 2016). The incidence of PE in developing countries is about seven times greater than in developed countries (WHO, 2015). The majority of maternal deaths are said to be caused by eclampsia rather than pre-eclampsia especially where maternal mortality rates are high (Jeyabalan, 2013). Specifically in KwaZulu-Natal, South Africa, the prevalence of PE is approximately 12% (Thakoordeen et al., 2017).

**2.3 Risk Factors**

The wide spectrum of risk factors associated with PE is largely reflected in the epidemiology of the disease. These risk factors are generally grouped into pregnancy-specific and maternal pre-existing characteristics. Pregnancy related risk factors include new paternity, primiparity and excessive placental volume. Nulliparity has been said to increase the risk of PE by almost three times (Jeyabalan, 2013, Chaiworapongsa *et al.,* 2014a). Maternal characteristics are inclusive of age, race, smoking, obesity, a family history of PE and pre-existing conditions such as hypertension, obesity and vascular disorders (Jeyabalan, 2013, Chaiworapongsa *et al.,* 2014a, Håpnes, 2014).

**3. PATHOGENESIS OF PRE-ECLAMPSIA**

Despite years of research, the exact cause of PE remains obscure. It is however postulated to be part of an exaggerated maternal immune response initiated by abnormal placentation. PE is associated with reduced placental perfusion possibly as a result of placental hypoxia, inadequate trophoblast invasion and defective maternal vascular transformation (Gathiram and Moodley, 2016, Jeyabalan, 2013, Chaiworapongsa *et al.,* 2014a). This is followed by the release of excess placental debris and various substances including apoptotic cells, proinflammatory cytokines, angiogenic [vascular endothelial growth factor (VEGF), placental growth

factor (PIGF)] and anti-angiogenic [soluble fms-like tyrosine-1(sFlt-1), soluble endoglin (sEng)] factors into the maternal circulation (Kalumba *et al.,* 2013, Gathiram and Moodley, 2016). In normal pregnancy, adequate placentation and angiogenesis is achieved through a state of equilibrium between angiogenic and anti-angiogenic factors. In PE however, the levels of circulating sFlt-1 are elevated while VEGF and PlGF levels are decreased therefore resulting in a shift in the angiogenic-antiangiogenic balance. Consequentially, this results in endothelial dysfunction with hypertension and proteinuria, the clinical symptoms of the disorder (Levine *et al.,* 2004 and Mbhele *et al.,* 2017)

## 4  INFLAMMATION AND PRE-ECLAMPSIA

More recently, an exaggerated maternal inflammatory response has been proposed as one of the causes of pre-eclampsia. However, the elucidation of a cause-effect association between the two phenomena has not been established (Ramma and Ahmed, 2011). Nonetheless, elevated levels of proinflammatory cytokines have been linked with decreased angiogenesis, reduced renal function, elevated blood pressure and endothelial dysfunction, all which have been implicated in the pathogenesis of PE (Teran, 2001, Udenze, 2015). Furthermore, studies using animal models have also shown abnormal expression of cytokines are with clinical signs of hypertension, proteinuria and decreased production of nitric acid (Mustafa, 2012, Ramma and Ahmed, 2011).

## 5    INFLAMMATORY MARKERS

### 5.1   C-Reactive Protein

The pentraxin, C-reactive protein (CRP) is a 224 amino acid residue protein with a molecular mass of approximately 25k Daltons (Saljoughian, 2008). It is a calcium dependent binding protein with specificity for phosphocholine residues on microorganisms (Chandrashekara, 2014). CRP was first described in 1930 by William Tillet and Thomas Francis (Gruys *et al.,* 2005) through its binding with the C-polysaccharide

of a pneumococcus bacterium in the serum of patients with an acute inflammation (Saljoughian, 2008). It is a positive acute phase protein and important marker of overall systemic inflammation whose concentrations rise dramatically in response to inflammation, infection, tissue injury and trauma (Chandrashekara, 2014, Gruys *et al.,* 2005). CRP is expressed mainly in liver hepatocytes in response to proinflammatory cytokines, interleukin-6 and tumour necrosis factor-α following stimulation by macrophages. Lymphocytes, monocytes, Kupffer cells and neurons can also produce this protein, however, in smaller concentrations (Kristensen *et al.,* 2009, Chandrashekara, 2014). CRP binding to pathogens results in activation of the complement system thereby promoting opsonisation and clearance of the pathogen (Gruys *et al.,* 2005).

## 5.2    Serum Amyloid A

Human Serum Amyloid A (SAA) is a member of a multigene family of four genes all located on chromosome 11 (Urieli-Shoval, 2000). The four genes encode the isoforms, SAA1 and SAA2 which are predominantly synthesized during the acute phase response, SAA3, a pseudogene and SAA4 which is constitutively produced in normal tissue and cells such as the large intestine, endothelial cells, spleen etc. (Hua *et al.,* 2009, Sun, 2015). SAA differs from its acute-phase homologue CRP, in that it has a half-life of one day and diminishes rapidly in serum (Hua *et al.,* 2009). SAA is also a high-density apolipoprotein involved in the cholesterol transport system during inflammation. This protein is known to have many functions which include adhesion to and migration of monocytes, modulation of neutrophils, upregulation of metalloproteinase, production of collagen, chemoattractant function and inhibition of the respiratory burst of leukocytes (Gruys *et al.,* 2005, Bargagli *et al.,* 2011, Erdoğan *et al.,* 2012). SAA is also involved in the development of systemic Amyloid A amyloidosis (AA amyloidosis), a complication of rheumatoid arthritis where it acts as a precursor protein of amyloid AA fibrils (Bargagli *et al.,* 2011).

Both CRP and SAA correlate with other known markers of inflammation. However, data on the role of these acute phase proteins in the pathogenesis of PE is contradictory (Kristensen, 2009, Erdoğan et al.,

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2012). Altered expression of these markers have been demonstrated in pre-eclamptic versus normotensive pregnant women (Hubel, 2008, Engin-Üstün et al., 2007, Qiu et al., 2004). In contrast, other studies have reported decreases and similarities in pre-eclampsia (Kristensen, 2009, Erdoğan et al., 2012). Moreover, the inhibition of nitric oxide synthesis in pregnant rats, due to excessive inflammation, causes proteinuria and intensifies endotoxin-induced glomerular thrombosis (Teran, 2001). Similarly, elevated CRP levels infused into mice were shown to cause hypertension, proteinuria, glomerular endotheliosis and atherosclerosis in the placenta of mice (Mohaupt, 2015). Furthermore, increased SAA levels occur in many inflammatory disorders such as atherosclerosis and thrombosis implicating its potential role in endothelial dysfunction and in the development of cardiovascular disease (Hua et al., 2009, Sun, 2015). Taken together, these findings suggest a potential pathogenic role of CRP and SAA and other markers of inflammation in the development of PE. Thus, in the duality of the high maternal deaths due to HIV infection and PE in South Africa, the role of these acute phase proteins in HIV associated PE is worthwhile being investigated.

**6. AIM**

To evaluate the concentration of the acute phase proteins, serum C-reactive protein and serum amyloid A in HIV-associated pre-eclamptic pregnancy

**6.1 Specific Objectives**

- To determine serum C-reactive protein and serum amyloid A levels in normotensive versus pre-eclamptic women, irrespective of their HIV status

- To determine serum C-reactive protein and serum amyloid A levels in HIV-infected versus uninfected women, regardless of pregnancy type (normotensive versus pre-eclampsia)

- To determine serum C-reactive protein and serum amyloid A levels across the study groups

**CHAPTER TWO**

**MANUSCRIPT**

**The Role C-Reactive Protein and Serum Amyloid A in HIV-associated Pre-Eclampsia**

**To be submitted to the Journal of Hypertension in Pregnancy**

**TITLE**

**THE ROLE OF C-REACTIVE PROTEIN AND SERUM AMYLOID A IN HIV ASSOCIATED PRE-ECLAMPSIA**

**Authors:**  [a]Akhona Sikutshwa, [b]Jagidesa Moodley and [a]Thajasvarie Naicker

**Affiliations:**  [a]Optics and Imaging Centre, Doris Duke Medical Research Institute, College of Health Sciences, University of KwaZulu-Natal, Durban, KwaZulu-Natal, South Africa, [b]Women's Health and HIV Research Group, Department of Obstetrics and Gynaecology, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, KwaZulu-Natal, South Africa

**\*Corresponding author:**

Akhona Sikutshwa

Optics & Imaging Centre,

Doris Duke Medical Research Institute,

College of Health Sciences,

University of KwaZulu-Natal,

Durban, South Africa.

Postal address:  Private Bag 7
Congella
KwaZulu-Natal
4013
South Africa

Email address: dantesikutshwa@gmail.com; naickera@ukzn.ac.za

**ABSTRACT**

***Objective***: Several inflammatory markers have been implicated in the development of pre-eclampsia. This study aimed to assess C-reactive protein (CRP) and serum Amyloid A (SAA) levels in HIV-associated pre-eclamptic pregnancies. ***Method***: The study population (n = 76) was divided based on pregnancy type ie., normotensive (n = 38) and pre-eclamptic (n = 38) and further stratified by HIV status. CRP and SAA levels were quantified using a Bioplex immunoassay. ***Results***: CRP was significantly different in pre-eclampsia compared to normotensive pregnancies, irrespective of HIV status (p = 0.0073). However, CRP did not differ by HIV status (p = 0.74). In contrast, SAA did not differ by pregnancy type, HIV status and across all study groups respectively (p = 0.07; p = 0.44; p = 0.76). ***Conclusion***: This study demonstrates significant reduction of CRP and non-significant elevation of SAA in PE versus healthy pregnant women. Decreased CRP is suggestive of a neutralized immune response in HIV associated pre-eclampsia.

**Keywords**: C-reactive Protein, serum amyloid A, HIV, Pre-eclampsia, Inflammation

**Running title**: CRP and SAA levels in HIV-associated pre-eclampsia

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**INTRODUCTION**

In normal pregnancy, a mild maternal inflammatory response enables adequate placentation, fetal tolerance and maternal resistance to bacterial and viral infections [1, 2]. In contrast, pre-eclampsia (PE) exhibits an excessive maternal immune response which results in a cascade of pathophysiological events and subsequent clinical manifestation of the disorder [3, 4].

Pre-eclampsia is considered an inflammatory disorder; and several studies suggest a link with intravascular inflammation and endothelial dysfunction [5]. Intravascular inflammation is a feature of PE whilst endothelial dysfunction is postulated to be a secondary phenomenon [6]. Nevertheless, the elucidation of a cause-effect relationship of PE remains unclear [5]. Regardless of these uncertainties, evidence supports an upregulation of circulating activated leukocytes and pro-inflammatory cytokines, chiefly interleukin- 6 (IL-6) and Tumour necrosis factor-α (TNF-α) in PE compared to normotensive pregnancy [4].

The acute-phase proteins, serum amyloid A (SAA) and C-reactive protein (CRP) increase 1000-fold reaching a maximum of approximately 500-1000μg/ml during inflammation, infection and trauma [3, 7]. Both SAA and CRP are secreted and synthesized by hepatocytes in response to the cytokines, interleukin-6 (IL-6) and TNF-α [5, 8]. However, the role of these markers in PE is conflicting [3, 5, 9, 10]. Moreover, CRP levels is a potential and independent predictor of cardiovascular disease (CVD) and HIV disease progression [11-13].

South Africa (SA) is the epicenter of the HIV global pandemic with devastating consequences on women of reproductive age [14-16]. The prevalence of HIV in pregnancy is estimated at 37%, specifically in the province of KwaZulu-Natal whilst 83% of all maternal deaths due to hypertensive disorders is attributed to PE development [15, 16]. The opposing immune responses reflected in HIV-associated PE suggest neutralization of the immune hyper- reactivity exhibited by PE with a lowered predisposition to PE development in HIV positive individuals. However, the use of highly active antiretroviral therapy

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(HAART), provides conflicting results [16-18]. Therefore, the aim of this study was to evaluate the role of SAA and CRP in HIV-associated PE.

## METHOD AND MATERIALS

### Study Population

Permission to conduct this study was obtained from the Department of Health, the hospital manager and from the institutional Biomedical Research Ethics Committee (Reference Number: BE211/17). Women were purposively recruited at Prince Mshiyeni Memorial Hospital in KwaZulu-Natal, Durban, South Africa. All women gave informed consent for participation in the study. The study population was inclusive of 76 pregnant women divided into two groups based on pregnancy type ie., normotensive (n=38) and pre-eclamptic (n=38, new onset blood pressure of $\geq$ 140/90 mmHg and proteinuria of $\geq$ 300/24 hr). The study population was further stratified based on HIV status, ie., HIV-positive normotensive (n=19), HIV-negative normotensive (n=19), HIV-positive pre-eclamptic (n=19) and HIV-negative pre-eclamptic (n=19).

*Exclusion criteria*: women with chronic diabetes, cardiac disease, chronic hypertension, gestational diabetes, chronic diabetes, pre-existing seizure disorders, chronic renal disease, sickle cell disease, abruption placentae, antiphospholipid antibody syndrome, chorioamnionitis, polycystic ovarian syndrome, eclampsia, thyroid disorder, unknown HIV status, no antenatal care as well as those who did not give informed consent were excluded.

*Inclusion criteria*: diagnosis of pre-eclampsia based only on hypertension and proteinuria, known HIV status and singleton pregnancy.

The inclusion and exclusion criteria of the cases matched those of the controls except for a diagnosis of pre-eclampsia. Both cases and controls were sub-stratified based on HIV status ie., HIV positive and HIV negative groups.

*Bioplex Assay*

Maternal serum samples were stored at -80$°$C until use. Samples aliquots were centrifuged at 1000 rpm for 10 min at 25 $°$C and the supernatant used for the measurement of serum CRP and SAA. A multiplex immunoassay was performed using a ProcartaPlex Human Basic Kit (cat. number: EXP010-10420-901); ProcartaPlex Human SAA (cat. number: EPX01A-12136-901) and a human CRP Simplex Kit (cat. Number: EPX010-10420-901) as per the manufacturer's instructions. Coupled magnetic beads were added into each well of the assay plate. Four-fold serial dilutions of both the standard and sample were then prepared and dispensed into designated wells followed by washing. Incubation allowed for the interaction of the antigen (SAA and CRP) with the capture antibody-coupled beads. Biotinylated detection antibodies were added with subsequent addition of streptavidin-phycoerythrin (SAPE) to complete the reaction. Plates were read on a Bio-Plex® MAGPIX™ Multiplex system (Bio-Rad Laboratories Inc., USA). Data from the multiplex analysis were obtained using the Bio-Plex Manager™ version 4.1 software.

**Statistical Analysis**

IBM SPSS Statistics (version 24) and Graphpad Prism (version 5, Software Inc., 2007) were utilised to analyse data. Descriptive statistics for continuous data were reported as median, interquartile range and mean ± standard deviation. Non-parametric Mann Whitney U and Kruskal-Wallis H tests were used to assess statistical significance within the study population. A value of $p < 0.05$ was considered statistically significant.

**RESULTS**

*Clinical Characteristics*

A summary of patient demographics of the overall study population are outlined in Table 1. Gestational age, diastolic and systolic blood pressures were significantly different between the PE compared to the normotensive pregnancy groups (p < 0.0001 each). Additionally, maternal age (p = 0.15) and weight (p= 0.21) were similar albeit both slightly higher in the PE group compared to the normotensive pregnant group.

Parity was similar between the normotensive pregnant vs PE group [median- 1.00 (1.0-2.0); p = 0.66] albeit non-significantly. The mean Body Mass Index (BMI) was $29.64 \pm 5.68$ kg/m$^2$ in the normotensive group and was slightly higher in the PE group ($31.65 \pm 6.43$ kg/m$^2$) albeit non-significantly (p = 0.11). The mean birth weight of the infants was significantly different between the normotensive pregnant ($3.26 \pm 0.36$ kg) vs PE group ($2.33 \pm 0.96$ kg) (Mann-Whitney U= 242.0, p< 0.0001).

**Serum concentrations of CRP and SAA**

Serum concentrations (pg/mL) of CRP and SAA are summarised in Table 2 and illustrated in Figures 1-5.

*CRP levels based on pregnancy type*: Irrespective of HIV status, a significant difference in CRP expression were noted between the normotensive pregnant vs PE groups (Mann-Whitney U = 463.5; p = 0.0073). A reduction of CRP levels in PE (mean = 113 pg/mL; 95% CI: 31.3-194.7) vs the normotensive pregnant (mean = 238.3 pg/mL; 95% CI: 112.8-363.9) group was evident.

*CRP levels based on HIV status*: In contrast, there was no statistical significance in CRP concentration between HIV-positive vs HIV-negative pregnant groups (p > 0.05), regardless of pregnancy type. Nonetheless, elevated CRP levels were observed in the HIV-positive (mean = 181.9 pg/mL; 95% CI: 66.48-

297.4) compared to the HIV-negative group (mean = 140.4 pg/ml; 95% CI: 53.7-227.0) albeit non-significantly (Mann-Whitney U = 690.0; p = 0.7434).

*CRP levels across all study groups*: Decreased CRP levels were noted in the HIV-positive PE compared to the HIV-negative PE pregnant group, however these did not differ significantly (Mann-Whitney U = 164.0; p = 0.64). Additionally, CRP were significantly reduced in HIV-positive PE compared to the HIV-positive normotensive pregnant group (Kruskal-Wallis H = 10.45; p < 0.0151).

*SAA levels based on pregnancy type*: There was no significant difference between PE vs normotensive pregnant women irrespective of HIV status (Mann-Whitney U = 546.5; p = 0.0690).

*SAA levels based on HIV status*: The level of SAA was similar between HIV positive vs HIV negative groups (Mann-Whitney U = 648; p = 0.44) regardless of pregnancy type.

*SAA levels across all study groups*: There was no significant difference in the serum concentration of SAA across study groups (Kruskal-Wallis H = 6.884; p = 0.76). Although serum SAA levels did not differ, an increasing trend was noted with regards to HIV status ie., in HIV- positive (mean = 73.5 pg/mL; 95% CI: 52.6-94.4) vs HIV -negative (mean = 67.97 pg/mL; 95% CI: 59.8-76.2) and PE-positive (mean = 90.1 pg/mL; 95% CI: 52.4-127.8) vs PE-negative (mean = 69.6 pg/mL; 95% CI: 59.7-79.4) pregnant groups.

**DISCUSSION**

In the duality of HIV associated PE, a discrepancy with regard to immune response and antiretroviral usage exists [16-18]. In this study, the pentraxin, CRP was significantly downregulated in PE compared to normotensive pregnancies. However, SAA remained similar between groups.

CRP is a major acute-phase protein and marker of overall systemic activity during inflammation [19, 20]. CRP together with other pro-inflammatory cytokines have been implicated as mediators of inflammation and endothelial dysfunction [12, 18]. Therefore, our results are unexpected as excessive inflammatory response and endothelial dysfunction are both characteristic features of PE [5]. Disturbances in the normal functioning of the endothelium results in altered coagulation, loss of vascular integrity thereby creating a pro-inflammatory environment [9, 21]. In contrast to normal pregnancy, where there is increased nitric oxide synthesis, nitric oxide synthetase expression is downregulated by CRP and TNF-α which results in decreased production of nitric oxide [9, 22]. The consequence is widespread endothelial dysfunction which is one of the clinical manifestations reflected in PE due to maternal endothelium injury [22]. Furthermore, since PE is an inflammatory disorder, CRP, IL-6 and TNF-α levels are expected to rise in PE compared to normal pregnancy.

In contrast to our findings, Qui *et al.,* [4], showed increased CRP levels that correlate with pre-pregnancy BMI. Pre-pregnancy BMI could be an independent predictive tool for PE development in lean women; however, they failed to demonstrate this association in overweight women. In our study, a higher pregnancy BMI occurred in PE compared to the normotensive groups. Baseline concentrations of CRP correlate with body mass index (BMI) and obesity is one of the risk factors of PE and cardiovascular diseases (CDVs) [3, 4, 23]. Similarly, Engin-Ustun *et al.,* [5], demonstrated increased levels of CRP in PE compared to normotensive pregnancy. Moreover, in a cross-sectional study of a high PE-risk population, CRP together with pro-inflammatory cytokines, IL-6 and TNF-α, were higher in women with PE compared with non-pregnant controls and normotensive pregnant women [10]. Furthermore, Udenze *et al.,* [24], reported

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similar results in IL-6, TNF-α and CRP in patients with severe PE compared to healthy pregnant women. These studies however, were not confounded by HAART usage. Notably, the discrepancy of CRP may be due to immune reconstitution due to the dual antiretroviral therapy rollout in South Africa (nevirapine plus HAART administration).

However, Kristensen *et al.,* [9] found no significant difference in CRP between PE and normotensive pregnant women. It is plausible to suggest that the downregulation of CRP levels observed in this study may also be due to impaired functioning of hepatocytes which are enhanced by adipocytes and steroid hormones, hence the influence of BMI.

Our study also demonstrates no significant difference in SAA expression between normotensive pregnant and pre-eclampsia, albeit with an elevated trend in PE. In contrast to our finding, SAA has been reported to be significantly elevated in PE compared to healthy pregnant women with a positive significant correlation with CRP level [5]. Since SAA strongly correlates with CRP expression, a known inflammatory marker, increased SAA would therefore contribute in the pathogenesis of PE. Nevertheless, similar to our findings, Kristensen *et al.,* [9], reported no significant difference in SAA levels between PE versus both normotensive and non-pregnant controls. Data on SAA levels in PE is limited and conflicting results have been reported [3, 5]. Our findings of similar serum SAA levels between PE and healthy pregnant women are corroborated by Erdogan and colleagues [3]. However, they emphasize minor differences of SAA levels between the maternal blood and cord blood in both PE and normotensive pregnant women, suggesting a lack of SAA transfer via the placenta.

SAA has a short half-life in serum, hence concentrations decline rapidly during recovery following a quick rise after an inflammatory stimulus [25]. Both HAART therapy and the short half-life of SAA may explain the non-significant increase noted in our study. Moreover, Gatt *et al.,* [26] showed that SAA was involved in the modulation of neutrophil function. Furthermore, SAA has an inhibitory effect on leukocytes [27] verifying a modulatory role for SAA in inflammation.

South Africa remains the epicenter of the global HIV pandemic, specifically in women of reproductive age and a high prevalence of pre-eclampsia (12%) [15, 16]. An increasing body of research interest is still focused on the interaction of HIV infection and PE as there is no concordance on the relationship between these two diseases [18]. It is possible that the suppressed immune status in HIV infection is slightly neutralised during pregnancy with further intensification of the neutralisation by PE. However, data is controversial with regard to immune reconstitution following the use of highly active antiretroviral therapy (HAART) in normal pregnancy, and the predisposition to PE development [2, 17, 28].

In our study, we demonstrate no significant difference in both CRP and SAA based on HIV status. A plausible explanation may be the immune restoration brought about by the antiretroviral therapy. Particularly, HAART regimens are associated with endothelial dysfunction and long term complications such as cardiovascular diseases and lipodystrophy [29]. In the study of Machado and colleagues [17], the authors observed that the use of HAART at conception was a risk factor for the development PE and eclampsia.

A limitation of our study is the duration of antiretroviral (ARV) treatment ie., pre-pregnancy or intra-pregnancy, which was incomplete for the study population. Moreover, ARV treatment type was variable. Some women were on triple [HAART, zidovudine (AZT) and nevirapine] whilst others received dual ARV (AZT and nevirapine) therapy. Viral load was not assessed as it is not a standard of care practice in South Africa. The standard practice guidelines for HIV treatment during pregnancy in South Africa, recommends all HIV positive pregnant women to start ARV therapy immediately regardless of CD4 count.

We were unable to correlate CRP and SAA expression with severity of HIV infection due to the lack of CD4 T cell count. However, the study of Lau *et al.,* [30], noted that patients who progressed to Acquired Immunodeficiency Virus (AIDS) had higher CRP levels than those who remained AIDS free and increasing inflammation was associated with disease progression. Nevertheless, the decreased CRP levels in the HIV-

positive PE group in our study are suggestive of some neutralization of the exaggerated immune response in PE by HIV infection.

## CONCLUSION

Finally, this study demonstrates significant reduction of CRP with a concomitant non-significant increase of SAA in PE compared to normotensive pregnant women. We suggest immune neutralization in HIV associated PE with immune reconstitution in HAART treated individuals. However, further studies with a larger sample size are required to evaluate these biomarkers as potential predictive tools of PE and their prognostic role in HIV associated PE.

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Table 1: Demographic and clinical characteristics of the study population

|  | N- | N+ | PE- | PE+ |
|---|---|---|---|---|
| Number | 19 | 19 | 19 | 19 |
| Maternal age (years) | 24 ± 5.26 | 27.42 ± 6.52 | 27.05 ± 6.63 | 27.62 ± 4.11 |
| Gestational age (weeks) | 38.50 ± 1.34 | 38.69 ± 1.30 | 35.22 ± 4.55 | 35.00 ± 4.34 |
| Maternal Weight (kg) | 77.10 ±15.97 | 68.11 ± 7.97 | 75.47 ± 13.27 | 77.45 ± 16.78 |
| Systolic BP (mmHg) | 119.53 ±8.77 | 118.58±10.66 | 170.95±18.16 | 166.62 ± 22.11 |
| Diastolic (mmHg) | 69.32 ±11.88 | 74.68 ± 10.24 | 104.05±14.25 | 103.69 ± 15.41 |

*Values are presented as mean ± standard deviation; n= 76; BP: Blood Pressure; N-: Normotensive HIV negative; N+: Normotensive HIV positive; PE-: Pre-eclamptic HIV negative; PE+: Pre-eclamptic HIV positive;*

The mean systolic and diastolic blood pressure was higher in the pre-eclamptic (169.0mmHg ±19.32 and 104.3mmHg ±14.29) compared with the normotensive (119.1mmHg ±9.64 and 72.0mmHg ±11.28) pregnant group respectively. Furthermore, the mean gestation age was higher in the normotensive versus the pre-eclamptic pregnant groups.

Table 2: Serum concentrations of acute phase reactants, CRP and SAA, across the study groups

| | Normotensive pregnancy | | Pre-eclamptic pregnancy | |
|---|---|---|---|---|
| | HIV - (n= 19) | HIV +(n= 19) | HIV - (n= 19) | HIV + (n= 19) |
| CRP (pg/mL) | 53.4(18.09-228.4) | 68.8(30.76-379.1) | 21.02(13.76-60.25) | 17.36(8.85-68.80) |
| SAA (pg/mL) | 71.64(39.22-86.08) | 41.48(30.89-84.20) | 74.21(50.31-91.15) | 86.04(52.13-86.04) |

Values are represented as median and interquartile range.  Key: HIV - : HIV negative; HIV +: HIV positive; pg: picogram; mL: millilitre; CRP: C-reactive protein; SAA: serum amyloid A; n: participants per group.
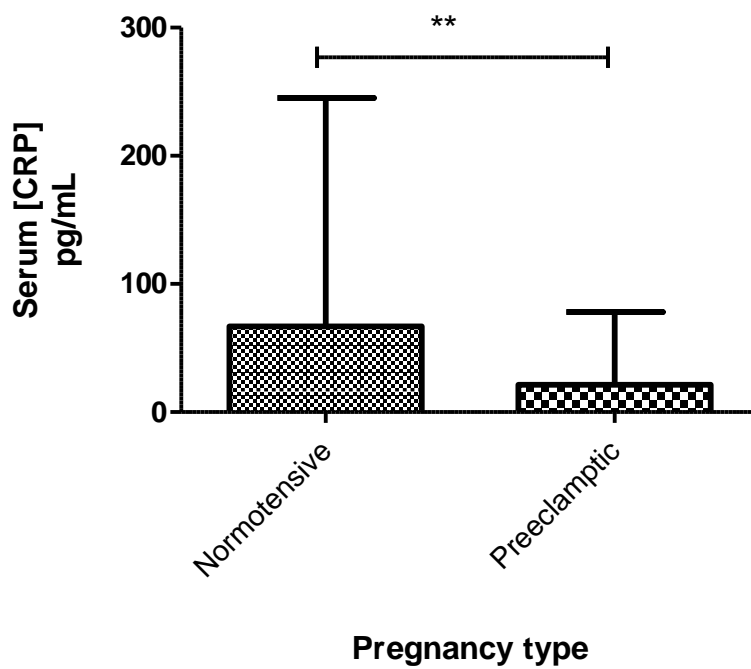
**Figure 1:** Serum levels of C-reactive protein (pg/mL), in normotensive and pre-eclamptic pregnancy, irrespective of HIV status. Results are presented as median and interquartile range.**Serum concentrations are significantly different between the normotensive and pre-eclamptic pregnancy groups, *p* = 0.0073.
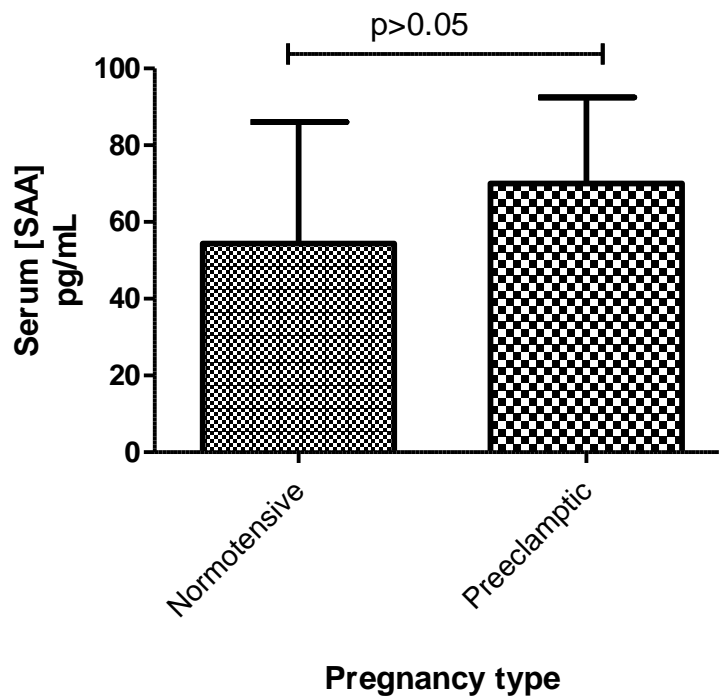
**Figure 2**: Serum concentrations of circulating maternal serum amyloid A (pg/mL) between normotensive and pre-eclamptic pregnancy groups irrespective of HIV status. Results are presented as median and interquartile range. No significant difference in serum concentrations was observed between the two groups, $p = 0.0690$.
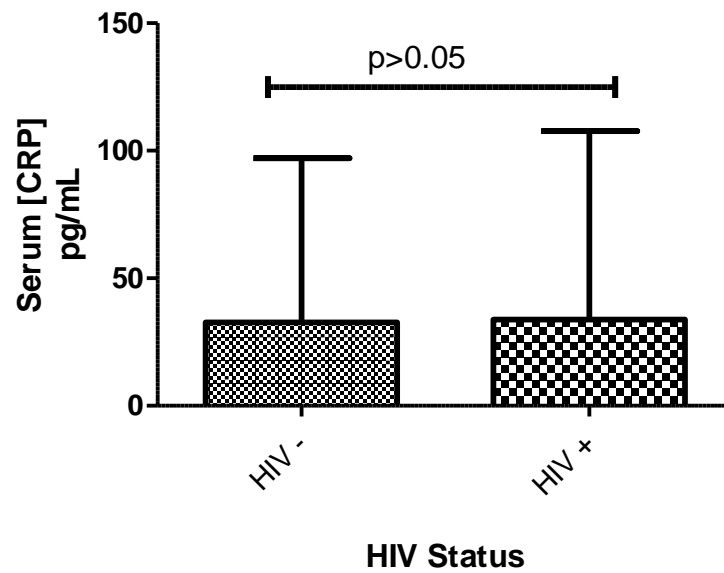
**Figure 3**: Serum concentrations of circulating maternal C-reactive protein (pg/mL) between HIV negative and HIV positive groups irrespective of pregnancy type. Results are presented as media and interquartile range. No significant difference in serum concentrations was observed between the two groups, $p = 0.7434$
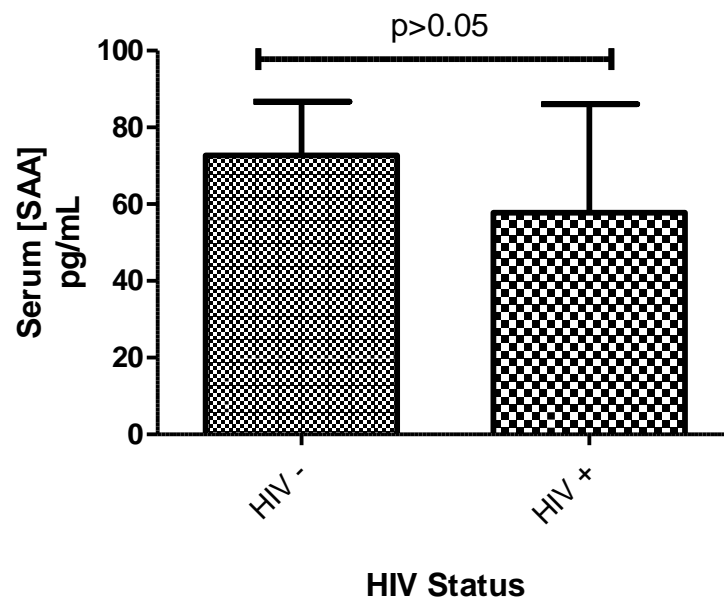
**Figure 4**: Serum concentrations serum amyloid A (pg/mL) between HIV negative and HIV positive groups irrespective of pregnancy type. Results are presented as median and interquartile range. Serum concentrations between the two groups are not significantly different, $p = 0.4448$.

A
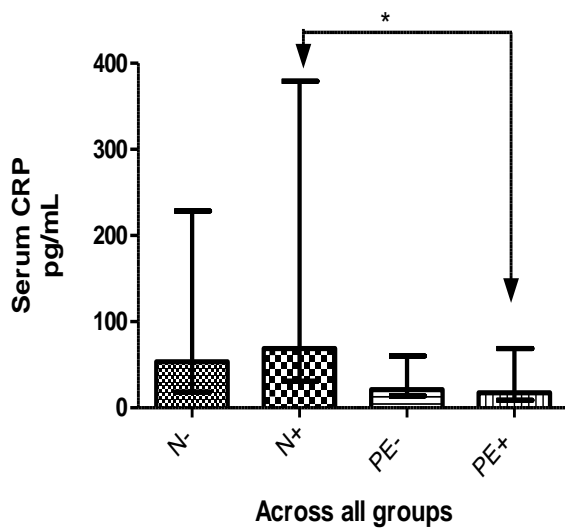


B
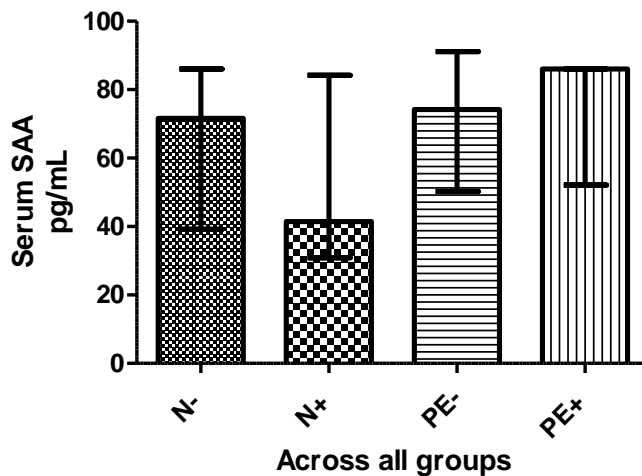


**Figure 5.** Serum concentrations (median and interquartile range) of (A) C-reactive protein (pg/mL) and (B) serum amyloid A (pg/mL) in HIV-negative normotensive (N-), HIV-positive normotensive (N+), HIV-negative pre-eclamptic (PE-) and HIV-positive pre-eclamptic (PE+) groups. *Serum concentrations of CRP between N+ and PE+ groups were significantly different, $p = 0.0151$. Serum concentrations of C-reactive protein between N- and PE- groups did not differ significantly, $p > 0.05$.

**CHAPTER 3**

**SYNTHESIS**

Pre-eclampsia, an obstetric disorder specific to human pregnancy remains a major cause of maternal mortality and morbidity globally (Chaiworapongsa et al., 2014a, Levine et al., 2004). In South Africa, the high maternal mortality rates are attributable to HIV infection, haemorrhage and hypertensive disorders with 83% of the latter deaths emanating from pre-eclampsia development (Moodley, 2011, Mothers, 2014). Furthermore, in the province of KwaZulu-Natal the prevalence of PE is 12% (Mothers, 2014) whilst HIV in pregnancy is estimated at 37.7% (Thakoordeen et al., 2017). While there is no cure for this disorder, the only resolution is delivery of the placenta and fetus (Chaiworapongsa et al., 2014b, Moodley, 2011).

One of the clinical manifestations reflected in PE is widespread endothelial dysfunction, postulated to be enhanced by an increased proinflammatory environment normally provoked by inflammation (Mustafa, 2012, Udenze, 2015). Although a dose-response relationship between PE and inflammation is yet to be established, it has been proposed that the latter may intensify the effects of the imbalance in circulating angiogenic factors reflected in PE. Thus inflammation alone, is not sufficient to cause the disorder (Ramma and Ahmed, 2011).

Marked increase of acute phase proteins and other known markers of inflammation have been reported in pre-eclamptic versus normotensive pregnant women (Maharaj, 2017, Hubel, 2008). However, conflicting results have been reported with regard to CRP and SAA levels in PE (Qiu et al., 2004, Erdoğan et al., 2012). Notably, an increase, decrease and similarities in their circulating concentration have been demonstrated in both PE and normotensive pregnancy (Teran, 2001, Kristensen, 2009, Erdoğan et al., 2012).

Both CRP and SAA levels increase in response to inflammation, acute and chronic infections. In contrast however, viral infections are thought to correlate with lower CRP expression compared with those elicited by bacterial infections (Saljoughian, 2008, Lau et al., 2006). CRP is known to bind with high affinity to

phosphocholine on microbes thereby inducing phagocytosis, destruction and clearance of the microbe (Samikkannu et al., 2013, Chandrashekara, 2014).

SAA production has been shown to induce the proinflammatory cytokines, IL-6, IL-1 and TNF-α (Samikkannu et al., 2013, Engin-Üstün et al., 2007). Elevated SAA occur in many inflammatory disorders such as atherosclerosis and thrombosis implicating it's potential role in endothelial dysfunction and in the development of cardiovascular disease (Sun, 2015, Hua et al., 2009). Although the main physiological role of SAA is poorly understood, various functions have been demonstrated which includes inducing adhesion and migration of monocytes and neutrophils, chemoattractant and cytokine-like functions, involvement in the cholesterol transport system and tissue infiltration of polymorphonuclear leukocytes (Urieli-Shoval, 2000, Thorn et al., 2004, Sun, 2015).

Whilst the data on SAA in the pathogenesis of PE is limited, the relationship of acute phase proteins in HIV-associated pre-eclampsia remains obscure and is investigated in the duality of HIV infection and PE (HIV-associated PE).

Pre-eclampsia is initiated by poor placentation, (Mustafa, 2012). In PE, defective trophoblast invasion results in failure of spiral artery remodelling with subsequent reduced organ perfusion and placental ischaemia (Chaiworapongsa et al., 2014a, Gathiram and Moodley, 2016). The resultant release of various maternal circulating factors creates an increased proinflammatory environment (TNF-α, IL-6 and 8) which can mediate and contribute to the widespread endothelial dysfunction exhibited by PE (Udenze, 2015, Maharaj, 2017). High cytokine levels are known to elevate blood pressure with a concomitant decline in renal function, in studies using pregnant animal models (Udenze, 2015). Also, the synthesis of CRP and SAA by liver hepatocytes is induced by increased levels of cytokines, mainly TNF-α and IL-6 (Udenze, 2015). Thus, the inflammatory relationship between acute-phase proteins and cytokines as depicted by increased concentrations, is expected in PE compared to normotensive pregnant women.

Nonetheless, this study demonstrates a significant downregulation of CRP with concomitant elevation of SAA in PE compared to normotensive pregnancy albeit non-significant. Although our results are unexpected, they are corroborated by the findings of Kristensen *et al.,*(2009) and colleagues who found no increase in both CRP and SAA levels in PE compared to healthy pregnant women (Kristensen, 2009). It must be noted that in our study all HIV infected women received anti-retroviral therapy which is a standard of care in South Africa. Nonetheless, in two previous studies the levels of proinflammatory cytokines TNF-α, TNF-γ and IL-6 at the 18th week of gestation were not increased in women who developed PE compared to normotensive pregnant women (Ramma and Ahmed, 2011). In our study, despite SAA expression been similar between PE and normotensive pregnant women, an elevated trend of SAA expression was noted in PE versus healthy pregnant women. These findings are consistent with those reported by Erdogan *et al., (*2012).

SAA is also a high-density lipoprotein and precursor of Amyloid A fibrils in systemic Amyloid A amyloidosis, an autoimmune disease with characteristics of chronic inflammation (Targonska-Stepniak and Majdan, 2014). The functions of SAA are varied and recent studies have shown that this protein may be more regulatory than proinflammatory as it also displays anti-inflammatory functions (Sun, 2015). Moreover, SAA declines rapidly during recovery due to its short-half life in serum (Hillström et al., 2010). The use of HAART in our study in combination with the short half-life of SAA may have resulted in the similar SAA levels observed between the groups.

In contrast to our findings, Engin-Ustun *et al.,* (2007) reported elevated CRP and SAA levels in PE pregnant women and this interrelation was suggestive of a potential role in the pathogenesis of PE (Engin-Üstün et al., 2007). Moreover, in the study of Teran *et al., (*2001*),* increased CRP and proinflammatory cytokine levels were observed in pre-eclamptic versus normotensive pregnant controls.

Increasing levels of proinflammatory cytokines impair endothelial function and inhibit expression of nitric oxide synthetase which results in decreased nitric oxide production with subsequent endothelial

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dysfunction, a hallmark of PE (Teran, 2001). Moreover, in animal studies the downregulation of nitric oxide synthetase can produce similar characteristics as those displayed in PE (Mustafa, 2012).

Our results were confounded by the use of HAART, a fact not considered in the above studies. HAART-induced HIV suppression restores immune activity to some extent by increasing $CD4^+$ T cell count therefore, this alters the level of inflammatory markers and delays HIV disease progression (Maharaj et al., 2017)

Despite our demonstrating no significant difference in both CRP and SAA based of HIV status, an upward trend was observed in HIV positive compared to HIV negative women. Pre-eclampsia and HIV/AIDS infection are inflammatory conditions known to play significant roles in immune activation and dysfunction. The interaction of these two conditions however, is still contentious as data on the use of HAART in HIV-associated PE is conflicting (Maharaj et al., 2017, Machado et al., 2014, Suy et al., 2006). In the study of Suy and colleagues (2006), the authors found an increased risk of PE development in HIV infected women who were exposed to HAART treatment prior to pregnancy (Suy et al., 2006). Moreover, Sansone *et al.,* (2016) demonstrated that HIV infected pregnant women exhibited a similar risk of PE development than uninfected women possibly due to the use of HAART (Sansone et al., 2016). However, Boyajian *et al.,* (2012) reported no difference in the risk of PE development between HAART treated HIV positive and HAART-naive pregnant women (Boyajian et al., 2012).

Based on HIV status our findings of elevated CRP and SAA (non-significant) are in part corroborated by those of Samikkannu *et al.,* (2013). In their study, alcohol and illicit drug abusers infected with HIV had increased CRP and SAA concentrations implicating a role in viral replication and disease progression. However, the authors concluded that elevated concentrations of these proteins appear to be accelerated by alcohol and drugs of abuse (Samikkannu et al., 2013). Nevertheless, Lau *et al.,* (2006) associated increasing inflammation with disease progression and found that higher CRP levels were present in patients who later progressed later to AIDS compared to those who remained free of AIDS. A plausible explanation of the

similarities in CRP and SAA levels based on HIV status (HIV positive vs HIV negative) may be due to the immune reconstitution by HAART.

Additionally, across all study groups we demonstrate a significant decrease of CRP in HIV positive PE versus HIV positive normotensive pregnant women. Decreased CRP levels in the HIV-positive PE group are suggestive of some neutralization of the exaggerated immune response in PE by HIV infection.

**CONCLUSION**

In conclusion, this study demonstrates significant reduction of CRP with a concomitant non-significant increase of SAA in PE compared to normotensive pregnant women. Our results in part suggest immune neutralization in HIV associated PE in HAART treated individuals. However, further studies with a larger sample size are required to evaluate these biomarkers as potential predictive tools of PE and their prognostic role in HIV associated PE.

**CHAPTER 4**

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**APPENDIX**

16 May 2017

Ms A Sikutshwa (210522713)
Discipline of Optics and Imaging
School of Laboratory Medicine and Medical Sciences
dantesikutshwa@gmail.com

Dear Ms Sikutshwa

**Protocol:** The role of serum C-reactive protein and serum amyloid A in HIV associated pre-eclampsia.
**Degree:** MMedSc                                              **BREC reference number: BE211/17**

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received 20 March 2017.

The study was provisionally approved pending appropriate responses to queries raised. Your response received on 09 May 2017 to BREC correspondence dated 05 May 2017 have been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given **full ethics approval** and may begin as from 16 May 2017.

This approval is valid for one year from **16 May 2017.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be **RATIFIED** by a full Committee at its next meeting taking place on **13 June 2017.**

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

Prof V Rambiritch
Deputy Chair: Biomedical Research Ethics Committee

cc supervisor: naickera@ukzn.ac.za
cc administrator: dudhrajhp@ukzn.ac.za