



UNIVERSITY OF KWAZULU NATAL

**AN INVESTIGATION INTO THE CLINICAL, BIOCHEMICAL,
IMMUNOLOGICAL AND EPIGENETIC FACTORS IN BLACK SOUTH AFRICAN
WOMEN WITH PREECLAMPSIA AND HIV**

By

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and Medical Sciences, Discipline of Medical Biochemistry and Chemical Pathology, College
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Declaration

I, Niren Ray Maharaj declare that:

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DEDICATION

To:

My late mother Draupadi, whose selfless love and care, sacrifice and commitment, continues to provide me with the courage and strength needed to succeed in adversity,

My father Roopchunder, whose high moral values and meticulous approach to a lifetime of teaching has provided guidance in my life,

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“ GOD Almighty creates the toughest challenges in order to bring out the best He has bestowed upon us ”

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ABBREVIATIONS

HIV	Human Immunodeficiency Virus
AIDS	Acquired Immunodeficiency Syndrome
CD4	Cluster of differentiation 4
HAART	Highly active antiretroviral therapy
RNA	Ribonucleic acid
IRAK 1	Interleukin-1-receptor associated kinase
TRAF 6	TNF Receptor –Associated Factor 6
DNA	Deoxyribonucleic acid
IL-2	Interleukin 2
TNF- α	Tumour necrosis factor alpha
IFN- γ	Interferon gamma
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-8	Interleukin 8
CD8	Cluster of Differentiation 8
FGF-2	Basic Fibroblast Growth Factor
mRNA	messenger RNA
sFlt-1	Soluble fms –like tyrosine kinase-1
sEng	Soluble endoglin
VEGF	Vascular endothelial growth factor
PIGF	Phosphatidylinositol Glycan Anchor Biosynthesis Class F)
AT1-AA	Angiotensin II Type 1 Receptor Autoantibody
H ₂ S	Hydrogen sulfide
NO	Nitric oxide
RISC	RNA induced silencing complex
(Ago2)	Argonaute- 2

NF- kB	Nuclear Factor kappa B
SEMA6A	Semaphorin 6A
Tlr2	Toll like receptor 2
Tlr4	Toll like receptor 4
BMI	Body mass index
STBM	Syncytiotrophoblast molecules
LDH	Lactate Dehydrogenase
C	Cytosine
T	Thymine
G	Guanine
PPAR- γ	Peroxisome proliferator-activated receptor gamma
C/EBPa	C Enhancer Binding protein alpha
CXCR4	Chemokine (C-X-C Motif) Receptor 4
PE	preeclampsia
SNP	single nucleotide polymorphism
microRNA,	microribonucleic acid
HELLP	hemolysis, elevated liver enzymes, low platelets
RA	rheumatoid arthritis
hsa miR-27a	human miR-27a

ABSTRACT

Introduction:

Preeclampsia (PE) and HIV/AIDS contribute significantly to adverse maternal and perinatal outcomes globally. The treatment of PE remains empiric, however in HIV infection, highly active antiretroviral therapy (HAART) is used routinely for maternal health and the prevention of vertical transmission in pregnancy. The relationship between PE and HIV /HAART remains controversial and despite extensive research, the pathophysiology of both conditions is not fully understood. Contemporary and new research strategies include immunological and genetic aspects of PE and HIV, and clinical and biochemical effects associated with HAART.

Aims and Objectives:

The aims of the study were to determine: (1) the association of HIV and HAART with clinical and biochemical indices in women with PE, (2) the effects of HAART in relation to inflammatory cytokines in PE and HIV, and (3) the role of gene polymorphisms in preeclamptic women with HIV on HAART.

The specific objectives of the study were to identify significant differences ($p > 0.05$) in the routine clinical and laboratory indices between the preeclamptic groups, (2) to identify significant changes in pro-inflammatory cytokines IL2, TNF α , IFN γ and IL6 in relation to HIV / HAART and preeclampsia, and (3) to determine the association of single nucleotide polymorphisms (SNP) miRNA 27a (rs 895819T>C) and miRNA 146a (rs 2910164G>C) with preeclampsia risk and susceptibility to associated factors.

Materials and Methods:

A prospective cohort study was conducted between July 2013 and September 2014 at Prince Mshiyeni Memorial Hospital in Durban, South Africa. To maintain ethnographic and anthropometric consistency, a standard cohort of one hundred and ninety three (193) Black women of Zulu ethnicity comprising 4 groups: i.e. uninfected normotensive (50; 26%), infected normotensive (45; 23%), uninfected preeclamptic (53; 28%) and infected preeclamptic women (45; 23%) was recruited during pregnancy after ethical approval and informed consent and followed until delivery. Women with gestational hypertension, renal disease, diabetes mellitus, chronic hypertension and collagen vascular disease were excluded. Specific patient characteristics, clinical features, laboratory indices, and complications were analysed descriptively. Serum levels of pro-inflammatory cytokines TNF- α , IFN- γ , IL2 and IL6 were

determined, using commercially available kits and Cytometric Bead Array (CBA). Genotyping using PCR and the TaqMan® SNP genotyping assay for single nucleotide polymorphisms miRNA 27a rs895819T>C and miRNA 146a rs 2910164G>C was performed and analysed in relation to preeclampsia risk, susceptibility to related clinical features and pro-inflammatory cytokines. Comparative data was recorded and analysed descriptively.

Results:

Our results indicate that the clinical features, laboratory indices, and complications among HIV infected preeclamptic women on HAART is similar to uninfected preeclamptic women. However, gamma-glutamyl transferase (GGT), a hepatic enzyme, was markedly elevated ($p=0.001$) in HIV infected preeclamptic women on HAART. There were significant decreases in pro-inflammatory cytokines IL2 ($p=0.010$), TNF- α ($p=0.045$), and IL6 ($p=0.005$), in normotensive women on HAART compared with uninfected women and significant decreases in IL2 ($p=0.000$) and TNF α ($p=0.000$) in preeclamptic women on HAART compared with uninfected preeclamptic women. In the genotype analyses, we found that the variant genotypes (GC/CC) in miRNA 146a were significantly associated with lower severe preeclampsia risk (OR: 0.34, 0.12-0.99; $p=0.048$), especially in the presence of HIV/HAART (OR: 0.11; 0.02-0.68, $p=0.018$), and the variant genotype (TC/CC) in miRNA 27a was associated with an elevated BMI in preeclamptic women (32.57 vs 29.25; $p=0.06$), especially in the presence of HIV/HAART (33.47 vs 27.87; $p=0.005$).

Conclusion:

The effects of HIV and HAART influence biochemical, immunological and genetic aspects in women with preeclampsia. Gamma-glutamyl transferase, a hepatic enzyme, was markedly elevated and suggests a possible compounding effect on the liver, which is a target organ for preeclampsia and the side effects of HAART. Current management guidelines remain appropriate, however serial hepatic function tests are necessary in clinical practice. The prevention of obesity is also necessary to reduce long term cardiovascular complications associated with these conditions. The immune reconstitutive effects of HAART include a reduction in pro-inflammatory cytokines in normotensive women as well as in preeclamptic women, which may have a bearing on the clinical course of preeclampsia in women on HAART. MiRNA 27a and miRNA 146a are endogenous small RNAs that post transcriptionally regulate gene expression and are implicated in a plethora of pathophysiological processes related to preeclampsia and its related features. TC/CC and GC/CC genotype variants in the functional single

nucleotide polymorphisms (SNP) microRNA27a (rs 895819 T>C) and microRNA 146a (rs 2910164G>C) were associated with obesity and severe preeclampsia respectively. Obesity is a well-recognised risk factor for preeclampsia, and severe preeclampsia is associated with increased morbidity and mortality. These findings demonstrate the importance of molecular genetics and inflammatory mediators in preeclampsia, the pathogenesis of which remains multifactorial in origin.

CHAPTER 1

1.1. INTRODUCTION

Preeclampsia (PE) is a pregnancy specific syndrome accompanied by the *de novo* appearance of hypertension and significant proteinuria after mid-gestation (Katritsis, Gersh et al. 2013). It complicates 2-10% of pregnancies and is associated with significant maternal and neonatal morbidity and mortality (Backes, Markham et al. 2011). HIV/AIDS is caused by infection with the human immunodeficiency virus (HIV) and accounts for approximately 5 % of pregnancy related deaths worldwide and 25% in Sub-Saharan Africa (Calvert and Ronsmans 2013).

HIV, a ribonucleic acid (RNA) retrovirus, is associated with infection of the host immune cells particularly CD4+ T lymphocytes, (Williams and Hopper 2015), and can lead to the acquired immune deficiency syndrome (AIDS), if left untreated (Fauci 2003). Highly active antiretroviral therapy (HAART) has been shown to successfully reduce plasma HIV-1 viral load and vertical transmission rates in pregnancy (Autran, Carcelain et al. 1997), (Siegfried, van der Merwe et al. 2011) and is now incorporated into routine policy guidelines (Southern African 2013).

The pathophysiology of PE (PE) remains unknown and treatment remains empiric. Current theories include inter alia, an abnormal maternal inflammatory response, deranged immunity and endothelial cell damage with a deranged hemodynamic milieu (Eiland, Nzerue et al. 2012). Recently, epigenetic factors and single nucleotide polymorphisms (SNPs) have also been suggested in the pathophysiology of PE (Magee, Pels et al. 2014).

It is evident however, that inflammation is a common thread in the pathophysiology of both conditions (Deeks 2011, Catarino, Santos-Silva et al. 2012), and is mediated by a variety of soluble factors, including cytokines (Shaikh, Sharma et al. 2011). In PE, the release of pro-inflammatory cytokines may trigger the maternal disease (Sargent, Borzychowski et al. 2006). In the progression of HIV infection, a pro-inflammatory to eosinophilic/anti-inflammatory cytokine shift has been observed, which appears to be counteracted with the usage of HAART (Fiore, Newell et al. 2006).

Regarding epigenetic factors and inflammation, microRNAs (miRNAs), small non-coding RNA molecules, such as miRNA-146a (miR-146a), has been noted to play a critical role in immune responses, viral infection and inflammatory disease (Hill, Clement et al. 2015), by preventing overstimulation of the inflammatory response through its recognised target genes, interleukin-1 receptor

associated kinase 1 (IRAK1) and TNF receptor associated factor (TRAF)-6 (Saba, Sorensen et al. 2014). Similarly, miR-27a is associated with a plethora of pathophysiological processes in PE including angiogenesis (Urbich, Kaluza et al. 2012), inflammation (Xie, Cui et al. 2014), and obesity (Kang, Lu et al. 2013), (Bodnar, Ness et al. 2005), a risk factor directly related to PE. However, SNP's, a variation at a single position in a DNA sequence among individuals have been shown to affect miRNA target expression, possibly contributing to disease susceptibility (Clop, Marcq et al. 2006), (Yu, Li et al. 2007). A SNP variant of miR-27a and miR-146a, includes rs2910164 and rs895819 respectively, as reported in the dbSNP database Build 130 (NCBI 2009).

The relationship between PE, co-morbid HIV infection and treatment with HAART remains unclear despite recent research (Frank, Buchmann et al. 2004), (Mattar, Amed et al. 2004), (Kalumba, Moodley et al. 2013), (Cassidy-Matthews 2015), and there is no consensus on the risk that HIV infected pregnant women have of developing PE compared with the general population. In South Africa, PE and HIV occur commonly and continue to remain leading causes of maternal mortality (Moodley, Pattinson et al. 2014), (Gebhardt, Fawcus et al. 2015). Moreover, reducing maternal mortality by 75% by 2015 is part of the millennium development goals of the World Health Organization (WHO) Nations (Osungbade and Ige 2011), which therefore consolidates the need to further investigate and improve on the current management of these conditions.

1.2. LITERATURE REVIEW

1.2.1. Preeclampsia

PE is defined as a blood pressure greater than or equal to 140 mmHg systolic or greater than or equal to 90 mmHg diastolic on 2 occasions at least 4 hours apart after 20 weeks of gestation in a woman with previously normal blood pressure (Obstetricians and Gynecologists 2013). Globally, PE complicates approximately 2-10 % of pregnancies and is associated with 10-15% of direct maternal deaths overall (Duley 2009) . In South Africa, which is considered an upper middle income country, the prevalence of hypertensive disorders of pregnancy is approximately 18% (Moodley and Kalane 2006). Although the etiology of PE remains unknown, many investigators now acknowledge that the diverse maternal symptoms of PE is related to a generalized dysfunction of the maternal endothelium (Sargent, Borzychowski et al. 2006) which appears to be part of an exaggerated systemic inflammatory response involving maternal leukocytes and pro-inflammatory cytokines (Raghupathy 2013). The sequence of events is proposed in 2 stages: a placental stage (stage 1) and a systemic stage (stage 2) as shown in Figure 1.1.

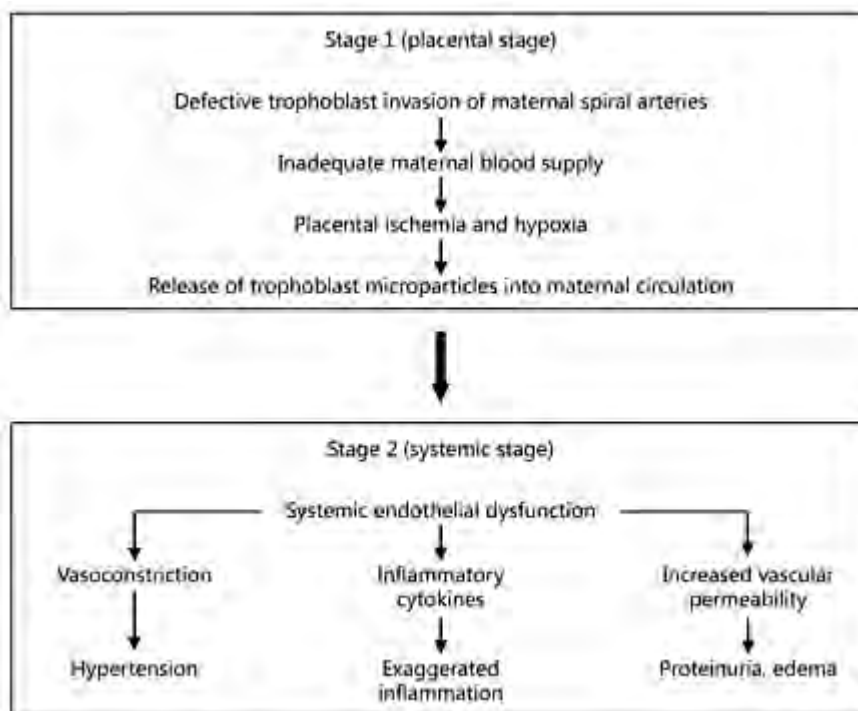


Figure 1.1: Proposed stages of events in PE (Raghupathy, 2013)

Severe maternal complications of PE include renal or hepatic failure, the “HELLP syndrome” (Haemolysis, Elevated Liver enzymes, Low Platelet count), eclampsia, stroke and death and perinatal complications include premature delivery, intra-uterine growth restriction (IUGR), neurological complications and foetal death (Lorquet, Pequeux et al. 2010). Despite extensive research, the treatment

of PE remains empiric and resolution is achieved after delivery of the placenta (Lorquet, Pequeux et al. 2010). Current management strategies includes screening during antenatal visits, administration of anti-hypertensive therapeutic agents, assessment of organ damage, antenatal fetal surveillance, antenatal corticosteroids and timeous delivery (NDoH2007). Currently, calcium supplementation, low-dose acetylsalicylic acid and magnesium sulphate are recommended for the prevention of PE and eclampsia respectively (WHO2011) .

1.2.2. HIV and HAART

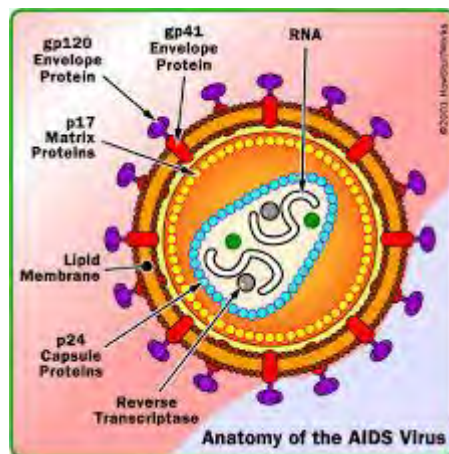


Figure 1.2: Structure of the Human Immunodeficiency virus:

(Source: science.howstuffworks.com)

Globally, 35.3 million people were infected with HIV in 2012, and more than half of this infection occurred in women and girls of reproductive age. (Browne, Schrier et al. 2015),(UNAIDS 2010). South Africa (SA) has the biggest and most high profile HIV epidemic in the world, with an estimated 6.3 million people living with HIV and 200,000 deaths from AIDS-related illnesses in 2013 (UNAIDS 2014). During pregnancy , approximately 29% of antenatal clinic attendees test positive for HIV during screening in SA (NDoH2012).

The transmission of the virus to the fetus can occur during pregnancy (intrauterine), labour and delivery (intrapartum), and after delivery (postpartum) mainly through breast feeding (De Cock, Fowler et al. 2000). Maternal complications of HIV include spontaneous abortions, ectopic pregnancies, systemic infections such as respiratory and urinary tract infections and co-infection with tuberculosis (UNAIDS 2012), (MacLeod and Rhode 1998). HIV has also been associated with obstetric haemorrhage, thrombocytopenia, anaemia and an increased risk of obstetric haemorrhage and complications

associated with caesarean section (MacLeod and Rhode 1998), (Adam 2015). Fetal complications include increased risk of stillbirth, infections, growth restriction and neonatal encephalopathy (Kennedy, Fawcus et al. 2012) (Pattinson, Hulsbergen et al. 2010).

The treatment of HIV using antiretroviral therapy (ART) began with the first clinical trial of zidovudine in 1986 (Fischl, Richman et al. 1987). Almost a decade later, the concept of 3-drug therapy showed benefit with a 60% to 80% decline in rates of AIDS, death, and hospitalization and was incorporated into clinical practice (Palella Jr, Delaney et al. 1998). The role of the nonnucleoside reverse transcriptase inhibitor (NNRTI) efavirenz, "the third drug" in combination with 2 nucleoside RTIs (NRTIs) that constitute HAART was established in 1999 (Study 006) (Staszewski, Morales-Ramirez et al. 1999). Thereafter, a rapid evolution in drug related research has led to the usage of a variety of drugs which include nucleoside/nucleotide reverse transcriptase inhibitors, such as emtricitabine, and tenofovir, nonnucleoside reverse transcriptase inhibitors (NNRTIs), such as efavirenz, protease inhibitors (PIs), such as ritonavir and integrase inhibitors, such as raltegravir, among others.

In South Africa, the use of HAART in pregnancy is guided by policy guidelines which include Fixed Dose combination (FDC). The FDC ART is one ARV pill which contains three drugs: tenofovir (TDF), emtricitabine (FTC) and efavirenz (EFV) (Southern African 2013). Although serious side effects such as dyslipidaemia, hepatotoxicity and nephrotoxicity may occur with these drugs, the risk for adverse events for both the mother and the baby are low in the short term. (Browne, Schrier et al. 2015). In pregnancy ART/HAART is used to reduce maternal viral replication and mother to child transmission of HIV (PMTCT) either by lowering plasma viral load in pregnant women or through post-exposure prophylaxis in their new-borns (Autran, Carcelain et al. 1997), (Siegfried, van der Merwe et al. 2011).

HAART, which usually comprises three drugs, has been advocated since 1996 and has successfully reduced the mother-to-child transmission rates to around 1-2% in some countries. (Siegfried, van der Merwe et al. 2011). However, it has been suggested that HAART could possibly influence the inhibitory effect of HIV on the development of hypertensive disease in pregnancy and this risk may be enhanced by the endothelial toxicity and vascular dysfunction associated with HAART (Browne, Schrier et al. 2015), (Wang, Chai et al. 2007), (Kline and Sutliff 2008). There has not been a systematic review regarding the risk of developing hypertensive disorders in pregnancy among HAART recipients and data on the relationship between PE and HIV/HAART is limited (Browne, Schrier et al. 2015). Challenges associated with HAART in the forthcoming years are likely to focus on more effective drugs with fewer side effects especially hepatotoxicity and to ensure provision of therapy to resource limited countries, notwithstanding that prevention is the hallmark in the management of HIV infection.

1.2.3. Cytokines

Cytokines are small secreted proteins and include lymphokines, monokines, chemokines and interleukins. They are produced predominantly by helper T cells (Th) and macrophages, and can act synergistically or antagonistically exerting an autocrine, paracrine or endocrine action (Zhang and An 2007) . The classification of cytokines is shown in Table 1.1.

Table 1.1 : Classification of cytokines .(Jun-Ming Zhang and Jianxiang , 2007)

Pro-inflammatory Th1-type, stimulatory	Anti-inflammatory Th2-type, inhibitory	Multifunctional
IFN γ	IL-4, IL-10, IL-1ra, IL-2	IL3, IL-1 β
TNF α	TNFsr	MCP-1
IP-10	TGF- β 2	sCD40L
IL-2, IL-6, IL-8, IL-12, IL-17		Growth factors: IL3 and G-CSF

Cytokines like interleukin (IL)-2, tumour necrosis factor (TNF)- α , and interferon (IFN)- γ are characteristic of T helper 1 (Th1)-type immunity and induce several cell-mediated cytotoxic and inflammatory reactions. T helper 2 (Th2)-type cells, on the other hand, secrete the Th2 cytokines IL-4, IL-5, IL-6, and IL-10 and are associated with humoral immunity (Romagnani 2000), (Mosmann and Sad 1996). A Th2-type immunity is associated with a normal pregnancy, whereas a strong Th1 reactivity is associated with proinflammatory cytokines such as IL-1, IL-2, IL-8, TNF- α , and IFN- γ and pregnancy related complications (Raghupathy 2013).

In PE, proinflammatory cytokines are increased in the blood and blood leukocytes, and serum from women with PE have increased Th1/Th2 cytokine ratios and increased levels of the cytokines IL-6 and TNF- α , (Szarka, Rigó et al. 2010). Levels of soluble TNF- α receptor, a more reliable marker for TNF activity, are also increased in PE as compared to normal pregnancies (Kupferminc, Peaceman et al. 1995). Additionally, a shift towards Th1-type immunity (expressed by the increased IL-2/IL-4 and IFN-gamma/IL-4 ratios), and circulating levels of pro-inflammatory cytokines IL-6 and TNF-alpha, amongst

others, are elevated in PE compared with healthy pregnancy (Szarka, Rigó et al. 2010). Elevations in IL-8, IL-6, and IFN- γ have also been demonstrated in PE (Pinheiro, Martins-Filho et al. 2013).

These findings suggest the overall pro-inflammatory systemic environment associated with PE. The relationship of inflammatory cytokines and endothelial dysfunction, a key feature in PE, is shown in Table 1.2.

Table 1.2: Inflammatory cytokines and endothelial dysfunction (Raghupathy, 2013)

TNF- α	<ul style="list-style-type: none"> Activates endothelial cells Increases coagulation Increases the expression of adhesion molecules Increases vascular permeability Induces microvascular leakage
IL-1	<ul style="list-style-type: none"> Increases thrombin production and coagulation Induces the production of platelet-activating factor Stimulates the expression of adhesion molecules
IL-6	Increases vascular permeability
IL-8	Activates endothelial cells

In HIV infection, the role of cytokines is complex as the rate of progression of the disease is variable and the pathophysiology is still not completely understood. However, elevated cytokine levels in HIV infection could have positive or negative effects on viral control or CD4+ T cell homeostasis (Keating, Golub et al. 2011). TNF- α and IL-2 have been shown to enhance HIV replication in *in vitro* studies (Folks, Clouse et al. 1989), (Kinter, Poli et al. 1995) whilst in contrast, IFN- α , IFN- γ (Shirazi and Pitha 1992), appear to decrease HIV replication in tissue culture. Earlier work has further demonstrated that chronic HIV infection, patients show elevations in serum or plasma TNF- α levels (Zangerle, Gallati et al. 1994).

HAART has been shown to correct much of the inflammation induced by HIV at the level of CD4+ and CD8+ T cells (Almeida, Cordero et al. 2007), however whilst abnormalities in many serum markers improve they do not necessarily resolve on HAART (French, King et al. 2009). Recent data from the SMART trial showed that elevated levels of C-reactive protein and IL-6 were associated with an increased risk of death in participants who received treatment (Kuller, Tracy et al. 2008). Changes in TNF- α and FGF-2 have been shown to be mitigated by HAART usage. Data from a population based study among women, showed HAART recipients to have fewer detectable differences in cytokines from

HIV uninfected persons when compared with untreated women, though TNF- α and FGF-2 were still different in the HAART compared to a negative group (Keating, Golub et al. 2011). Moreover, cytokine levels associated with effective HAART usage are not identical to those in HIV seronegative women, highlighting the limitations of HAART to wholly correct the systemic inflammation associated with HIV and the need for further research in this area (Keating, Golub et al. 2011).

1.2.4. Epigenetics and miRNA's

Epigenetics refers to changes in gene expression that occur without alterations in DNA sequence (Roman, Dafashy et al. 2014). These modifications can occur through various mechanisms, but most significantly, by post-transcriptional gene regulation (Roman, Dafashy et al. 2014). Epigenetic modifications, especially during pregnancy complications, are significant as they can lead to an altered placental phenotype (Chan, Fish et al. 2004).

Epigenetic factors within the placenta have been implicated in the pathogenesis of PE (Nelissen, van Montfoort et al. 2011). Both conventional and microarray approaches have identified a substantial number of differentially expressed (DE) genes in the transcriptome of human placentas from PE compared with normotensive healthy term deliveries (Sitras, Paulssen et al. 2009), (Mouillet, Chu et al. 2011), suggesting differential gene expression may be associated with the pathogenesis of PE.

HIV was among the first pathogens implicated in the induction of an epigenetic change in host cells. For example, terminating the transcription of IFN- γ may result in blocking the host immune response (Ay, Banati et al. 2013). Epigenetic mechanisms are also implicated in mechanisms of drug resistance in HIV infected cells. Mutant viral genomes cause alterations of the viral enzymes reverse transcriptase, protease and integrase resulting in the development of clinical resistance to antiretroviral drugs (Shafer, Rhee et al. 2007).

MicroRNAs have been linked with pregnancy complications in epigenetic studies (Choudhury and Friedman 2012). MiRNAs are endogenous, highly conserved, small noncoding RNAs (≈ 22 nucleotides) that regulate the expression of target mRNAs (Bartel 2004). They play an important role in mediating placental epigenetic changes, acting as silencers of post-transcriptional gene expression by base pairing with, rather than degrading, mRNA. By targeting mRNA, they are able to upregulate or downregulate gene expression, without altering the gene itself (Gheorghe, Goyal et al. 2010).

The biogenesis of miRNA's involves two main pathways – a canonical and an alternate pathway. The canonical pathway, driven by RNase III enzymes generates the majority of animal miRNAs (Ghildiyal and Zamore 2009). The biogenesis starts with RNA polymerase II-dependent transcription of a miRNA gene locus, generating a long primary transcript known as pri-miRNA that can fold into a hairpin structure (van Kouwenhove, Kedde et al. 2011). Cleavage occurs near the base of each pri-miRNA hairpin by the microprocessor complex that contains a RNase III enzyme called Drosha and its cofactor DGCR8 (DiGeorge syndrome critical gene 8) (Han, Lee et al. 2006). DGCR8 is a double-stranded RNA (dsRNA) binding protein that recognizes the proximal ~10 bp of stem of the pri-miRNA hairpin, positioning the catalytic sites of the RNase III enzyme Drosha (Han, Lee et al. 2006).

The alternative pathway for miRNA biogenesis are utilised by mitrons, a subset of miRNAs (Ruby, Jan et al. 2007) located within short introns, and once splicing is complete, a debranching enzyme generates the pre-miRNA-like hairpin structure. (Baley and Li 2012). These pre-miRNAs are recognised by a complex of exportin-5 (Exp5) and RAN-GTP (van Kouwenhove, Kedde et al. 2011) and then exported to the cytoplasm by Exp5, where they are cleaved into mature 20-25 nucleotide miRNA duplexes by another RNase III endonuclease, DICER, and its double stranded RNA binding cofactor the RNA-binding protein: trans- activation response (TAR) RNA binding protein (TRBP) (Winter, Jung et al. 2009), (Bronze-da-Rocha 2014).

The passenger strand is typically degraded, while the guide strand is incorporated into RNA- induced silencing complex (RISC) and then mediates mRNA degradation or translational inhibition (D'Anzeo, Faloppi et al. 2014). The Canonical and Major Alternative miRNA Biogenesis pathways involved in the biogenesis of miRNA's is illustrated in Figure 1.3.

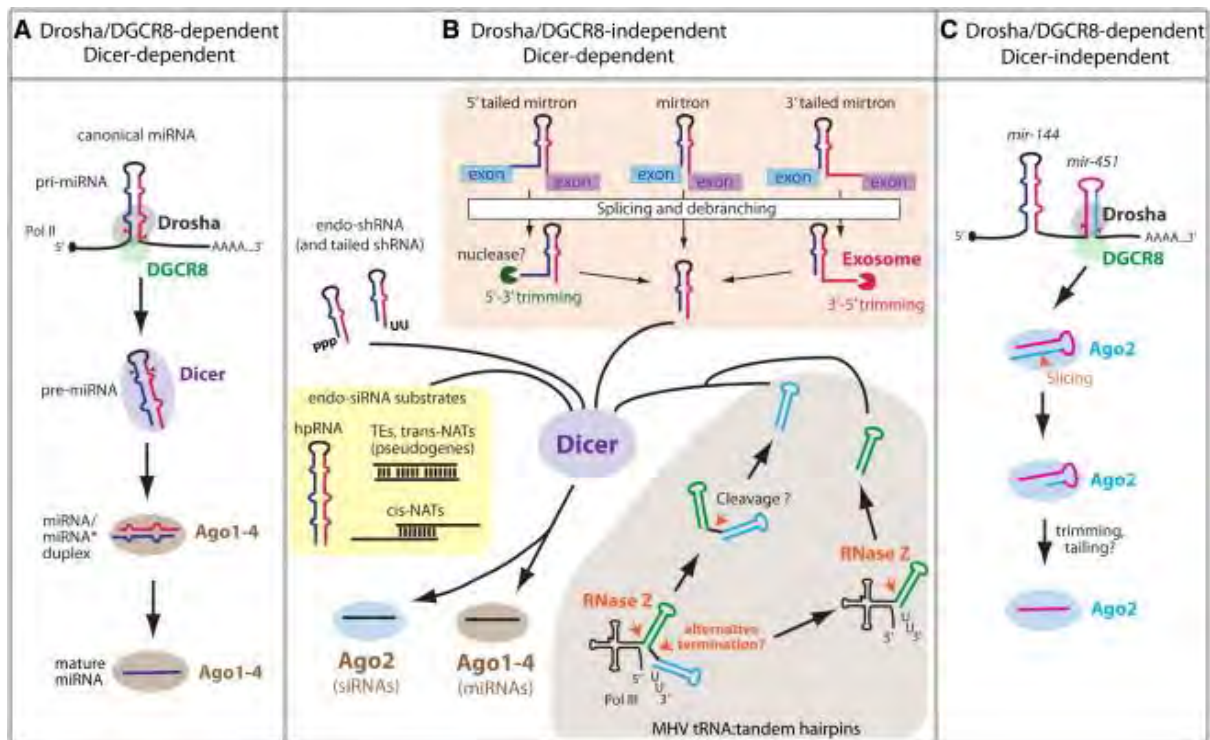


Figure 1.3: Canonical and Major Alternative miRNA Biogenesis Pathways in Animals. (Yang et al., 2011)

The regulation of placental development by miRNAs has been shown through the control of trophoblast cell proliferation, migration, invasion, apoptosis, and angiogenesis (Fu, Brkić et al. 2013). MiRNA's have diverse effects on PE pathogenesis and could be a potential causal factor in the pathophysiology of PE. The evidence from a systematic review on the role of miRNA's in PE revealed that miRNAs interfere with angiogenesis during early pregnancy by dysregulating angiogenic factors (sFlt-1, sEng, VEGF and PlGF) and their receptors (Harapan and Yeni 2015). Dysregulation of these angiogenic factors also induce hypertension during the clinical stage of PE. Additionally, miRNAs also induce hypertension by inducing the production of AT1-AA and targeting several vasodilators such as prostacyclin, 17 β -estradiol, H₂S and NO (Harapan and Yeni 2015).

MiRNAs also play an important role in modulating HIV-1 infection, which has evolved several mechanisms to promote its continued existence. Triboulet et al., demonstrated the role played by cellular miRNAs during HIV-1 infection: (1) by knocking down Drosha and Dicer, (miRNA processing proteins), (2) binding to and co-localizing with proteins of the RISC complex (Ago2) and (3) negatively regulating HIV-1 gene expression by preventing viral mRNA association with polysomes (Triboulet, Mari et al. 2007). Overall, HIV-1-mediated modulation of cellular miRNAs, although not directly essential for basic replication stages, could play a role in pathogenesis and disease progression (Swaminathan, Navas-Martín et al. 2014). The association of HIV on proteins involved in miRNA biogenesis pathway is shown in Figure 1.4.

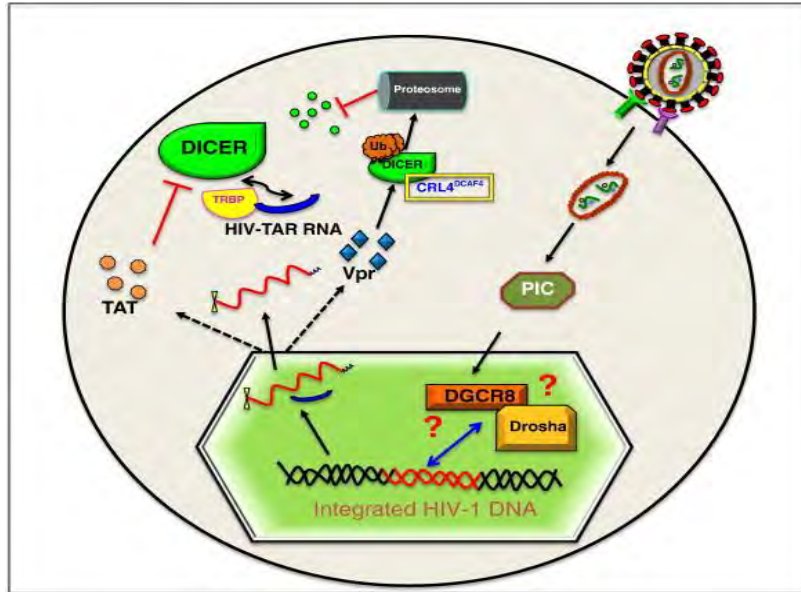


Figure 1.4. The association of HIV-1 proteins in the miRNA biogenesis pathway (Swaminathan et al., 2014)

MiR-27a is a member of the intergenic 23a ~ 27a ~24-2 cluster and is involved in cell cycle control, proliferation and differentiation of various cell types (Zhu 2013). It is ~ 22 nucleotides long and forms one strand of the RNA duplex (Ma and Chen 2012). It has been implicated in many metabolic and angiogenic processes as well as in oncogenesis. It promotes angiogenesis by targeting the angiogenesis inhibitor SEMA6A, which controls repulsion of neighbouring endothelial cells (Urbich, Kaluza et al. 2012). In the process of adipogenesis, it plays an anti-adipogenic role by targeting prohibitin and impairing mitochondrial function (Kang, Lu et al. 2013). MiR-27a also targets peroxisome proliferator-activated receptor gamma (PPAR- γ) to prevent the terminal differentiation of adipocytes and negatively regulates lipoprotein lipase in adipocytes (Bouvy-Liivrand, Heinäniemi et al. 2014), thus playing a role in lipid homeostasis. In inflammation, its role is demonstrated by enhanced expression of pro-inflammatory cytokines, such as IL10 when upregulated in Tlr2- or Tlr4-activated macrophages (Xie, Cui et al. 2014). In cardiovascular disease, it is associated with angiogenesis and endothelial apoptosis in cardiac ischemia (Bang, Fiedler et al. 2012). The diverse role of miR-27a is shown in Figure 1.5.

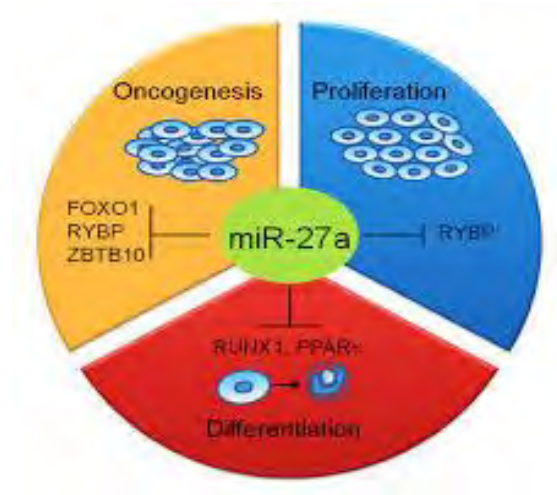


Figure 1.5: Importance of hsa-miR-27a in oncogenesis, proliferation and differentiation
(Source: molecular-cancer.biomedcentral.com)

The oncogenic role of miR-27a has been demonstrated in renal cell carcinoma (Gottardo, Liu et al. 2007), in cervical cancer (Wang, Tang et al. 2008), gastric adenocarcinoma (Liu, Tang et al. 2009) and in breast cancer (Guttilla and White 2009). In addition to the regulatory role of miR-27a in diverse processes, miR-27a is also influenced by viral infections. It has been observed that both host and virus contain mechanisms to regulate miRNA expression and/or activity (Buck, Perot et al. 2010). Polymorphisms of miR-27a have been associated with breast cancer risk and gastric mucosal atrophy (Yang, Schlehe et al. 2010), (Arisawa, Tahara et al. 2007).

Interestingly, a recent study showed that PPAR- γ also inhibits NF- κ B, which promotes pro-inflammatory cytokine production in the lungs of septic mice (Wang, Ruan et al. 2014). A knock down of miR-27a down regulated pro-inflammatory cytokines IL-6 and TNF- α and pulmonary inflammation, indicating decreased inhibition of PPAR- γ (Wang, Ruan et al. 2014), and may represent the interactive relationship between miR-27a and miR-146a.

MiR-146a (miR-146a ; has – miR – 146 a - 5p ; 5'- U G A G A A C U G A A U U C C A U G G G U U - 3'; 59 % A + U; [NR_029701](http://atlasgeneticsoncology.org/Genes/GC_MIR146B.html) ; [http:// atlasgeneticsoncology.org/Genes/ GC_MIR146B.html](http://atlasgeneticsoncology.org/Genes/GC_MIR146B.html)), is encoded from a single locus at chromosome 5q33.3 in humans and is a rapidly induced, pro-inflammatory miRNA with a relatively short half-life of about 1.5–2 h in humans (Alexandrov, Dua et al. 2014) . MiR-146a has been found to be inducible upon stimulation with lipopolysaccharide (LPS) in a NF- κ B dependent manner, and to target the TRAF 6) and IL-1 receptor associated kinase 1 (IRAK-1) gene. (Taganov, Boldin et al. 2006). A diagrammatic representation of this pathway is shown in Figure 1.6. MiR-146a may play important roles in many pathophysiological and physiological processes such as the innate immune and inflammatory response, virus infection and human diseases

such as autoimmune disease and cancer (Li, Chen et al. 2010). Manipulation of miR-146a expression may also represent potential new therapy for several human diseases and serve as a biomarker for disease diagnosis, prevention and treatment (Li, Chen et al. 2010).

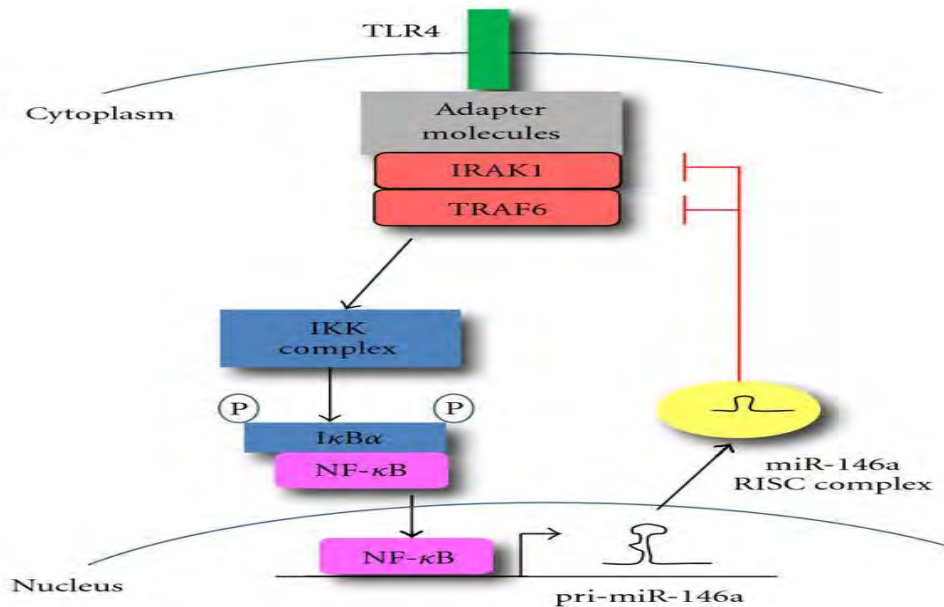


Figure 1.6. MiRNA-146a: TRAF6 and IRAK and NF-κB activation
(Source: krauthammerlab.med.yale.edu)

SNPs form the majority of genetic variations in the human population and are at the forefront of current human genomic, epidemiological and pharmacogenomic studies focusing on the identification of genetic variations responsible for common and complex diseases (Lee and Shatky 2008). A SNP is a DNA sequence variation occurring when a single nucleotide adenine (A), thymine (T), cytosine (C), or guanine (G) in the genome (or other shared sequence) differs between members of a species or paired chromosomes in an individual (ISOGG 2015).

SNP's may not cause a particular disorder, however maybe associated with certain diseases and may be associated with an individual's genetic predisposition to develop a disease (Sachidanandam, Weissman et al. 2001). SNPs, located either in the pre-miRNAs or within miRNA-binding sites, have been shown to affect miRNA target expression, thereby possibly contributing to disease susceptibility (Clop, Marcq et al. 2006), (Yu, Li et al. 2007). A common T/C miR-27a SNP (rs 895819), occurs in the terminal loop, of the pre-miRNA (Shi, Li et al. 2012) and a common G/C SNP (rs 2910164), located within the seed sequence of miR-146a (Jazdzewski, Liyanarachchi et al. 2009), may alter the expression of miR-27a and miR-146a respectively. However, whether genetic variants confer differences in the

expression of the miRNA's in PE, HIV or their associated features is unclear. Reliable identification of disease-causing SNPs is expected to lead to early diagnosis, personalized treatment and targeted drug therapy in the future (Lee and Shatkay 2008).

1.3. RESEARCH QUESTIONS: HYPOTHESIS, AIMS AND OBJECTIVES

Preeclampsia (PE) and HIV/AIDS contribute significantly to adverse maternal and perinatal outcomes globally. The relationship between PE and HIV /HAART remains controversial and despite extensive research, the pathophysiology of both conditions is not fully understood. This study therefore investigated the clinical and biochemical effects associated with HAART as well as immunological and genetic aspects of PE and HIV, with the following hypothesis:

1.3.1. Hypothesis:

- (1) HAART is associated with a potential restoration of the immune system during pregnancy and affects the clinical and biochemical features associated with co-morbid PE
- (2) HAART impacts on the pro-inflammatory environment in HIV infected women with PE through alterations in pro-inflammatory cytokines.
- (3) Gene polymorphisms contribute to PE susceptibility and/or some of the associated risk features and is differentially regulated in the presence of HIV infection and HAART.

1.3.2. Aims:

To test this hypothesis, four areas were investigated in this study, aimed at further analysing the relationship between PE and HIV/HAART, *viz.*, (1) clinical parameters, (2) biochemical/laboratory indices, (3) immunological factors (pro-inflammatory cytokines) and (4) epigenetic factors (microRNA SNPs: miR-27a and miR-146a), in 4 groups of Black South African women, considered to be high risk for these conditions, *viz.*, : (1) normotensive HIV negative , (2) normotensive HIV positive , (3) PE HIV negative and (4) PE HIV positive .

1.3.3. Objectives:

The specific objectives of the study were to:

- (1) To determine the association of HIV and HAART with clinical and routinely used biochemical /laboratory indices, and to assess complications in women with co-morbid PE.
- (2) To determine the effects of HAART on pro-inflammatory cytokines IL-2, TNF- α , IFN- γ and IL-6 in women with PE
- (3) To determine the role of 2 single nucleotide polymorphisms (SNP) miR-27a (rs895819 T>C), and miR-146a (rs2910164 G>C), in women with PE and HIV infection on HAART.

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CHAPTER 2

THE ASSOCIATION OF HIV AND HAART WITH CLINICAL AND BIOCHEMICAL INDICES IN AFRICAN WOMEN WITH PREECLAMPSIA

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Synopsis: HIV / HAART in pregnancy does not adversely affect clinical outcomes and laboratory indices associated with preeclampsia, except gamma glutamyl transferase, which requires serial estimations in pregnancy

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Abstract:

Title: The association of HIV and HAART with clinical and biochemical indices in African women with preeclampsia

Objective: To determine whether clinical and biochemical features associated with preeclampsia are significantly altered in the presence of HIV and HAART.

Methods: A prospective observational cohort study was conducted between July 2013 and September 2014 at Prince Mshiyeni Memorial Hospital in Durban, South Africa. One hundred and ninety three women (45 HIV infected preeclamptic women on HAART and 53 uninfected preeclamptic women and, 45 normotensive HIV infected women on HAART and 50 normotensive uninfected women) were enrolled and followed until delivery. Specific demographic data, clinical features, laboratory indices, and complications were analysed.

Results: There were no significant differences in the clinical features and laboratory indices between the groups, except for gamma glutamyl transferase which was significantly elevated in the preeclamptic HIV/HAART group ($p=0.001$). Perinatal and maternal complications were similar and there were no maternal deaths.

Conclusion: The clinical features, laboratory indices, and complications among HIV infected preeclamptic women on HAART is similar to uninfected preeclamptic women. Current guidelines remain appropriate, however serial hepatic function tests are necessary. The prevention of obesity is also necessary to reduce long term cardiovascular complications associated with these conditions.

Keywords: preeclampsia, HIV, HAART, clinical, laboratory

Introduction:

Preeclampsia is a multi-organ disorder of pregnancy associated with significant maternal and neonatal morbidity and mortality [1]. It complicates 2-10% of pregnancies, and is associated with 10-15% of direct maternal deaths overall [2]. Maternal manifestations of preeclampsia include multisystem involvement whilst adverse perinatal outcomes include small for gestational age (SGA), fetal growth impairment (FGI) and stillbirths.

AIDS-related illness is the leading cause of death and disease among women of reproductive age in low and middle income countries, particularly in Africa [3].

It is estimated that approximately 5 % of pregnancy related deaths worldwide, and 25% in Sub – Saharan Africa are attributable to HIV [4]. Although the use of anti-retroviral (ARV) therapy has significantly reduced the rate of mother to child transmission of HIV, it is also associated with perinatal complications [5].

In South Africa, a middle income country, preeclampsia (and other hypertensive disorders of pregnancy(HDP) , and HIV/AIDS, are implicated by the National Confidential Enquiries Committee on Maternal Deaths in South Africa(NCCEMD) as leading causes of maternal deaths. (Saving Mothers Reports, 2008 -2010 and 2011-2013) [6].

The relationship between HIV infection and preeclampsia remains unclear despite recent research. While some studies have found that preeclampsia is less common in HIV infected women, others have shown that HIV infected women develop a higher rate of preeclampsia [7, 8]. Currently, there is no consensus on whether pregnant women who are HIV infected are at lower, equal or higher risk of developing preeclampsia than the general population, and the treatment of these conditions still remains largely empirical. Furthermore, there is a lack of data on the clinical and biochemical alterations that may occur when preeclampsia and HIV infection co- exist.

We hypothesized that the clinical features , blood pressure measurements , biochemical indices and outcomes in HIV infected preeclamptic women on HAART is similar to uninfected preeclamptic women , and may be associated with HAART induced immune reconstitution. To elucidate the former, we analysed these parameters in a cohort of patients with preeclampsia and HIV, at a large regional hospital over a 14 month period.

Materials and Methods:

Study design:

The study is a prospective observational cohort study conducted between July 2013 and September 2014 at the Maternity Unit of Prince Mshiyeni Memorial Hospital. The hospital is a large regional hospital in southern Durban, KwaZulu-Natal that serves a semi –urban population of approximately 2 million people, where approximately 12 000 deliveries are conducted annually. The inclusion criteria included preeclamptic and normotensive women with and without HIV infection. Women with gestational hypertension, renal disease, diabetes mellitus, chronic hypertension and collagen vascular disease were excluded. Overall, one hundred and ninety three (193) women were recruited by convenience sampling on admission and followed until after delivery. Specific demographic, clinical and laboratory data were recorded and analysed in 4 groups i.e. HIV uninfected normotensive, HIV infected normotensive, HIV uninfected preeclamptic, HIV infected preeclamptic groups.

Study setting:

The majority of women who deliver at the unit are African women of Zulu ethnicity. The antenatal HIV seroprevalence rate in the area is approximately 37 % [9]. The incidence of hypertensive disease (HDP) in the region is approximately 12-18 % [10]. Following institutional and University (UKZN) ethical approval, written informed consent was obtained from all patients. The management of patients at the study site included a full clinical assessment, relevant investigations, antihypertensive medications, monitoring and timely delivery, as described in the Maternity Guidelines of South Africa [11].

Patient selection:

All patients enrolled in the study were referred from local clinics within the catchment area prior to booking at the hospital, or booked directly at the institution's Maternity Unit, where they were recruited and followed up until the early postpartum period. To maintain ethnographic and anthropometric consistency, all patients chosen were of Black African origin, resident in the same geographical location and of Zulu ethnicity. Patients selected in the study were non-smokers, did not consume alcohol or recreational drugs, and all HIV infected patients were on daily fixed dose combination (tenofovir 300mg, emtricitabine 200mg, efavirenz 600mg) of HAART (highly active antiretroviral therapy), in terms of the National guidelines [12]. Calcium supplementation was administered routinely to all patients attending the clinic.

Diagnostic criteria:

Preeclampsia was defined as a blood pressure greater than or equal to 140mmHg systolic or greater than or equal to 90 mmHg diastolic on 2 occasions at least 4 hours apart after 20 weeks of gestation in a woman with previously normal blood pressure [13]. All patients had proteinuria $\geq +1$ on urine dipstick testing. Data on all patients was obtained from the institution's maternity case records and laboratory data from the National Health Laboratory Services computerised database at the institution. HIV was diagnosed on a rapid test kit, and blood pressure recordings (highest BP) were taken at initial booking, and during the antenatal, intrapartum and postpartum periods. Weight was categorised as: normal weight (BMI: 18-<25), overweight (BMI: 25-<30) and obese (BMI: 30+). Early onset preeclampsia was defined as preeclampsia occurring before 34 weeks of gestation. Severe preeclampsia was diagnosed when any of the following was present: systolic BP ≥ 160 mmHg, diastolic BP ≥ 110 mmHg, impaired renal /liver function, thrombocytopenia, pulmonary oedema, cerebral /visual disturbances and persistent right upper quadrant/epigastric pain unresponsive to treatment, and fetal criteria including fetal growth impairment , oligohydramnios and fetal death [13].

Statistical analysis:

Statistical analysis was done using SPSS[®] version 22. The descriptive statistics are tabulated. Comparisons of mean across groups were done using the Kruskal– Wallis test, a non-parametric test for variables that were found not to be normally distributed. The student t test was used to compare difference in means between two groups mainly PE HIV negative and PE HIV positive. In all the tests, we used $\alpha = 0.05$ as the level of significance. The decision to reject the null hypothesis is based on the p value (probability of committing a Type 1 error). The Chi-square test was used to test association across groups.

Results:

Figure 2.1. shows the distribution of patients with preeclampsia and HIV infection. Ninety eight women (45 HIV infected preeclamptic women on HAART i.e.46%, and 53 uninfected preeclamptic women i.e.54%) were recruited into the study and followed until delivery and discharge from the institution. A control group of 95 women (50 normotensive uninfected i.e. 53% and 45 normotensive infected i.e.47%) was also included for comparison of patient characteristics.

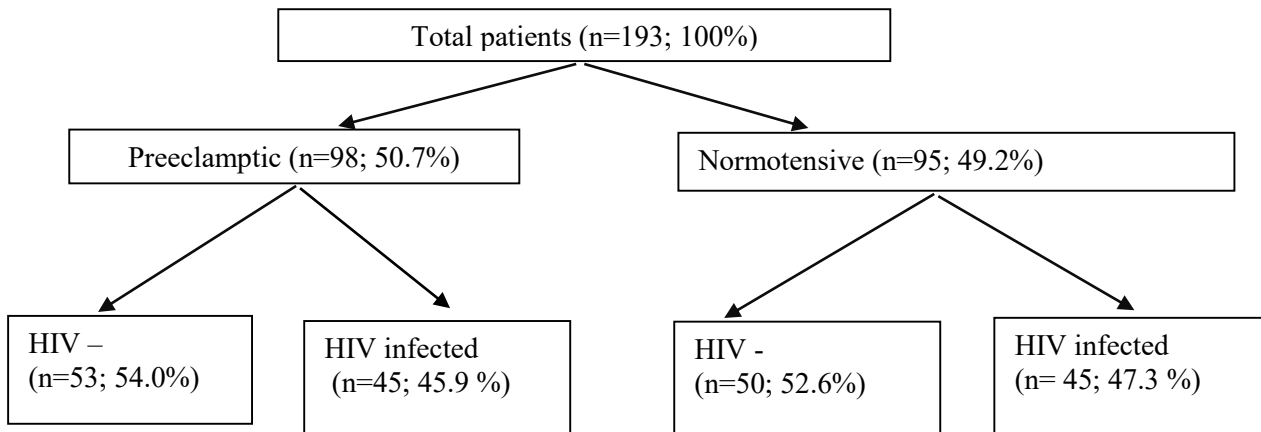


Figure 2.1: Flow diagram of the study population

The characteristics of the patients that were analysed in the study is shown in *Table 2.1*. The mean age of the women was 26.5 years and HIV infected women with pre-eclampsia were significantly older than uninfected women with preeclampsia. Most women i.e.197 (93%), booked at a local clinic at an average of 22 weeks of gestation and had 4 antenatal clinic visits. Overall, women in all groups were overweight /obese (BMI =31).The mean CD4 cell count between normotensive and pre-eclamptic HIV infected women was not significantly different.

The clinical and biochemical data of women with preeclampsia are shown in *Table 2.2* and *Table 2.3* respectively. The prevalence of early onset preeclampsia (EOPE) and severe preeclampsia was 32(33%) and 39(40%) respectively, and did not differ significantly in the HIV group.

Table 2.1. Clinical Characteristics of participants

Variable	PE HIV uninfected (Grp 1) n= 53	PE HIV infected (Grp 2) n=45	Normotensive uninfected (Grp 3) n=50	Normotensive HIV infected (Grp 4) n=45	Total (n,%)	p value . (all grps)	p value (Grp 1 vs 2)
Age (n, %) (mean, ± SD) Range	53(100) 24.8±5.3 16-40	44(98) 28.7±7.3 16-42	50(100) 24.6 ±6.4 16-42	45(100) 28 ± 6.4 17-46	192(99)	0.002	0.003
Parity (n, %) 0 1-5 >5	26(39.4) 27(22.0) 0(0)	18 (27.3) 26 (21.1) 1 (25.0)	17(25.8) 31(25.2) 2(50)	5 (7.6) 39(31.7) 1 (25.0)	193(100) 66 (34) 123(64) 4 (2)	0.006	0.400
Booking GA (wks.) Mean± SD Range	22 ±6.8 6-37	22 ±7.0 4-35	22 ±7.1 5-38	21 ±7.0 7-36	179(93)	0.581	0.837
CD4($\times 10^6/l$) (n,%) (mean, ±SD)	-	42(93) (436±181)	-	40(89) (432±220)		0.40*	
BMI (n, %) (mean, SD)	35(66) 24.3(±13.0)	39(87) 39.4(±12.4)	35(70) 29.7(±12.0)	36(80) 30.1(±15.4)	145(75) 31(±7.4)	0.73	0.50
MOD (n, %)						0.00	0.00
ELCS	9(17)	9(20)	25(53)	27(61)	70(37)		
EMCS	32(60)	23(52)	13(28)	11(25)	79(42)		
VD	12(23)	12(27)	9(19)	6(14)	39(21)		
TOTAL	53(100)	44(98)	47(94)	44(98)	188(97)		

Abbreviations: GA = gestational age, BMI = Body Mass Index, MOD= mode of delivery, ELCS= elective caesarean section, EMCS= emergency caesarean section, VD= vaginal delivery, SD=standard deviation PE=pre-eclampsia, n=total number (1): p = significance, * = Grp 2 vs Grp 4

In most cases, blood pressure was controlled using a single therapeutic agent (usually methyl dopa), with no significant difference the number of antihypertensive drugs used to control blood pressure between HIV infected and uninfected groups (Table 2.2) . There was no significant difference in the highest BP recorded at booking, and during the antenatal, intrapartum and postpartum periods.

The analysis of the biochemical indices investigated showed no significant differences apart from gamma glutamyl transferase (GGT), which was significantly raised among HIV infected preeclamptic women. CD4 counts were analysed in the HIV infected preeclampsia and normotensive women, as CD4 testing is not routinely performed in HIV uninfected women in the study setting. Table 2.4 shows the perinatal and maternal outcomes among the women with preeclampsia. The mean birth weight overall, including normotensive women was 2.82 kg, and more female babies were born compared with males (55.8 % vs 44.2%) in the study cohort.

Table 2.2: Clinical characteristics of women with preeclampsia

Variable	PE HIV uninfected (n= 53)	PE HIV infected (n=45)	Total (n,%)	P value
EOPE (n, %)	14(26)	18(40)	32(33)	0.153
Severe PE (n, %)	18(34)	21(47)	39(40)	0.200
No of AHT drugs (n, %)			97(99)	0.666
1	29(30)	24(25)	53 (55)	
2	15(15)	12(12)	27 (28)	
3	7 (7)	8 (8)	15 (15)	
4>	1(1)	1(1)	2 (2)	
Proteinuria(dipstix) (n,%) (mean)	53(100) 2+	45(100) 2+	-	-
BK SYS (mmHg) (n, %) (mean , ±SD) Range	53(100) 126± 22.4 91-198	45(100) 134± 18.4 94-194	98(100)	0.070
BK DIA (mmHg) (n, %) (mean, ±SD) Range	53(100) 79.8± 14.4 57-123	45(100) 84.8±13.2 50-131	98(100)	0.081
AN SYS (mmHg) (n, %) (mean, ±SD) Range	53(100) 157.1 ±17.1 99-195	45(100) 159 ±14.2) 133-195	98(100)	0.565
AN DIA (mmHg) (n, %) Mean, ±SD Range	53(100) 101.92 ±9.8 45-127	45(100) 104.5±9.8 88-131	98(100)	0.304
IP SYS (mmHg) (n, %) Mean, ±SD Range	21(40) 135.51±19.5 99-175	17(38) 134.0±15.4 112-172	39(40)	0.789
IP DIA (mmHg) (n, %) Mean, ±SD range	21(40) 87.16± 12.9 68-109	17(38) 83.32± 13.0 47-105	39(40)	0.368
PP SYS (mmHg) (n, %) Mean, ±SD Range	49(92) 145.0± 20.5 107-193	43(96) 142.95± 18.4 105-191	92(94)	0.615
PP DIA (mmHg) (n, %) Mean, ± SD Range	49(92) 91.5± 14.2 60-118	43(96) 91.05± 14.6 60-114	92(94)	0.881

Abbreviations: n=total number, EOPE=early onset pre-eclampsia, PE=preeclampsia
AHT=antihypertensive drugs, BK= booking, SYS=systolic. DIA=diastolic,
IP=intrapartum, PP=postpartum, AN=antenatal

Table 2.3: Laboratory Data:

Variable	PE HIV uninfected(n=53)	PE HIV infected (n=45)	p value	Total (n,%)
WCC($\times 10^9/l$) (n, %) Mean \pm SD	53(100) 9.1 \pm 3.4	45(100) 8.1 \pm 2.7	0.124	98(100)
Hb (g/dl) (n, %) Mean), \pm SD	53(100) 10.8 \pm 1.12	45(100) 10.9 \pm 1.31	0.560	98(100)
Platelets($\times 10^9$) (n, %) Mean, \pm SD	53(100) 232 \pm 66.6	45(100) 175 \pm 61.4	0.488	98(100)
Urea (mmol/l) (n,%) Mean, \pm SD Range	51(96) 2.3 \pm 1.2 0.4-5.5	43(96) 2.2 \pm 1.83 0.9-13.1	0.877	94(96)
Cr (umol/l) (n, %) Mean, \pm SD Range	51(96) 55.5 \pm 16.8 32-104	43(96) 58.9 \pm 31.9 32-249	0.514	94(96)
ALT (U/l) (n, %) Mean, \pm SD Range	43(81) 26.3 \pm 56.79 7-386	42(93) 26.4 \pm 53.0 9-360	0.993	85(87)
UA (mmol/l) (n, %) Mean, \pm SD	42(79) 0.29 \pm 0.086	44(98) 0.30 \pm 0.083	0.510	86(88)
AST (U/l) (n, %) Mean , \pm SD range	31(58) 33.4 \pm 34.73 9-140	33(73) 64.2 \pm 205.9 16-1227	0.429	64(65)
GGT (U/l) (n, %) Mean \pm SD Range	42(79) 17.1 \pm 14.0 5-75	41(91) 26.9 \pm 40.9 6-200	0.001*	83(85)
LDH (U/l) (n, %) Mean \pm SD Range	15(28) 630. \pm 207.2 460-1302	22(49) 1052.7 \pm 2047.6 374-10362	0.390	37(38)

Abbreviations: WCC= white cell count, Hb=haemoglobin, Cr=creatinine, ALT=alanine transaminase, AST=aspartate transaminase, GGT=gamma glutamyl transferase .LDH= lactate dehydrogenase, UA=uric acid, * = significant difference

Babies born to pre-eclamptic women were smaller compared with normotensive women (2.49 kg vs 3.14 kg), but similar between HIV infected and uninfected women with pre-eclampsia (2.4kg vs 2.5kg). Other outcomes were similar between these 2 groups. There were no maternal deaths during the duration of the study.

Table 2.4: Maternal and perinatal outcomes in preeclamptic women

Complications	HIV infected (n=45)	HIV uninfected (n=53)
<u>Perinatal: (n, %)</u>		
Preterm delivery	12 (27)	8 (15)
IUGR	7 (16)	9 (17)
Macerated stillbirths	1 (2)	1 (2)
Fresh stillbirths	1 (2)	0 (0)
Early neonatal death	1 (2)	2 (4)
Intrauterine death	2 (4)	0 (0)
IUGR	6 (13)	4 (8)
Apgars (mean) (1 min)	7 (16)	7 (13)
(5 min)	8 (18)	9 (17)
Birth weight (kg) (mean)	2.4	2.5
Ventilation (ICU)	0 (0)	0(0)
Birth defects (any)	0 (0)	0(0)
<u>Maternal (n, %)</u>		
Termination *	1(2)	1(2)
Abruptio placentae	2(4)	2(4)
HELLP **	1(2)	0(0)
Eclampsia	0(0)	1(2)
Maternal deaths	0(0)	0(0)

delivery before 28 weeks for maternal reasons

*** (haemolysis, elevated liver enzymes, low platelets)*

**** Intrauterine growth restriction*

Discussion:

Our data reveal that the clinical parameters, laboratory indices and complications associated with preeclampsia are not significantly altered in the presence of HIV infection and treatment with HAART. However, significant increases were found in gamma glutamyl transferase ($p=0.001$) among preeclamptic women on HAART. This may suggest a compounding effect on the liver, which is a target organ for preeclampsia and the side effects of HAART.

The literature on HIV infection, antiretroviral regimens and preeclampsia is controversial. Data on the impact of HIV on preeclampsia is inconsistent and currently there is no consensus on the risk that HIV infected women have of developing preeclampsia. In South Africa, Frank et al, [8] showed no reduction in the risk of developing preeclampsia/eclampsia among untreated HIV infected women compared with HIV negative women (5.7% vs 5.2% ; $p=0.61$). In contrast, Kalumba et al, [7] found that the rates of

preeclampsia are lower in HIV infected women. Internationally, a Spanish group reported a significantly higher risk for preeclampsia with HIV [14].

In view of the heterogeneity associated with the existing studies, we took a different approach to the investigation of HIV infection and preeclampsia by evaluating the biochemical indices and clinical parameters used frequently in the management HIV infected preeclamptic women on HAART compared with uninfected preeclamptic women. We further evaluated the maternal and fetal complications in these groups, with the aim of making recommendations for clinical management based on differences that may exist. To our knowledge, this is the first study to evaluate demographic, clinical and laboratory comparisons collectively in these conditions.

Preeclampsia has been associated with oxidative stress and cellular sources such as CD4⁺ T cells have been implicated [15]. CD4⁺ cells are generally decreased in preeclamptic women when compared to normal pregnancy [16]. In our comparison of CD4⁺ cells in HIV infected preeclamptic women with HIV infected controls, we did not find a significant difference (436 vs 432; $p=0.40$). Our findings differ from Kalumba *et al*, who showed significant increases in CD4⁺ among HIV positive women with preeclampsia (304 vs 208, $p=0.008$) [7]. In contrast to Kalumba *et al*. who suggest that the immunity was less affected in those who developed preeclampsia, our findings do not show an effect on immunity in those who developed preeclampsia. We also found a non-significant decreases in leucocyte counts in the HIV infected group. Leucocytes are however, also influenced by other factors e.g. disease progression, infections and drug treatment, which were not evaluated independently.

The utilisation of platelet counts, as well as renal and hepatic function tests remain major determinants in the clinical management and timing of delivery in patients with preeclampsia and associated complications like HELLP syndrome (haemolysis, elevated liver enzymes, and low platelets). Lower platelet counts were found in the HIV infected pre-eclamptic cohort and may represent a compounding effect, as both conditions are associated with reduced counts independently [17,18]. Laboratory tests of renal function (i.e. urea, creatinine, uric acid) were not significantly altered. Among the laboratory analytes of hepatic function evaluated, we found non-significant increases in LDH (630 vs 1052; $p=0.39$), but a significant increase in gamma glutamyl transferase (GGT) (26.9 vs 17.1 $p=0.001$). Our findings are consistent with other studies that show elevated levels of LDH and GGT in preeclampsia, and with antiretroviral therapy [19-21]. We did not find a significant difference in the haemoglobin levels between the groups (10.8 vs 10.9, $p=0.56$). The association of anaemia in pregnancy with lower CD4 counts in HIV infected women on antiretroviral therapy has been shown in a recent study [22].

We did not find a significant association of HIV with either early or severe preeclampsia, however further studies which evaluate the onset of infection, timing of HAART, and stage of disease progression are required in future long term studies to provide more specific data. HIV infection did not significantly affect the highest blood pressure levels at booking or in any of the antenatal,

intrapartum or postpartum periods of confinement. The use of additional antihypertensive drugs to control blood pressure was also not required in these patients (Table 2) Intrapartum blood pressure values, however, are limited by confounding variables such as pain, the stage of labour and analgesia used during labour, and did not apply to patients undergoing elective surgery.

Of concern, is the higher though non-significant BMI values (39.4 and 30.1) in both HIV infected groups (Table 1). Preeclamptic women with HIV had the highest BMI (39.4) compared with other groups. These findings may be associated with dietary habits, lack of exercise, genetic factors and diet related counselling offered during anti-retroviral treatment. Our findings are consistent with Wand et al, who recently showed a high prevalence of obesity among HIV infected women in our province [23]. Older age and lack of education were also found to be significant predictors of obesity [23].

Data on the association of antiretroviral therapy and preeclampsia are also inconsistent [24]. The use of HAART has also been associated with increased morbidity and adverse outcomes in earlier studies [13]. We did not find a significant association of HIV with maternal and perinatal outcomes in the cohort we analysed. (Table 4) Marginal differences were observed with slightly more preterm deliveries in the positive group, presumably due to higher rates of underlying infection, but fewer cases of growth impairment. Our findings are consistent with other studies that did not confirm adverse associations [25], however, our study was limited by a small sample size and larger, longer term studies are required to provide more accurate data. Further limitations in our study were the absence of an untreated HIV infected group, the inclusion of which would be unethical under the existing National Guidelines [12]. Further larger studies that evaluate the immune status of infection preconceptually, and track changes throughout pregnancy in relation to the development of preeclampsia and its sequelae will provide significant information on the relationship between these conditions.

Conclusion:

HIV infection treated with HAART is not associated with significant differences in the biochemical indices, clinical features or maternal and fetal outcomes in women with preeclampsia. The restoration of the immune system associated with the use of HAART, may facilitate the immunological responses in preeclampsia similar to that occurring in HIV uninfected women, and may explain the similarities in the clinical and laboratory manifestations in these patients. Current clinical guidelines on the management of HIV infected preeclamptic women appear to remain appropriate, however frequent evaluation of hepatic function in these women will enable early detection of hepatic toxicity. Strategies for the prevention of obesity, which may complicate long term cardiovascular and metabolic effects associated with preeclampsia, are necessary. Further research on immune restoration associated with HAART in pregnancy, will improve our understanding of these conditions, which still remain a significant health care challenge.

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CHAPTER 3

THE EFFECTS OF HAART ON PRO-INFLAMMATORY CYTOKINES IN AFRICAN WOMEN WITH PREECLAMPSIA

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Synopsis: HIV and HAART are associated with significant decreases in pro-inflammatory cytokines IL2, TNF α and IL6 in African women with preeclampsia.

Abstract

Introduction:

Preeclampsia and HIV/AIDS are inflammatory conditions that contribute significantly to adverse maternal and fetal outcomes. The immune reconstitution effects of HAART on inflammatory mediators has not been adequately studied in pregnancy and may impact on the inflammatory cytokine network in women with co-morbid preeclampsia.

Aim:

To evaluate the changes in pro-inflammatory cytokines IL-2, TNF- α , IFN- γ and IL-6 in HIV infected preeclamptic women on HAART.

Methods:

A prospective experimental study was conducted at Prince Mshiyeni Memorial Hospital between July 2013 to September 2014. One hundred and ninety three pregnant women were recruited into 4 groups : uninfected normotensive (50;26%), infected normotensive(45;23%), uninfected preeclamptic (53;28%) and infected preeclamptic women (45;23%). Serum levels of cytokines TNF- α , IFN- γ , IL2 and IL6 were determined, using commercially available kits and Cytometric Bead Array (CBA). Comparative data was recorded and analysed descriptively.

Results:

In the control group i.e. normotensive groups, significantly lower values were found in IL-2 ($p=0.010$), TNF- α ($p=0.045$), and IL-6 ($p=0.005$), and a non-significant decrease was observed in IFN- γ ($p=0.345$) in HIV infected women on HAART compared to uninfected controls. In the experimental group i.e. preeclamptic women, significantly reduced levels were observed in IL-2 and TNF- α ($p=0.000$; $p=0.000$) and non-significant decreases were observed in IFN- γ and IL-6 ($p=0.023$; $p=0.086$) in HIV infected women on HAART compared with uninfected preeclamptic women. Non-significant differences were observed between uninfected preeclamptic and normotensive women.

Conclusion:

In uncomplicated/normotensive pregnancies, HAART is associated with significant decreases in IL-2, TNF- α and IL-6, and in preeclamptic women, it is associated with significant decreases in IL-2 and TNF- α . These findings suggest that HIV/HAART impacts on pro-inflammatory cytokines in women with co-morbid preeclampsia and provides a platform for further research on immune reconstitution effects of HAART during pregnancy, and the development of potential immune modulation therapies for the management of preeclampsia.

Key words: Preeclampsia; HAART; IL2/6; TNF- α ; IFN- γ

Introduction:

Preeclampsia(PE), a multi-organ hypertensive disorder of pregnancy and HIV/AIDS (human immunodeficiency virus/acquired immunodeficiency syndrome) are associated with significant maternal and perinatal morbidity and mortality, especially in poor resourced countries(WHO 2009, Backes, Markham et al. 2011, Moodley 2014). The treatment of preeclampsia (PE) still remains empiric, and resolution is achieved by delivery (Nääv, Erlandsson et al. 2015). Highly active antiretroviral therapy (HAART) has been shown to successfully reduce plasma HIV-1 viral load and vertical transmission rates in pregnancy (Autran, Carcelain et al. 1997, Volmink, Siegfried et al. 2007), and is now integrated with the management of HIV infection in pregnancy (NDH 2013).

However, there is no consensus on the relationship between these conditions as data on the impact of HIV on the rate of PE are conflicting (Kalumba, Moodley et al. 2013). HAART may influence the postulated inhibitory effect of HIV on the development of hypertensive disorders during pregnancy (Browne, Schrier et al. 2015). Although the underlying immunological changes in PE and HIV is not completely understood, it is generally accepted that both conditions are associated with inflammation (Deeks 2011, Catarino, Santos-Silva et al. 2012). Inflammation is mediated by a variety of soluble factors, including a group of secreted polypeptides known as cytokines (Shaikh, Sharma et al. 2011).

Cytokines can be classified as pro and anti –inflammatory and are considered as important initiators and mediators of inflammation and endothelial dysfunction (Raghupathy 2013). Cytokines such as interferon gamma (IFN- γ) and tumour necrosis factor alpha (TNF- α), are characteristic of T helper 1 (Th1) type immunity and mediate several cell mediated cytotoxic and inflammatory reactions (Mosmann and Sad 1996, Romagnani 2000). Interleukin (IL)-2 plays a critical role in regulating cellular and humoral chronic inflammatory responses; whilst IL-6, which also has anti-inflammatory properties, is also observed in many chronic inflammatory and autoimmune disorders and serves as a marker for the systemic activation of pro-inflammatory cytokines (Hirano and Kishimoto 1989, Shaikh, Sharma et al. 2011).

Preeclampsia is associated with a generalized systemic inflammatory response and subsequent release of pro-inflammatory cytokines that may trigger the maternal disease (Sargent, Borzychowski et al. 2006). Increases in the IL-2/IL-4 and IFN- γ /IL-4 ratios, as well as elevated circulating levels of IL-6 and TNF- α , has been reported suggesting a pro-inflammatory systemic environment when compared to normal pregnancy (Martínez-Varea, Pellicer et al. 2014).

In the progression of HIV infection, a Th1 (pro-inflammatory) to Th2 (eosinophilic/anti-inflammatory) cytokine shift has been observed, which appears to be counteracted with the usage of HAART (Fiore, Newell et al. 2006). Data on the immune reconstitution effects of HAART on pro-inflammatory cytokines in HIV infected women with PE is lacking. To further understand the effects of HAART on

the inflammatory cytokine network in HIV infected pre-eclamptic women, we investigated IL-2, TNF- α , IFN- γ and IL-6 in women during the third trimester of pregnancy.

Materials and methods:

Study population and sample collection

Institutional ethical and hospital regulatory permission was obtained for the study (Biomedical Research Ethics Committee, University of KwaZulu-Natal, South Africa; reference number BE 119/11). After informed consent was obtained, participants were recruited over a 14 month period from July 2013 to September 2014 from the maternity unit at Prince Mshiyeni Memorial Hospital in Durban, South Africa. This hospital is a regional level facility and serves a predominantly semi-urban African population from where the participants were recruited. Normotensive (n=95, age range: 16-46 years) and PE patients (n= 98, age range: 16-42 years) were enrolled into the study. Maternal venous blood samples were then taken. To maintain ethnographic and anthropometric consistency, all patients recruited were of African descent, resident in the same geographical location and of Zulu ethnicity. All patients were non-smokers, non-consumers of alcohol or recreational drugs, and all HIV infected patients were on HAART (tenofovir, emtricitabine, efavirenz) as per the National guidelines (NDH 2013). Calcium supplementation was administered routinely to all patients attending the clinic. Women with gestational hypertension, renal disease, diabetes mellitus, chronic hypertension and collagen vascular disease were excluded. Preeclampsia was defined as a blood pressure \geq 140mmHg systolic or \geq to 90 mmHg diastolic on 2 occasions at least 4 hours apart after 20 weeks of gestation in a woman with previously normal blood pressure (Roberts, August et al. 2013). All patients had proteinuria \geq +1 on urine dipstick testing. Data on all patients was obtained from the institution's maternity case records and laboratory data from the National Health Laboratory Services computerised database at the institution, and HIV was diagnosed on a rapid test kit. Weight was categorised as: normal weight (BMI: 18-<25), overweight (BMI: 25-<30) and obese (BMI: 30+). A summary of the clinical characteristics of the participants is shown in Table 1.

Cytokine quantification:

The BD Cytometric Bead Array Human Th1/Th2/Th17 Cytokine kit was used to measure the IL-2, TNF- α , IFN- γ and IL-6, protein levels in a serum samples. Briefly, lyophilized standards were prepared by reconstitution and serial dilution (1:2 – 1:256) in assay diluent immediately before staining with Capture Beads and PE Detection Reagent. All serum samples were also diluted in assay diluent (1:4)

before staining with Capture Beads and PE Detection Reagent. For the staining procedure, 50µL of each standard and unknown sample was added to appropriately labeled sample tubes followed by 50µL of the Human Th1/Th2/Th17 PE Detection Reagent and incubated (3 h, RT, protected from light). Following incubation 1 mL of Wash Buffer was added to each assay tube and centrifuged at 200xg for 5 minutes. The supernatant from each assay tube was then carefully aspirated and 300µL of Wash Buffer was added to each assay tube to resuspend the bead pellet. Flow cytometric data was acquired using the BD AccuriC6 Sampler counting 2100 gated events. This ensures that the sample file contains approximately 300 events per Capture Bead. Data analysis was performed using the FCAP Array analysis software. All cytokines are represented as pg/mL as extrapolated from standard curves.

Statistical analysis

Statistical analysis was done using SPSS® version 22. Correlation between continuous variables was assessed using the Spearman rank correlation coefficient. The Wilcoxon rank-sum (Mann-Whitney) test was used to compare difference in sum of ranks (i.e. cytokine concentration) by dichotomous group, mainly between PE HIV– and PE HIV+. Comparisons of mean across 3 or more groups were done using the Kruskal–Wallis test. Non-parametric approaches were employed above as cytokine distributions were not normally distributed with evidence of asymmetry. The Pearson Chi-square (χ^2) test was used to test association between group(s) and categorical explanatory variables. In the determination of significance, a p-value < 0.05 was deemed statistically significant.

Results:

The clinical characteristics of participants are shown in Table 3.1. The average duration of HAART was 16.6 weeks in the control group and 14.5 weeks in the preeclamptic group, however the duration of exposure is not accurate. All women were in the third trimester of pregnancy and the mean gestational age was 36.5 weeks of gestation. There was a significant difference in the parity across all groups ($p=0.006$) but not among the preeclamptic women ($p=0.400$).

Table 3.1. Clinical characteristics of participants

Variable	PE HIV uninfected (Grp 1) n= 53	PE HIV infected (Grp 2) n=45	Normotensive uninfected (Grp 3) n=50	Normotensive HIV infected (Grp 4) n=45	Total (n,%)	p value. (all grps)	p value (Grp 1 vs 2)
Age (yrs)(n,%) (mean, ± SD) Range	53(100) 24.8±5.3 16-40	44(98) 28.7±7.3 16-42	50(100) 24.6 ±6.4 16-42	45(100) 28 ± 6.4 17-46	192(99)	0.002	0.003
Parity (n, %) 0 1-5 >5	26(39.4) 27(22.0) 0(0)	18 (27.3) 26 (21.1) 1 (25.0)	17(25.8) 31(25.2) 2(50)	5 (7.6) 39(31.7) 1 (25.0)	193(100) 66 (34) 123(64) 4 (2)	0.006	0.400
Booking GA (wks.) Mean± SD Range	22 ±6.8 6-37	22 ±7.0 4-35	22 ±7.1 5-38	21 ±7.0 7-36	179(93)	0.581	0.837
CD4 (x10 ⁶ /l) (n,%) (mean, ±SD)	-	42(93) (436±181)	-	40(89) (432±220)		0.40*	
BMI (n,%) (mean, SD)	35(66) 24.3(±13.0)	39(87) 39.4(±12.4)	35(70) 29.7(±12.0)	36(80) 30.1(±15.4)	145(75) 31(±7.4)	0.73	0.50
MOD (n, %) ELCS EMCS VD TOTAL	9(17) 32(60) 12(23) 53(100)	9(20) 23(52) 12(27) 44(98)	25(53) 13(28) 9(19) 47(94)	27(61) 11(25) 6(14) 44(98)	70(37) 79(42) 39(21) 188(97)	0.00	0.00

Abbreviations: GA = gestational age, BMI = Body Mass Index, MOD= mode of delivery, ELCS= elective caesarean section, EMCS= emergency caesarean section, VD= vaginal delivery, SD=standard deviation PE=preeclampsia, n=total number (1): p = significance, * = Grp 2 vs Grp 4

There was also a significant difference in age across the groups, however there was little or no correlation between cytokine levels and age i.e. suggesting little confounding influence of age in this regard. Hence age was not factored into the cytokine comparison by group. The overall quantitative evaluation of cytokines and group sub-analysis is shown in Table 3.2 and 3.3 and Fig.3.1 and 3.2 respectively. Significant differences were found in many of the circulating cytokines investigated. In the control groups i.e. normotensive groups, significant decreases were found in IL-2 ($p=0.010$), TNF- α ($p=0.045$), and IL-6 ($p=0.005$), and a non-significant decrease in IFN- γ ($p=0.345$) in HIV infected women on HAART ^{4} compared to uninfected controls ^{3}.

Table 3.2: Quantitative evaluation of cytokines:

CYTOKINE	Statistic	PE+HIV+ {2} vs PE+HIV- {1}	PE- HIV- {3} vs PE +HIV- {1}	PE – HIV+ {4} vs PE – HIV- {3}
(1) IL2 (pg/ml)	p-value ⁱ	0.0008	0.9384	0.0104
	Group { } Median [] (IQR)	{2} [268.7] (263.8,272.1) {1} [273.2] (269.5,278.6)	{3} [274.4] (261.9,284.3) {1} [273.2] (269.5,278.6)	{4} [257.6] (245.1,269.5) {3} [274.4] (261.9,284.3)
(2) TNFa (pg/ml)	p-value ⁱ	0.0001	0.2792	0.0453
	Group { } Median [] (IQR)	{2} [172.2] (161.2,181.3) {1} [185.2] (178.7,196.2)	{3} [198.8] (175.4,247.5) {1} [185.2] (178.7,196.2)	{4} [174.8] (165.7,189.1) {3} [198.8] (175.4,247.5)
(3) IFNg (pg/ml)	p-value ⁱ	0.0233	0.5534	0.3451
	Group { } Median [] (IQR)	{2} [214.1] (205.1,218.3) {1} [218.3] (208.7,226.1)	{3} [217.1] (212.3,244.1) {1} [218.3] (208.7,226.1)	{4} [214.1] (202.1,227.3) {3} [217.1] (212.3,244.1)
(4) IL6 (pg/ml)	p-value ⁱ	0.0865	0.7743	0.0051
	Group { } Median [] (IQR)	{2} [139.7] (131.8,163.2) {1} [147.0] (138.4,173)	{3} [149.8] (138.8,182.7) {1} [147] (138.4,173)	{4} [133] (122.7,138.4) {3} [149.8] (138.8,182.7)

Abbreviations: PE= preeclampsia, HIV = Human Immunodeficiency Virus, IL-2 = interleukin 2, TNFa=tumour necrosis factor alpha, IFNg=interferon gamma, i: Wilcoxon rank-sum (Mann-Whitney) test, IQR: interquartile range, vs=versus

In the experimental group i.e. preeclamptic women, significant decreases were observed in IL-2 and TNF- α ($p=0.000$; $p=0.000$) and non-significant decreases in IFN- γ and IL-6 ($p=0.023$; $p=0.086$) in HIV infected women on HAART ^{2} compared with uninfected preeclamptic women ^{1}. No significant differences were observed in uninfected preeclamptic women ^{1} when compared with uninfected normotensive women ^{3}.

Table 3.3: Comparative analysis of cytokine perturbations by groups

CYTOKINE	HIV+ vs HIV - (Group 4 vs 3) (Δ in median pg/ml) (p value in brackets)	PE vs NORMOTENSIVE (Group 1 vs 3) (Δ in median pg/ml) (p value in brackets)	PE/ HIV+ vs PE/ HIV - (Group 2 vs 1) (Δ in median pg/ml) (p value in brackets)
IL2	-16.8 (0.010)*	-1.20 (0.938)	-4.5 (0.000)*
TNF α	-24.0 (0.045)*	-13.60 (0.279)	-13.0 (0.000)*
IFN γ	-3.0 (0.345)	+1.20 (0.553)	-4.2 (0.023)
IL6	-16.8 (0.005)*	-2.80 (0.774)	-7.3 (0.086)

**(p > 0.05 is deemed statistically significant); Δ = difference in values; PE= preeclampsia*

The relationship between cytokine estimations in the respective groups are depicted in Figures 3.1 and 3.2 below. Differences in concentrations and spread of the cytokines shown in Table 3.2 can be graphically observed in Figure 3.2.

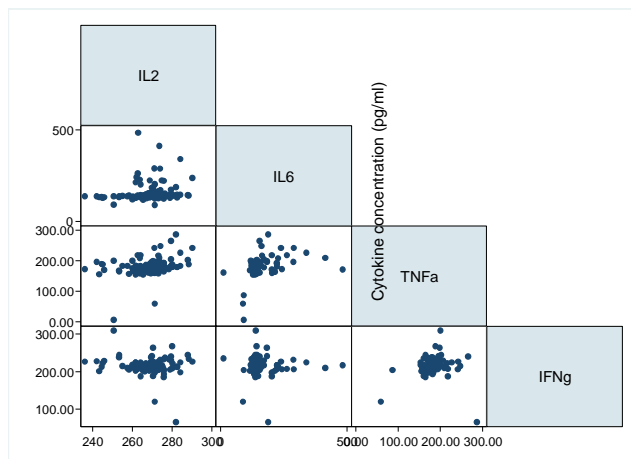


Figure 3.1: Relationship between individual cytokine concentration

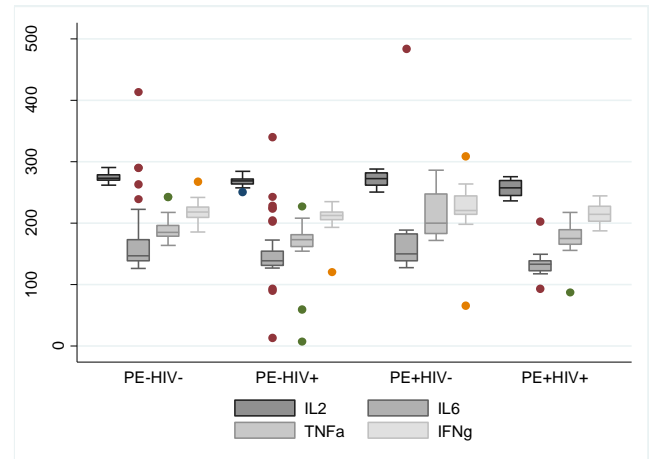


Figure 3.2: Cytokine concentrations by group comparison

Discussion:

Our study demonstrates that decreases in inflammatory cytokines occur in association with HAART in normotensive pregnancies and in preeclampsia. HAART may have an attenuating effect on the pro-inflammatory environment generally associated with preeclampsia (Saito, Umekage et al. 1999, Bates, Quenby et al. 2002, Cemgil Arikan, Aral et al. 2012), similar to that occurring in normotensive women, and may inhibit the pro-inflammatory sequelae of preeclampsia.

Cytokines have been implicated as potential mediators in preeclampsia, where endothelial dysfunction is considered the hallmark of the syndrome (Szarka, Rigó et al. 2010). Cytokines may also be involved with abnormal inflammatory responses caused by syncytiotrophoblast molecules (STBM) shed into

maternal blood in preeclampsia. (Raghupathy 2013). The alteration between pro-inflammatory/regulatory responses does not occur in preeclampsia, or may be reverted in very early stages of the disease, leading to a pro-inflammatory state (Pinheiro, Martins-Filho et al. 2013). In our study, we did not find significant differences in IL-2, TNF- α , IFN- γ or IL-6 in pre-eclamptic women compared to normotensive women. Our findings do not reflect the pro-inflammatory environment in PE shown in other studies (Saito, Umekage et al. 1999, Bates, Quenby et al. 2002, Cemgil Arikan, Aral et al. 2012), however differences in study designs exist in relation to sample population, pregnancy status, and sampling techniques.

HIV is known to infect T cells that have CD4⁺ receptors present on their surface, which, among others in the immune system, secrete cytokines (Tudela, Singh et al. 2014). Studies conducted earlier have revealed that HIV-infected individuals have a weaker immune system and the inability of CD4⁺ T cells to proliferate, due to the decrease in the levels of IL-12 (Noble, Thomas et al. 2001). As a result, decreases in IL-2 and IFN- γ occur, leading to immunosuppressive effects and opportunistic infections, a marker of advanced disease. In a recent study of the cytokine milieu in untreated HIV infection, pro-inflammatory cytokines, IL-2, IL-12, and IFN- γ were shown to be significantly decreased. CD4 cell counts are an important biomarker for HIV progression, and are lower in HIV participants versus healthy participants (Tudela, Singh et al. 2014). In our study, we did not find a significant difference between the CD4⁺ counts in normotensive HIV infected women and those with preeclampsia, however, both groups were on HAART in terms of existing guidelines (NDH 2013).

Because HIV has immune – depressive effects, an association between preeclampsia and HIV has been suggested (Hall, Gebhardt et al. 2014). Highly active antiretroviral therapy (HAART) suppresses HIV viremia, increases CD4⁺ cell counts and is suggested to counteract the Th1 to Th2 shift in the disease progression of HIV (Fiore, Newell et al. 2006). Its use in pregnancy provides significant benefits in delaying HIV disease progression and reducing the risk of mother-to-child-transmission, and has been integrated into policy (NDH 2013).

Our data shows significant decreases in the pro-inflammatory cytokines IL-2 ($p=0.010$), TNF- α ($p=0.045$), and IL6 ($p=0.005$), and a non-significant decrease in IFN- γ ($p=0.345$) among HIV positive normotensive women on HAART compared with uninfected normotensive pregnant women, highlighting the suppressive effect of HAART on these inflammatory cytokines. A similar significant effect on IL-2 and TNF- α ($p=0.000$; $p=0.000$) and non-significant decreases in IFN- γ and IL-6 ($p=0.023$; $p=0.086$) was observed with HAART in HIV infected preeclamptic women when compared to uninfected preeclamptic women, suggesting a potentially attenuating effect of HAART on

inflammatory cytokines in uncomplicated and preeclamptic HIV infected pregnancies. . Similar findings were observed in a local study among Black African participants, receiving HAART, although this study involved non –pregnant participants (Malherbe, Steel et al. 2014). Our data suggests that immune reconstitution by HAART in these conditions includes alterations in pro-inflammatory cytokines. Although some clinical data is associated with a lower rate of preeclampsia among HIV positive women who receive HAART (Mattar, Amed et al. 2007), further randomised control trials are necessary to determine the clinical association. Currently there is a paucity of data on cytokine mediated immune reconstitution effects associated with HAART in pregnancy, possibly due to differences in drug regimens ,patient profiles, study settings and rapidly evolving therapeutic regimens.

Our study was limited by the lack of knowledge on the precise duration of HAART, and detailed knowledge of drug adherence. Moreover, pregnancy is inherently immunogenic and is associated with longitudinal variation, posing further challenges to accurately contextualise changes. In our study, we did not include patients with untreated HIV infection, which is unethical under the current guidelines. Our study was conducted among African women, and, therefore, the results may not be generalizable to other HIV-infected populations. In the context of racial variation on cytokine responses, African–Americans have been shown to have higher baseline levels of inflammatory cytokines (Slopen, Lewis et al. 2010). Differences in sample sizes, patient selection and techniques used, further contribute to heterogeneity among studies relating to preeclampsia and HIV. Furthermore, functional pleiotropy and redundancy are characteristic features of cytokines, and may show overlapping activities depending on the type and developmental state of the target cells involved (Scheller, Chalaris et al. 2011).

Conclusion:

The effects of HAART in pregnancy include alterations in pro-inflammatory cytokines IL-2, TNF- α , IFN- γ and IL-6. In uncomplicated/ normotensive pregnancies, HIV/HAART was associated with significant reductions in IL-2, TNF- α and IL-6. In pre-eclampsia, HIV/HAART was associated with significant decreases in IL-2 and TNF- α . These findings highlight the need for further investigation on the immune reconstitution effects of HAART during pregnancy, and the potential of immune modulation therapy for the management of preeclampsia, where treatment still remains empiric.

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CHAPTER 4

MiRNA-27a rs895819 T>C single nucleotide polymorphism is associated with obesity in HIV infected preeclamptic Black South African women on HAART

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Synopsis: MiR-27a rs895819 polymorphism may not be associated with increased susceptibility to preeclampsia (PE); however, the miR-27a TC/CC genotype increases susceptibility to elevated BMI in PE, which may be significantly influenced by Human Immunodeficiency virus (HIV) infection and highly active antiretroviral therapy (HAART).

Abstract

Preeclampsia (PE) and HIV/AIDS present a major health challenge globally. South Africa has the highest disease burden of both HIV/AIDS and PE in the world. Despite extensive research, the pathophysiology of these conditions is not completely understood, however a genetic predisposition in women may affect susceptibility. MiRNA-27a regulates adipogenesis and glucose metabolism. A single nucleotide polymorphism (SNP) in miRNA-27a (rs895819T>C) has shown to have disparate effects in various populations. This study investigated the frequency of rs rs895819T>C in pregnant normotensive and preeclamptic Black South African women. The cohort was genotyped and the association of the SNP to PE in the presence of co-morbid HIV infection treated with HAART was determined.

Enrollment into the study included: normotensive (n=95; 80 analysed for rs895819T>C, age range: 16-46 years) and PE patients (n= 98; 56 analysed for rs895819T>C), age range: 16-42 years). DNA was isolated from peripheral blood mononuclear cells (PBMCs). Genotyping and the polymorphism of miRNA-27a (rs895819) was detected using a TaqMan[®] SNP Genotyping assay. The cytokines Interleukin-2 (IL-2), Tumor Necrosis Factor (TNF)- α , Interferon- γ (IFN- γ) and Interleukin-6 (IL-6) were measured using the BD Cytometric Bead Array Human Th1/Th2/Th17 Cytokine kit. All other clinical parameters were obtained from institutional records. We did not find a significant association of miR-27a polymorphism with PE susceptibility in our data. However, in the subgroup analysis, the variant genotypes (TC/CC) were associated with higher body mass index (BMI) among PE women (32.57 vs 29.25, $p=0.064$), significantly in the presence of HIV infection (33.47 vs 27.8, $p=0.005$). The results of this study suggests that miR-27a rs895819 may not be associated with PE susceptibility; however, the miR-27a TC/CC genotype increases susceptibility to elevated BMI in PE, which may be significantly influenced by co-morbid HIV infection among pregnant women on HAART.

Keywords: miR-27a, preeclampsia, Black South African women, single nucleotide polymorphism, HIV, BMI, HAART

1. Introduction:

Preeclampsia (PE) is a pregnancy-specific multi-organ syndrome recognized by the new onset of hypertension and proteinuria after 20 weeks of gestation (Jeyabalan, 2013). Globally, PE complicates approximately 2-10 % of pregnancies and is associated with 10-15% of direct maternal deaths overall (Duley, 2009). Perinatal complications include premature delivery, intra-uterine growth restriction, hypoxic neurological lesions and foetal death (Lorquet et al., 2010). The overall risk of PE is further increased by obesity (Bodnar et al., 2005) and features of the metabolic syndrome (obesity, hypertension, insulin resistance, impaired glucose tolerance, and dyslipidaemia) occur more commonly in women with PE (Kaaja, 1998). Furthermore, PE has also been associated with cardiovascular disease in later life (Gynecologists, 2013).

The pathogenic mechanisms underlying PE remain to be elucidated; however, immune maladaptation, inadequate placental development and trophoblast invasion, placental ischaemia, oxidative stress and thrombosis are all thought to represent key factors in the development of disease (Williams and Pipkin, 2011). All of these components have genetic factors that may be involved in the pathogenesis of PE (Williams and Pipkin, 2011).

MicroRNAs (miRNAs) are endogenous small RNAs that post transcriptionally regulate gene expression and have been shown to have important roles in numerous disease processes (Hilton et al., 2013). Interestingly, many miRNA regulated pathways are co-incident with pathophysiological processes related to PE. For instance, miRNAs regulate pathways in adipose tissue that control adipogenesis, insulin resistance and inflammation (Hilton et al., 2013), and regulate endothelial cell function and angiogenesis by regulating pro- and anti-angiogenic activity (Urbich et al., 2012). They have also been shown to regulate vascular integrity in angiogenesis induced by ischemia (Fish et al., 2008; Solingen et al., 2009).

More specifically, evidence shows the involvement of miRNA-27a (miR-27a), a member of the miR-23~27~24 cluster (Zhu and Fan, 2013), in the regulation of many of these processes. MiR-27a promotes angiogenesis by targeting the angiogenesis inhibitor SEMA6A, which controls repulsion of neighbouring endothelial cells (Urbich et al., 2012). It plays an anti-adipogenic role by influencing prohibitin and impairing mitochondrial function (Kang et al., 2013) and in cardiovascular disease, it is associated with angiogenesis and endothelial apoptosis in cardiac ischemia (Bang et al., 2012). Its role in inflammation is demonstrated by enhanced expression of pro-inflammatory cytokines, such as IL-10 when up-regulated in Tlr2- or Tlr4-activated macrophages (N Xie et al., 2014). More recently, a knock down of miR-27a, has been shown to down regulate pro-inflammatory cytokines IL-6 and TNF- α , which are associated with PE (Wang et al., 2014) (Martínez-Varea et al., 2014).

Genetic polymorphisms involve one of two or more variants of a particular DNA sequence and can affect miRNA expression, maturation or mRNA recognition and may represent an important risk determinant of disease susceptibility (Li et al., 2015). The miR-27a single nucleotide polymorphism (SNP) rs895819 is located in the terminal loop of pre-miR-27a (Shi et al., 2012). Given the biological functions associated with miR-27a and the role of the SNP rs 895819 in the promotion of cell growth and proliferation (Stenholm et al., 2013), this study investigated the association of rs 895819 with PE, related clinical features and cytokines among Black South Africa women, where the prevalence of PE is very high (Moodley and Kalane, 2006). Due to the associated high rate of co-morbid HIV infection in this population (HealthSystemsTrust, 2012) we included HIV infected women on HAART to identify differential associations.

2. Materials and Methods

2.1. Study population and sample collection

Institutional ethical and hospital regulatory permission was obtained for the study (Biomedical Research Ethics Committee, University of KwaZulu-Natal, South Africa; reference number BE 119/11). After informed consent was obtained, participants were recruited over a 14 month period from July 2013 to September 2014 from the maternity unit at Prince Mshiyeni Memorial Hospital in Durban, South Africa. This hospital is a regional level facility and serves a predominantly semi-urban African population from where the participants were recruited. Normotensive [n=95, (80 analysed for rs895819T>C), age range: 16-46 years] and PE patients [n= 98 (56 analysed for rs895819T>C), age range: 16-42 years] were enrolled into the study. Maternal venous blood samples were then taken. To maintain ethnographic and anthropometric consistency, all patients recruited were of African descent, resident in the same geographical location and of Zulu ethnicity. All patients were non-smokers, non-consumers of alcohol or recreational drugs, and all HIV infected patients were on highly active antiretroviral therapy (HAART viz. tenofovir, emtricitabine, efavirenz) as per the National guidelines (NDoH, 2013). Calcium supplementation was administered routinely to all patients attending the clinic. Women with gestational hypertension, renal disease, diabetes mellitus, chronic hypertension and collagen vascular disease were excluded. PE was defined as a blood pressure \geq 140mmHg systolic or \geq to 90 mmHg diastolic on 2 occasions at least 4 hours apart after 20 weeks of gestation in a woman with previously normal blood pressure (Gynecologists, 2013). All patients had proteinuria \geq +1 on urine dipstick testing. Data on all patients was obtained from the institution's maternity case records and laboratory data from the National Health Laboratory Services® computerised database at the institution. HIV was diagnosed on a rapid test kit, Weight was categorised as: normal weight (BMI: 18-<25), overweight (BMI: 25-<35). Early

onset preeclampsia was considered as ≤ 34 weeks of gestation (Tranquilli, 2014). Severe preeclampsia was diagnosed when features included any of the following: systolic blood pressure ≥ 160 mmHg or diastolic blood pressure ≥ 110 mmHg; maternal neurological disorders such as persistent headaches and brisk reflexes, eclampsia, acute pulmonary edema, proteinuria ≥ 5 g/day, oliguria < 500 cc/day, creatinine > 120 $\mu\text{mol/L}$, features HELLP syndrome and thrombocytopenia $< 100,000/\text{mm}^3$, fetal criteria including intrauterine growth retardation, oligohydramnios, or fetal death in utero. (Obstetricians and Gynecologists 2013), (Sibai, Dekker et al. 2005).

2.2 DNA Extraction & Genotyping

DNA from PBMCs of 56 PE patients and 80 normotensive subjects was extracted using the Quick-gDNA MiniPrep kit (Zymo Research, catalogue no. D3006) and FlexiGene DNA kit (Qiagen, catalogue no. 51204) as per the manufacturer's protocol. DNA was quantified using the Nanodrop2000 spectrophotometer. All samples were standardised to a concentration of 10ng/ μL .

All subjects were genotyped for miR-27a rs895819 using a TaqMan[®] Pre-designed SNP genotyping assay (Life Technologies, catalogue no. 4351379), following the manufacturer's protocol. A final reaction mixture consisted of 40 \times TaqMan[®] Predesigned genotyping assay, 2 \times TaqMan[®] Genotyping Master Mix, nuclease-free water, and a 10ng genomic DNA template. The experiment was performed using the Applied Biosystems[®] ViiA[™] 7 Real-Time PCR System.

The TaqMan Predesigned Genotyping Assay contains two primers for amplifying the sequence of interest, and two TaqMan[®] minor-groove binding (MGB) probes for detecting alleles. The presence of two probe pairs in each reaction allows genotyping of the two possible alleles at the SNP site in a DNA target sequence. The genotyping assay determines the presence or absence of a SNP based on the change in fluorescence of the dyes associated with the probes. Each probe is labelled with a VIC[®] dye-labelled probe and FAM[™] dye-labelled probe - assigned specifically to either the ancestral or variant allele.

2.3. Cytokine quantification:

The BD Cytometric Bead Array Human Th1/Th2/Th17 Cytokine kit was used to measure the Interleukin (IL)-2, IL-6, IL-4, IL10, IL 17a, Tumour Necrosis Factor (TNF)- α and Interferon- γ (IFN- γ) levels in each patient serum sample. Briefly, lyophilized standards were prepared by reconstitution and serial dilution (1:2 – 1:256) in assay diluent immediately before staining with Capture Beads and PE Detection Reagent. All serum samples were also diluted in assay diluent (1:4) before staining with Capture Beads and PE Detection Reagent. For the staining procedure, 50 μL of each standard and

unknown sample was added to appropriately labelled sample tubes followed by 50µL of the Human Th1/Th2/Th17 PE Detection Reagent and incubated (3h, RT, protected from light). Following incubation 1mL of Wash Buffer was added to each assay tube and centrifuged at 200g for 5 minutes. The supernatant from each assay tube was then carefully aspirated and 300µL of Wash Buffer was added to each assay tube to resuspend the bead pellet. Flow cytometric data was acquired using the BD AccuriC6 Sampler counting 2100 gated events. This ensured that the sample file contained approximately 300 events per Capture Bead. Data analysis was performed using the FCAP Array analysis software. All cytokines are represented as pg/mL as extrapolated from standard curves.

2.4 Statistical analysis:

Statistical analyses were done using GraphPad prism software (version 5.0). The Hardy–Weinberg equilibrium was used to test for deviation of allele/genotype frequency. Allele and genotype frequencies were calculated using the Fisher’s exact and Chi square tests, respectively, and the Odd’s ratio and confidence intervals were determined. To assess difference in the clinical parameters (not grouped according to genotype), the t-test with Welch’s correction or one-way ANOVA tests were used. The correlations between the clinical parameters, grouped per genotype, were assessed for PE and normotensive patients, which were also further tested under the HIV positive and negative subsets. The t-test with Welch’s correction and Fisher’s exact test were used for these analyses. A p-value <0.05 was deemed statistically significant.

3. Results:

The clinical characteristics of all study subjects are shown in Table 4.1. The study cohort was categorised into 4 groups: (1) uninfected PE women (PEHIV-), (2) infected PE women (PEHIV+), (3) uninfected normotensive women (Normo-) and (4) infected normotensive women (Normo+). All women were in the third trimester of pregnancy and the mean gestational age was 36.5 weeks. There was a significant difference in the parity across all groups but not between the PE women ($p=0.400$). There was also a significant difference in age across the groups, however there was little or no correlation between cytokine levels. The average duration of HAART was 16.6 and 14.5 weeks in the normotensive and PE groups respectively, however this is not a precise duration of exposure. There were significant differences in the mode of delivery, based on obstetric related indications. CD4 counts were not routinely available in uninfected women as per institutional standard of care.

Table 4.1: Clinical characteristics of participants:

Variable	PE HIV- (n= 53)	PE HIV+ (n=45)	NORMO HIV- (n=50)	NORMO HIV + (n=45)	Total (n=193)	p value. (all grps)	p value PE HIV- vs PE HIV+
Age (n, %)	53(100)	44(98)	50(100)	45(100)	192(99)	0.002	0.003
(mean, ± SD)	24.8±5.3	28.7±7.3	24.6 ±6.4	28 ± 6.4			
Range(yrs)	16-40	16-42	16-42	17-46			
Parity (n, %)						0.006	0.400
0	26(39.4)	18 (27.3)	17(25.8)	5 (7.6)	66 (100)		
1-5	27(22.0)	26 (21.1)	31(25.2)	39(31.7)	123(100)		
>5	0(0.0)	1 (25.0)	2(50)	1 (25.0)	4 (100)		
CD4(x10⁶/l)						0.399*	---
(n,%)	-	42(93)	-	40(89)			
(mean, ±SD)		(436±181)		(432±220)			
BMI (n, %)	35(66)	39(87)	35(70)	36(80)	145(76)		
(mean, SD)	24.3(±13.0)	39.4(±12.4)	29.7(±12.0)	30.1(±15.4)	31(±7.4)	0.391	0.229
MOD (n,%)						<0.001	<0.001
ELCS	9(17)	9(20)	25(53)	27(61)	70(37)		
EMCS	32(60)	23(52)	13(28)	11(25)	79(73)		
NVD	12(23)	12(27)	9(19)	6(14)	39(21)		
TOTAL	53(100)	44(98)	47(94)	44(98)	188(98)		

Abbreviations: GA = gestational age, BMI = Body Mass Index, MOD= mode of delivery, ELCS= elective caesarean section, EMCS= emergency caesarean section, NVD= normal vaginal delivery, SD=standard deviation PE=preeclampsia, NORMO=normotensive, n=total number (l): p = significant at <0.05, * = PEHIV+ vs NORMOHIV+

The genotype and allele frequencies of the ancestral TT, heterozygous TC and homozygous CC variants are shown in Table 4.2. There was no significant difference in the genotype or T/C allele frequencies when compared between all normotensives and all PE women ($p= 0.834$, $p=0.806$). The genotype distribution was compatible with the Hardy–Weinberg equilibrium in the study sample ($p=0.972$; $p=0.882$).

Table 4.2: miR-27a genotype and allele frequency distribution in controls and pre- eclamptic patients

	†Normotensive (<i>n</i> = 80)	‡Preeclamptic (<i>n</i> = 56)	p-value (Odds ratio; 95% CI)
Genotypes <i>n</i> (%)			
TT	18 (23)	13 (23)	0.834
TC	41 (51)	26 (46)	
CC	21 (26)	17 (31)	
Alleles <i>n</i> (%)			0.806 (0.9342; 0.5758 - 1.516)
T	77 (48)	52 (46)	
C	83 (52)	60 (54)	
HWE p-value	0.972	0.882	

Abbreviations: HWE = Hardy Weinburg Equilibrium; CI =confidence interval; †= all cases; ‡=all cases; $p<0.05$ is statistically significant; T=thymine, C= cytosine

Table 4.3 represents a sub-analysis of the genotype and allele frequencies among women stratified according to HIV status (i.e. negative or positive). No significant differences were noted in the genotype or allele frequencies among all groups. No significant differences were noted between groups ($p= 0.834$; 0.806) respectively. Further analysis by group comparison also did not show any significant differences.

Table 4.3: Genotype and allele frequencies between groups

	Norm HIV negative (n = 42)	Norm HIV positive (n = 38)	PE HIV negative (n = 29)	PE HIV positive (n = 27)
Genotypes n (%)				
TT	8 (19)	10 (26)	8 (28)	5 (19)
TC	23 (55)	18 (48)	11 (38)	15 (56)
CC	11 (26)	10 (26)	10 (34)	7 (25)
Alleles n (%)				
T	39 (46)	38 (50)	27 (47)	25 (46)
C	45 (54)	38 (50)	31 (53)	29 (54)
HWE	0.8076	0.9487	0.4405	0.8306
	Genotypes p-values	Alleles p-values (OR; CI)		
All groups	0.8338	0.9636		
PE HIV- vs PE HIV+	0.4131	1.000 (0.9898; 0.4707 - 2.082)		
Norm HIV- vs Norm HIV+	0.7113	0.7515 (1.154; 0.6198 - 2.148)		
Norm HIV- vs PE HIV-	0.3737	1.0000 (1.005; 0.5137 - 1.966)		
Norm HIV + vs PE HIV +	0.7316	0.7238 (0.8621; 0.4285 - 1.734)		

Abbreviations: PE= preeclampsia; norm+ normotensive; HWE= Hardy Weinburg Equilibrium; $p < 0.05$ is statistically significant

Table 4.4 represents the association between the ancestral and variant genotypes with clinical features in all (a) PE women, and (b) according to HIV status. Correlations were made with respect to mean concentrations of cytokines TNF- α , IFN- γ , and IL-2, IL-17a, IL-6, IL-10 and IL-4, in HIV infected and uninfected women with PE relating to the genotypes, however no significant differences were found between the groups. Of note was the significant association of the variant genotype (TC/CC) with body mass index (BMI) in the PE HIV+ group (27.8 ± 0.69 vs 33.47 ± 1.66 ; $p=0.0059$). Moreover, a similar but non-significant trend of higher BMI values were also noted among PE HIV- women (29.5 ± 1.45 vs 31.7 ± 2.11 ; $p=0.388$), and among all PE women overall (29.2 vs 32.6 , $p=0.064$), in association with the TC/CC genotype.

Table 4.4. Association of rs895819T/C genotypes with clinical parameters in preeclampsia and HIV co-infection

(a) PREECLAMPSIA (n=56)				
Variable	TT vs TC/CC			p-value
BMI (kg/m^2)	29.25 ± 1.084 vs 32.57 ± 1.344			0.0643
Age (years)	25.85 ± 2.186 vs 27.28 ± 1.045			0.5621
CD4 ($\times 10^6/l$)	446.0 ± 110.9 vs 471.9 ± 37.00			0.8357
EOPE (%)	31 vs 72			0.5346
Severe PE(5)	38 vs 49			0.5453
No of AHT drugs	1.692 ± 0.2371 vs 1.595 ± 0.1081			0.7141
SYS BP(mmHg)	154.5 ± 3.890 vs 160.8 ± 2.342			0.1810
DIA BP(mmHg)	100.0 ± 2.614 vs 105.0 ± 1.692			0.1268
(b)	PE HIV- (n=29)		PE HIV+ (n=27)	
Variable	TT vs TC/CC	p-value	TT vs TC/CC	p-value
BMI	29.5 ± 1.45 vs 31.7 ± 2.11	0.3882	27.8 ± 0.69 vs 33.47 ± 1.66	0.0059*
Age	24.00 ± 2.18 vs 25.4 ± 1.11	0.5736	28.8 ± 4.51 vs 29.05 ± 1.682	0.9613
CD4	N/A	N/A	446 ± 110.9 vs 453 ± 36.4	0.9551
EOPE (%)	25 vs 29	1.000	40 vs 55	0.6483
Severe PE (%)	38 vs 27	1.000	40 vs 59	0.6280
No of AHT drugs	2.0 ± 0.32 vs 1.40 ± 0.112	0.1212	1.20 ± 0.200 vs 1.77 ± 0.173	0.0533
SYS BP(mmHg)	153 ± 5.91 vs 161.7 ± 5.1	0.2946	156 ± 4.986 vs 163 ± 3.224	0.2764
DIABP (mmHg)	99 ± 3.51 vs 102 ± 2.38	0.5116	102 ± 4.250 vs 108 ± 2.274	0.2507

Abbreviations: BMI =body mass index, EOPE= early onset preeclampsia, PE=preeclampsia; AHT=antihypertensive drugs; SYS/DIA BP= systolic and diastolic blood pressure;

Table 4.5 shows a sub-analysis of the BMI in all the groups. Among normal pregnancies (normotensive), the variant genotype was associated with a significantly lower BMI (29.0 vs 34.3, $p=0.047$), which appears unaffected by HIV infection. In contrast, the variant genotype was associated with a higher BMI among all women with PE ($p=0.064$). This however, was significantly higher in PE women with co-morbid HIV infection on HAART ($p=0.005$). Due to policy guidelines, a cohort of HAART naive women was not available to differentiate the impact of HAART.

Table 4.5: Sub-analysis of Body Mass Index and rs895819 genotypes in all groups

Groups	TT vs TC/CC	BMI Trend	P value
All PE	29.25 ± 1.084 vs 32.57 ± 1.344	↑	0.0643
PE HIV -	29.50 ± 1.458 vs 31.77 ± 2.111	↑	0.3882
PE HIV+	27.87 ± 0.6960 vs 33.47 ± 1.666	↑↑	0.0059*
All NORMO	34.31 ± 2.258 vs 29.03 ± 1.019	↓↓	0.0473*
NORMO HIV-	32.14 ± 2.512 vs 29.48 ± 1.535	↓	0.3971
NORMO HIV+	35.51 ± 3.260 vs 28.56 ± 1.359	↓	0.0774

Abbreviations: ↓ : decreasing trend; ↑ : increasing trend; ↑↑ : significant increase; $p < 0.05$ = significant

Figures 4.1 and 4.2 show a graphic analysis of BMI grouped per genotype for all PE patients. The mean BMI in the ancestral group is 29.25 ± 1.084 compared with 32.57 ± 1.344 in the variant types, demonstrating the elevated BMI associated with the variant genotype. This observation is in contrast to that observed in the normotensive women that showed a lower BMI in relation to the variant genotype (TT: 34.31 ± 2.258 ; TC/CC: 29.03 ± 1.019).

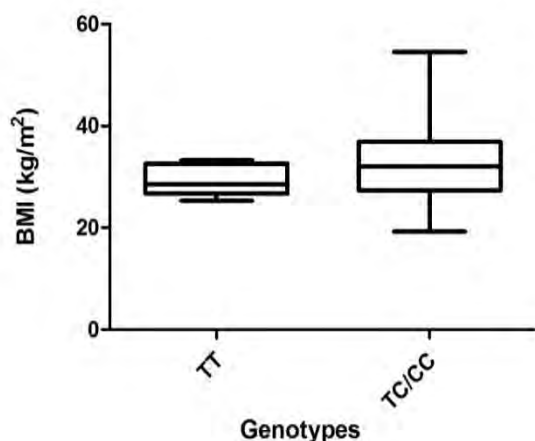


Figure 4.1. BMI per genotype for all PE women

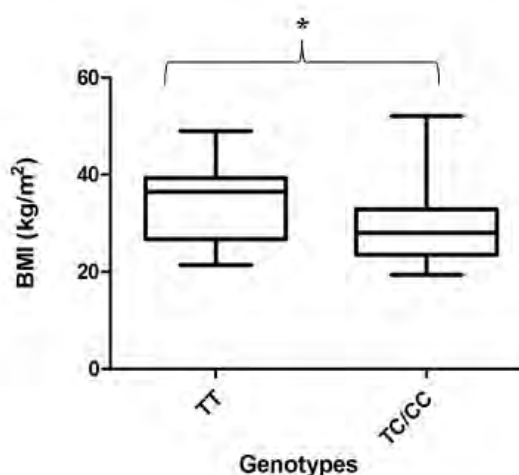


Figure 4.2: BMI per genotype for all normotensive women

Figures 4.3 and 4.4 demonstrate the BMI and genotype relationship in PE women with and without co-morbid HIV infection. There is a significant difference in the HIV infected PE group compared with the HIV uninfected group which shows a non-significant increase (27.87 ± 0.6960 vs 33.47 ± 1.666 ; $p=0.0059$ vs. 29.50 ± 1.458 vs 31.77 ± 2.111 ; $p=0.3882$). Figure 4.5 represents the overall BMI grouped per genotype for all subjects in the cohort.

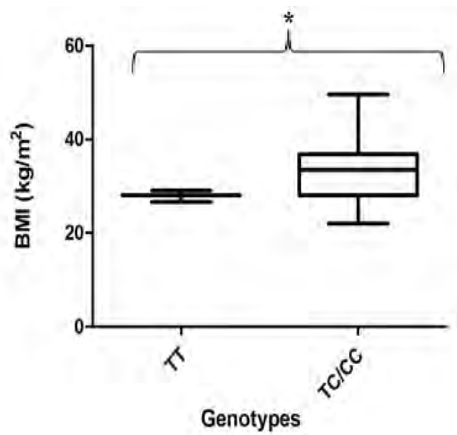


Figure 4.3. BMI per genotype in PE HIV+ women

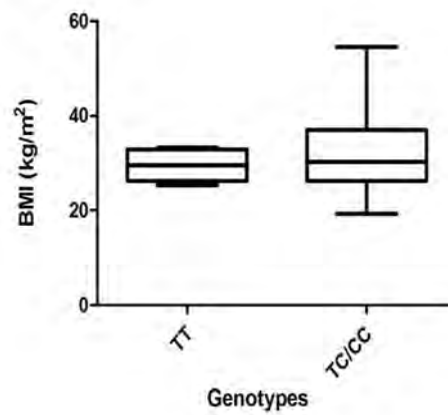


Figure 4.4. BMI per genotype in PE HIV- women

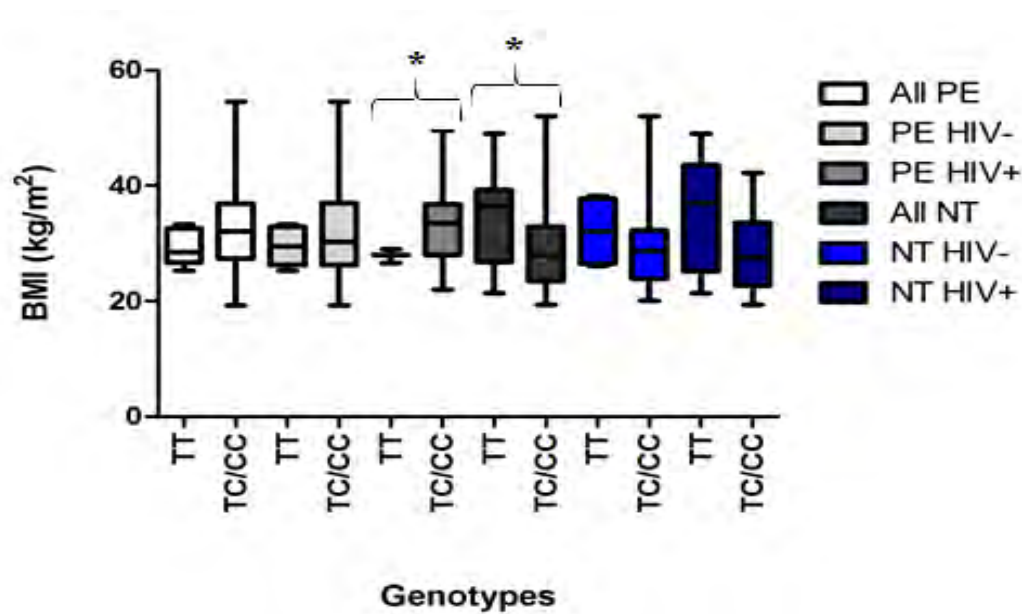


Figure 4.5 BMI's grouped per genotype for all subjects

Figure 4.6 shows the divergent association of the variant genotype with normotensive, PE and HIV infected PE women. In pregnant women with the variant genotype, the BMI is lower in the normotensive groups, but rises in the PE group. The BMI in this group specifically, is significantly higher in the presence of HIV/HAART (27.87 ± 0.69 vs 33.47 ± 1.66 ; $p= 0.0059$). This trend is diametrically opposed to the group of women with the ancestral genotype, who had a higher BMI in normotensive groups, but lower BMI with PE, lowered further by the presence of co-morbid HIV infection.

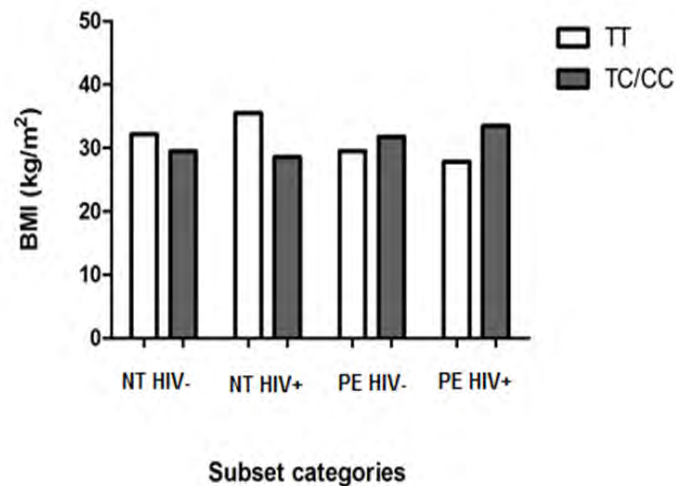


Figure 4.6: BMI's in different genotypes in all groups

4. Discussion

The morbidity and mortality associated with PE and HIV/AIDS remains a global health concern. In developing countries, and South Africa in particular, hypertensive disorders of pregnancy and HIV/AIDS have remained leading causes of mortality despite sustained interventions (NCCEMD, 2011-2013). Existing research aimed at better understanding the pathophysiology of PE and the associated complications still remains inconclusive. Contemporary and new studies now extend to include both genetic and epigenetic aspects of PE.

In this study, the findings do not show significant differences in the ancestral and variant genotype and allele frequencies among the groups and in relation to the parameters described. However, of relevance is the observation that the variant TC/CC genotype is associated with higher BMI in the PE women studied (TT: 29.25 ± 1.084 vs TC/CC: 32.57 ± 1.344 , $p= 0.06$), in contrast to lower BMI values in normotensive pregnancies (TT: 34.31 ± 2.258 vs TC/CC: 29.03 ± 1.019 $p=0.04$). In the normotensive women, the presence of HIV infection is associated with a decrease in BMI in the variant group,

however in PE, it is associated with a significant increase among carriers of the variant genotypes (TT: 27.87 ± 0.6960 vs TC/CC: 33.47 ± 1.666 , $p=0.005$). A relationship of susceptibility to increased BMI may therefore exist in women with PE who carry the variant genotypes. This relationship may be further potentiated by the presence of co-morbid HIV infection (on HAART), through complex differential regulation of miR-27a.

The regulatory activity of miR-27a has also been associated with viral infections. Buck et al. (2010) observed that both host and virus contain mechanisms to regulate miRNA expression and/or activity (Buck et al., 2010). As the expression of miR-27a is associated with reduced adipogenesis, the variant genotype may be associated with dysregulation in PE, which is possibly further potentiated by co-existing HIV disease through complex mechanisms.

The regulatory activity of miR-27a in adipogenesis has been demonstrated previously (Lin et al., 2009) - the overexpression of miR-27a specifically inhibited adipocyte formation, and expression of miR-27a results in blockade of expression of peroxisome proliferator-activated receptor gamma (PPAR γ) and CCAAT/enhancer-binding protein (CEBP) α , the two master regulators of adipogenesis. MiR-27a, has also been shown to inhibit adipogenic differentiation of 3T3-L1 preadipocytes (Lin et al., 2009). In animal models, mature adipocytes from obese mice had lower miR-27a expression as compared to lean mice, indicating miR-27a downregulation may be necessary for adipocyte hypertrophy (Kim et al., 2010). The regulatory activity of miR-27a in relation to adipogenesis and obesity in PE and HIV infection, is however complex and warrants expansive investigation.

Interestingly, adipose tissue is considered to be hormonally active, producing cytokines (Briana and Malamitsi-Puchner, 2009) that demonstrate the association of obesity with increased inflammation, insulin resistance and oxidative stress (Berg and Scherer, 2005, Greenberg and Obin, 2006). Inflammatory cytokine release from adipose tissue and elevated inflammatory cytokine levels including TNF- α and IL-6 have been associated with obesity (Cummings and Schwartz, 2003). The investigation of cytokines in relation to obesity was not the focus of the study, however, the study did not find significant differences with IL-2, IL-4, IL6, IL-10, IL17a, TNF- α , and IFN- γ concentrations in relation to rs895819 T>C polymorphism between the groups.

Obesity is a major epidemic in developed countries, and the trend is now extending to developing countries (Misra and Khurana, 2008). The prevalence of obese and overweight women (BMI ≥ 25 kg/m 2) in South Africa is estimated to be 69% by the (WHO, 2010). Although the relationship of obesity to increase Type 2 diabetes and cardiovascular disease is well recognized, evidence suggests a three-fold increase in the risk of PE associated with obesity (Bodnar et al., 2005). In the United States, it appears that obesity is the leading attributable risk for PE (Bodnar et al., 2005), and in several other populations

around the world the relationship of obesity and increased risk of PE has also been reported (Mahomed et al., 1998, Hossain et al., 2007, Hauger et al., 2008). Interestingly, increases in BMI in the normal range has also been shown to be associated with an increased risk of PE (Bodnar et al., 2005).

The clinical relevance of this study relates to the prevalence of obesity in South Africa, particularly among women and young girls (Hauger et al., 2008, Labadarios et al., 2005) which is progressively increasing (Labadarios et al., 2005, Senekal et al., 2003), however the study is limited by a small sample size, and racial and gender bias which require future large scale population based studies. The exclusion of a HAART naïve group limits comparisons on its effects, however the inclusion of such a group is unethical in current practice. The promotion of weight loss activities in pregnancy is not feasible, therefore effective obesity prevention strategies are needed which incorporate healthy diet and life style messages to those who are at risk for HIV infection and other non-communicable diseases (Wand and Ramjee, 2013) to reduce morbidity and mortality.

Taken together, the study provides an insight into the association of miR-27a and obesity and the association of miR-27a rs895819 polymorphism in relation to BMI, PE and the influence of HIV infection and HAART in pregnant women. Further scientific investigation is required in the long term to unravel the cross regulatory mechanisms that may be involved.

5. Conclusion:

MiR-27a has an important regulatory function in the development of obesity. The functional rs895819 SNP may negatively regulate the adipogenic activity of miR-27a, and possibly increase the susceptibility to obesity in preeclamptic Black South African women on HAART. The data provides new insight into the role of miR-27a polymorphism in the triad of PE, HIV/HAART and obesity, and has potentially important future therapeutic implications.

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CHAPTER 5

MiRNA 146a rs2910164 G>C Polymorphism is associated with severe preeclampsia in Black South African women on HAART

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Synopsis: MiR-146a GC/CC genotype may reduce susceptibility to severe preeclampsia, which may be further influenced by the presence of co-morbid Human Immunodeficiency virus (HIV) infection among pregnant women on highly active antiretroviral therapy (HAART).

Abstract

Objectives:

We investigated the association of the reference and variant genotypes of miR-146a rs2910164 G>C polymorphism in association with preeclampsia (PE), related features and cytokines and include a further analysis among preeclamptic women with HIV infection on HAART.

Study Design:

This hospital-based, case-control study was conducted by genotyping Black South African women, where the risk of both PE and HIV is high. Sixty women with PE and eighty normotensive women were genotyped by PCR-RFLP. Serum cytokine levels (Th1 related cytokines) and (Th2 related cytokines) were also determined in 4 groups of pregnant women, viz : normotensive, HIV infected, preeclamptic and HIV infected preeclamptic women, using a Cytometric Bead Array assay.

Results:

There was no significant association of miR-146a polymorphism with PE susceptibility in our data. However, in the subgroup analyses, the variant genotypes (GC/CC) were significantly associated with lower severe PE risk (OR: 0.34, 0.12-0.99; p=0.048), more especially in the presence of HIV and HAART (OR: 0.11; 0.02-0.68, p= 0.018).

Conclusions:

Our study suggests that miR-146a rs2910164 polymorphism might not be associated with PE susceptibility, cytokines or related features. However, the miR-146a GC/CC genotype might reduce susceptibility to severe PE, which might be further influenced by the presence of co-morbid HIV infection among pregnant women on HAART.

Keywords: MiR-146a, preeclampsia, cytokines, HIV, HAART

Introduction:

Preeclampsia and HIV infection contribute significantly to adverse maternal and perinatal outcomes globally (Backes, Markham et al. 2011),(Calvert and Ronsmans 2013) . In South Africa, and other developing countries, the prevalence of both conditions remains high and these co-morbidities are important causes of mortality (Moodley and Kalane 2006),(Nel, Mabude et al. 2012), (Gebhardt, Fawcus et al. 2015). Despite extensive research, the pathophysiology of HIV infection and preeclampsia is not completely understood.

Preeclampsia is heterogeneous in nature, and is associated with differences in the timing of disease, clinical manifestations, severity of organ damage, maternal and fetal outcomes and complications. The diverse nature of preeclampsia is further evident in the severity in which it can manifest i.e. mild and severe disease (Sibai, Dekker et al. 2005). Although the definition of severe preeclampsia varies, it is generally associated *inter alia* with markedly elevated blood pressure, maternal neurological complications, seizures, signs of hepatic and renal dysfunction, and fetal affectation (Obstetricians and Gynecologists 2013). In the developing countries, severe forms of preeclampsia and eclampsia are more common, and range from a low of 4% of all deliveries to 18% in parts of Africa (Villar, Say et al. 2003).

Preeclampsia is associated with a pro-inflammatory milieu, in which cytokines play a significant role as mediators (Raghupathy 2013). Other pathogenic mechanisms such as immune maladaptation, inadequate placental development and trophoblast invasion, placental ischaemia, oxidative stress and thrombosis are all thought to represent key factors in the development of preeclampsia (Williams and Pipkin 2011). All of these components have genetic factors that may be involved in the pathogenesis (Williams and Pipkin 2011), which may alter susceptibility to preeclampsia or its complications. The genetic basis of preeclampsia is further supported by epidemiological findings that show a 2- 5 fold increased risk of pre-eclampsia in women with a maternal history of this disease. (Uzan, Carbonnel et al. 2011).

MicroRNAs (miRNAs) are small (+- 22 nucleotides), endogenous, non-coding RNA's that regulate gene expression by modulating the expression of multiple target mRNA's, inducing either translational inhibition or mRNA degradation (Li, Du et al. 2015). They are involved in various pathological (Li, Du et al. 2015) processes, including the regulation of innate and adaptive immune responses (Saba, Sorensen et al. 2014). MiRNA-146a, is involved in modulating the negative regulation of Toll like receptor (TLR) signalling, and inflammatory cytokines. (Hill, Clement et al. 2015). MiRNA146a lies at the crossroads of biological processes that involve innate-immune responses, viral-infection and inflammatory disease (Hill, Clement et al. 2015). It prevents an overstimulation of the inflammatory response through its recognised target genes, IRAK1 and TRAF6 (Hébert and De Strooper 2007), (Saba,

Sorensen et al. 2014), and its dysregulation is associated with many inflammatory diseases including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (Li, Du et al. 2015) . Its role in preeclampsia has not been established.

Polymorphisms may affect miRNA expression, maturation or mRNA recognition, and alter disease susceptibility (Li, Du et al. 2015). MiRNA 146a has been associated with the single nucleotide polymorphism (SNP) rs 2910164 G>C , which is located within the seed sequence of pre-miRNA 146a , which is the miRNA 146a precursor (Mehanna, Ghattas et al. 2015) ,(Kogo, Mimori et al. 2011). This functional polymorphism has been associated with various inflammatory diseases including rheumatoid arthritis and SLE. (Yang, Schlehe et al. 2010), (Löfgren, Frostegård et al. 2012)

Based on (1) the inflammatory milieu in preeclampsia and HIV, (2) the regulatory role of miR-146a in inflammatory responses, and (3) the association of miRNA 146a SNP rs 2910164 G>C polymorphism with inflammatory diseases, this study investigated the role of rs2910164 with preeclampsia risk , related features and associated cytokines . Due to the high prevalence of HIV (Nel, Mabude et al. 2012), we included women with HIV infection on HAART in order to identify a possible differential influence. To the best of our knowledge, this is the first study to investigate this association in high risk Black South African women during pregnancy.

2. Materials and Methods:

2.1. Study population and sample collection

Institutional ethical and hospital regulatory permission was obtained for the study (Biomedical Research Ethics Committee, University of KwaZulu-Natal, South Africa; reference number BE 119/11). After informed consent was obtained, participants were recruited over a 14 month period from July 2013 to September 2014 at the maternity unit at Prince Mshiyeni Memorial Hospital in Durban, South Africa. This hospital is a regional level facility and serves a predominantly semi –urban African population. Normotensive [n=95, (80 genotyped) age range: 16-46 years] and preeclamptic patients [n= 98, (60 genotyped) age range: 16-42 years] were enrolled overall into the study. To maintain ethnographic and anthropometric consistency, all patients recruited were of African descent, resident in the same geographical location and of Zulu ethnicity. All patients were non-smokers, non-consumers of alcohol or recreational drugs, and all HIV infected patients were on HAART (tenofovir, emtricitabine, efavirenz) as per the National guidelines (Southern African 2013). Calcium supplementation was administered routinely to all patients attending the clinic. Women with gestational hypertension, renal disease, diabetes mellitus, chronic hypertension and collagen vascular disease were excluded. Preeclampsia was defined as a blood pressure \geq 140mmHg systolic or \geq to 90 mmHg diastolic on 2

occasions at least 4 hours apart after 20 weeks of gestation in a woman with previously normal blood pressure, consistent with guidelines (Obstetricians and Gynecologists 2013). All patients had proteinuria $\geq +1$ on urine dipstick testing. Data on all patients was obtained from the institution's maternity case records and laboratory data from the National Health Laboratory Services® computerised database at the institution. HIV was diagnosed on a rapid test kit and weight was categorised as: normal weight (BMI: 18-<25), overweight (BMI: 25-<30), obese (BMI: 30+). Early onset preeclampsia was considered as ≤ 34 weeks of gestation (Tranquilli, 2014). Severe preeclampsia was diagnosed when features included any of the following: systolic blood pressure ≥ 160 mmHg or diastolic blood pressure ≥ 110 mmHg; maternal neurological disorders such as persistent headaches and brisk reflexes, eclampsia, acute pulmonary edema, proteinuria ≥ 5 g/day, oliguria < 500 cc/day, creatinine > 120 $\mu\text{mol/L}$, features HELLP syndrome and thrombocytopenia $< 100,000/\text{mm}^3$, fetal criteria including intrauterine growth retardation, oligohydramnios, or fetal death in utero. (Obstetricians and Gynecologists 2013), (Sibai, Dekker et al. 2005).

2.2. Cytokine quantification:

The BD Cytometric Bead Array Human Th1/Th2/Th17 Cytokine kit was used to measure Interleukin (IL) -2, IL-4, IL-6, IL-10, IL17a and Tumor Necrosis Factor (TNF- α) protein levels in a serum samples. Briefly, lyophilized standards were prepared by reconstitution and serial dilution (1:2 – 1:256) in assay diluent immediately before staining with Capture Beads and PE Detection Reagent. All serum samples were also diluted in assay diluent (1:4) before staining with Capture Beads and PE Detection Reagent. For the staining procedure, 50 μL of each standard and unknown sample was added to appropriately labelled sample tubes followed by 50 μL of the Human Th1/Th2/Th17 PE Detection Reagent and incubated (3 h, RT, protected from light). Following incubation 1 mL of Wash Buffer was added to each assay tube and centrifuged at 200g for 5 minutes. The supernatant from each assay tube was then carefully aspirated and 300 μL of Wash Buffer was added to each assay tube to resuspend the bead pellet. Flow cytometric data was acquired using the BD AccuriC6 Sampler counting 2100 gated events. This ensures that the sample file contains approximately 300 events per Capture Bead. Data analysis was performed using the FCAP Array analysis software.

2.3. Genomic DNA extraction

Genomic DNA was extracted from whole blood samples of the study subjects (80 normotensive and 60 PE). Cells were transferred to 600 μL lysis buffer (0.5% SDS, 150mM NaCl, 10mM EDTA, 10mM Tris-HCl (pH 8.0)). To this, RNase A (100 $\mu\text{g}/\text{mL}$; DNase free) was added to the solution and incubated

(37°C, 1h). Proteinase K (200µg/mL) was then added and incubated (50°C, 3h). Protein contaminants were then precipitated by adding 5mM 0.1% potassium acetate before centrifugation (5,000xg; 15min). Supernatants containing genomic DNA were transferred to fresh tubes and extracted with 100% isopropanol on ice, and thereafter washed with 70% ethanol. DNA samples were dissolved in 10mM Tris and 0.1mM EDTA (pH 7.4, 4°C). DNA concentration was determined using the Nanodrop2000 spectrophotometer, and all samples were standardised to a concentration of 10ng/µL.

2.4. Genotyping

An optimised PCR was used to obtain the highest specificity and yield of the 147bp PCR product. This was achieved by amplification of the genomic DNA using 40pmol of each primer (Forward Primer: 50 – CATGGGTTGTGTCAGTGTTCAGAGCT – 30; Reverse Primer: 50 - TGCCTTCTGTCTCCAGTCTTCCAA-30). A no-template sample was run with the positive samples as a quality control measure against PCR contamination. The 30µL reaction consisted of 200mM of each dNTP, 2.5mM MgCl₂, 19 Green GoTaq Flexi buffer, 0.2U Go-Taq DNA polymerase (Promega) and 30ng genomic DNA template. PCR was performed under the following cycling conditions: 94°C for 10min (initial denaturation), followed by 30 cycles of 94°C for 30s, 65°C for 30s (annealing) and 72°C for 7min (final extension). PCR products were electrophoresed on agarose gel (1.8 %) and visualised using the Uvitech image documentation system (Uvitech Alliance 2.7).

PCR–RFLP was used to determine the miR-146a rs2910164 genotypes. 15µL of each PCR product was subjected to restriction by 1.5µL (10u/µL) *Sac* I and 2µL 10x Buffer-*Sac* I (Fermentas). Overnight restriction occurred at 37°C, and thereafter restriction products were electrophoresed on agarose gel (3%) and visualised as was the PCR product. Presence of the wild-type G-allele resulted in no cleavage of the PCR product. The variant C-allele yielded two fragments of 122 and 25bp. The homozygous genotype yielded three bands of 147, 122 and 25 bp. Restriction products were run alongside a DNA ladder for accurate reading of fragment sizes, thus enabling correct analysis of genotypes.

2.3. Statistical analysis

Statistical analysis was done using SPSS® version 22. Correlation between continuous variables was assessed using the Spearman rank correlation coefficient. Comparisons of mean across 3 or more groups were done using the Kruskal– Wallis test. The Pearson Chi-square (χ^2) test was used to test association between group(s) and categorical explanatory variables. The Hardy–Weinberg equilibrium was used to test for deviation of allele/genotype frequency. Allele and genotype frequencies were calculated using the Fisher's exact and Chi square tests, respectively. Other calculations were done using a one-way ANOVA. In the determination of significance, a p-value < 0.05 was deemed statistically significant.

3. Results:

The clinical characteristics of participants are shown in Table 5.1. The study cohort was divided into 4 groups: (1) uninfected preeclamptic women (PEHIV-), (2) infected preeclamptic women (PEHIV+), (3) uninfected normotensive women (Normo -) and (4) infected normotensive women (Normo +). All women were in the third trimester of pregnancy and the mean gestational age was 36.5 weeks of pregnancy. There was a significant difference in the parity across all groups ($p=0.006$) but not between the preeclamptic women ($p=0.400$). There was also a significant difference in age across the groups, however there was little or no correlation between cytokine levels. The average duration of HAART was 16.6 and 14.5 weeks in the normotensive and preeclamptic group respectively, however this is not a precise duration of exposure. There were differences in the mode of delivery, however these were based on obstetric related indications. CD4 counts were not routinely performed on uninfected women.

Table 5.1: Clinical characteristics of participants

Variable	PE HIV- (Grp 1) (n= 53)	PE HIV+ (Grp 2) (n=45)	Normo – (Grp 3) (n=50)	Normo + (Grp 4) (n=45)	Total (n=193)	<i>p</i> <i>value.</i> (all grps)	<i>p value</i> (Grp 1 vs 2)
Age (n, %) (mean, ± SD) Range(yrs)	53(100) 24.8±5.3 16-40	44(98) 28.7±7.3 16-42	50(100) 24.6 ±6.4 16-42	45(100) 28 ± 6.4 17-46	192(99)	0.002	0.003
Parity (n, %)						0.006	0.400
0	26(39.4)	18 (27.3)	17(25.8)	5 (7.6)	66 (100)		
1-5	27(22.0)	26 (21.1)	31(25.2)	39(31.7)	123(100)		
>5	0(0.0)	1 (25.0)	2(50)	1 (25.0)	4 (100)		
CD4 ($\times 10^6/l$) (n,%) (mean, ±SD)	-	42(93) (436±181)	-	40(89) (432±220)		0.399*	---
BMI (n, %) (mean, SD)	35(66) 24.3(±13.0)	39(87) 39.4(±12.4)	35(70) 29.7(±12.0)	36(80) 30.1(±15.4)	145(76) 31(±7.4)	0.391	0.229
MOD (n,%)						<0.001	<0.001
ELCS	9(17)	9(20)	25(53)	27(61)	70(37)		
EMCS	32(60)	23(52)	13(28)	11(25)	79(73)		
NVD	12(23)	12(27)	9(19)	6(14)	39(21)		
TOTAL	53(100)	44(98)	47(94)	44(98)	188(98)		

Abbreviations: GA = gestational age, BMI = Body Mass Index, MOD= mode of delivery, ELCS= elective caesarean section, EMCS= emergency caesarean section, NVD= normal vaginal delivery, SD=standard deviation PE=preeclampsia, NORMO=normotensive, n=total number (1): p = significant at <0.05, * = PEHIV+ vs NORMOHIV+

The genotype and allele frequencies of the ancestral / reference GG, heterozygous GC and homozygous CC variants are shown in Table 5.2 .There were no significant differences in the GC genotype ($p=0.589$), CC genotype ($p=0.491$) or G /C allele frequencies ($p=0.671$) when compared between all preeclamptic and all normotensive women. The genotype distribution was compatible with the Hardy –Weinberg equilibrium in the study sample ($p=0.905$; $p=0.197$).

Table 5.2: miR-146a genotype and allele frequency distribution in controls and pre- eclamptic patients

	† Controls: (n = 80)	‡ Pre-eclamptic (n =60)	p-value (Odds ratio; 95% CI)
Genotypes n (%)			
GG	31 (38.8)	24 (40.0)	1 (ref)
GC	38 (47.5)	24 (40.0)	0.589 (0.82; 0.34- 1.71)
CC	11 (13.8)	12 (20.0)	0.491 (1.41; 0.53- 3.74)
Alleles n (%)			
G	100 (62.5)	72 (60.0)	0.671 (1.11; 0.66- 1.86)
C	60 (37.5)	48 (40.0)	
HWE p-value	0.905	0.197	0.439

Abbreviations: HWE = Hardy Weinburg Equilibrium; CI =confidence interval; †= all normotensives; ‡=all preeclamptics; $p<0.05$ is statistically significant; n= cases genotypes G=guanine, C=cytosine,

Table 5.3 represents a sub-analysis of the genotype and allele frequencies among women stratified according to HIV status (i.e. negative or positive). No significant differences were noted in the genotype or allele frequencies by group comparison.

Table 5.3: Genotype and allele frequencies between groups

	NORMO HIV- (n =42)	NORMO HIV+ (n =38)	PE HIV- (n =31)	PE HIV+ (n =29)
Genotypes n (%)				
GG	17 (40.5)	14 (36.8)	13 (41.9)	11 (37.9)
GC	20 (47.6)	18 (47.4)	12 (38.7)	12 (41.4)
CC	5 (11.9)	6 (15.8)	6 (19.4)	6 (20.7)
Alleles n (%)				
G	54 (64.3)	46 (60.5)	38 (61.3)	34 (58.6)
C	30 (35.7)	30 (39.5)	24 (38.7)	24 (41.4)
HWE	0.810	0.957	0.305	0.428
Group comparison:				
	Genotypes <i>p</i> -values (OR; CI)		Alleles (G/C) <i>p</i> -values (OR; CI)	
Norm - vs Norm +				
GG	1 (<i>ref</i>)		1 (<i>ref</i>)	
GC	0.855 (1.09; 0.42- 2.83)		0.624 (1.17; 0.59- 2.34)	
CC	0.593 (1.46; 0.37- 5.80)			
Norm - vs PE HIV-				
GG	1 (<i>ref</i>)		1 (<i>ref</i>)	
GC	0.640 (0.78; 0.28-2.17)		0.711 (1.14; 0.54- 2.36)	
CC	0.525 (1.57; 0.39- 6.30)			
Norm + vs PE HIV+				
GG	1 (<i>ref</i>)		1 (<i>ref</i>)	
GC	0.765 (0.85; 0.29- 2.49)		0.824 (1.08; 0.51- 2.30)	
CC	0.732 (1.27; 0.32-5.06)			

Abbreviations: PE= preeclampsia; norm+ normotensive; HWE= Hardy Weinburg Equilibrium; $p < 0.05$ is statistically significant; n= genotyped cases GG = reference genotype G= reference allele

Table 5.4 represents the odds ratios (OR) and p values for the cytokines and selected features associated with preeclampsia. There were no significance differences noted with any of the mean cytokine levels or the other variables assessed in the groups, except for severe preeclampsia, where a significant difference was noted across the genotype groups. ($p = 0.048$). In a further sub-analysis according to HIV infection, the odds ratio for severe preeclampsia was significantly lower for the genotypes in the HIV + group ($p = 0.018$). Taken together, the OR for severe preeclampsia was lower among the GC/CC genotypes compared with the ancestral genotype GG in all preeclamptic women (OR: 0.34 (0.12 -0.99) ($p = 0.048$) and even lower in HIV infected preeclamptic women with the GC/CC genotypes (OR: 0.11; 0.02-0.68 $p = 0.018$).

Table 5.4: Odds ratios for variables in all preeclamptic women and according to HIV status.

Variable	N 60	GC/CC vs GG (ref) (n=36) (n=24)		p-value
		OR (95%, CI)		
BMI	46	0.98 (0.9-1.06)		0.612
Age	60	0.98 (0.91-1.06)		0.569
CD4	30	1 (0.99-1.0)		0.135
EOPE	60	0.79 (0.27-2.28)		0.665
Severe Preeclampsia	60	0.34 (0.12-0.99)		0.048*
No of AHT drugs	60	1.1 (0.69 -1.7)		0.676
SYS BP	59	1.01(0.99-1.03)		0.263
DIA BP	59	1.01 (0.98-1.04)		0.421
IL2	17	0.96 (0.9-1.02)		0.199
TNF- α	18	1 (0.98-1.02)		0.907
IL-17 α	17	1 (1.0-1.0)		0.642
IL-6	18	1 (0.99-1.01)		0.976
IL-10	17	1 (1-1.01)		0.658
IL-4	18	1.01 (0.98-1.04)		0.582
	GC/CC vs GG PE HIV⁻(n=31) OR(95% CI)	<i>p value</i>	GC/CC vs GG PE HIV⁺(n= 29) OR(95% CI)	<i>p value</i>
Severe PE	0.74 (0.18-3.15)	0.686	0.11 (0.02-0.68)	0.018*
Cytokines				
IL2	0.96 (0.88-1.04)	0.271	0.95 (0.89-1.02)	0.165
TNF α	0.89 (0.72-1.09)	0.250	0.99 (0.89-1.1)	0.873
IL17a	0.99 (0.96-1.02)	0.444	1 (1.0-1.0)	0.88
IL6	1.01 (0.99-1.02)	0.392	1 (1.0-1.0)	0.327
IL10	1.01 (0.99-1.02)	0.464	0.94 (0.83-1.06)	0.286
IL4	1.0 (1-1.01)	0.351	0.94 (0.86-1.03)	0.202

Abbreviations: OR= odds ratio, BMI =body mass index (kg/m²), EOPE= early onset preeclampsia, AHT = antihypertensive drugs, SYS= systolic, DIA= diastolic, BP= blood pressure (mmHg), IL+ interleukin, TNF α =tumour necrosis factor alpha, *p<0.05

Discussion:

The dysregulation of miRNA146a has been associated with inflammatory diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (Yang, Chen et al. 2012) ,(Löfgren, Frostegård et al. 2012). A downregulation of miRNA 146a has been found in placentas of preeclamptic women compared with normal women (Chen and Wang 2013), therefore suggesting a potential role for miRNA146a in the pathogenesis of preeclampsia. Based on the presence of an increased inflammatory environment in preeclampsia (Murphy, Tayade et al. 2015), (Garovic and August 2013), (Ramma and

Ahmed 2011), polymorphisms in the miRNA146a gene may therefore affect susceptibility to preeclampsia. The rs 2910164 G>C polymorphism of miRNA146a has recently been studied in inflammatory conditions (Yang, Chen et al. 2012), however its role in preeclampsia and co-morbid HIV infection has not been evaluated.

Inflammation is mediated by a variety of soluble factors, including cytokines (Shaikh, Sharma et al. 2011), which were evaluated in this study. Preeclampsia is characterised by significantly higher levels of pro-inflammatory cytokines such as IL-6 and TNF- α , when compared with normal pregnant women (Catarino, Santos-Silva et al. 2012). In HIV infection, the move away from a pro-inflammatory cytokine milieu that occurs as disease progresses, is counteracted with the usage of HAART (Fiore, Newell et al. 2006).

Our data did not show any significant association in the frequency of the variant genotypes (GC/CC) in women with preeclampsia and there was no significant association in the groups stratified according to HIV. Furthermore, we did not find a significant relationship of the variant genotypes with cytokines or the features of preeclampsia that were assessed when stratified according to HIV status. However the odds ratio (OR) for the presence of severe disease was significant (GC/CC) (0.34: 0.12-0.99; $p=0.048$), suggesting a reduced susceptibility for the development of severe disease with the variant genotype. The OR for severe preeclampsia was also significantly lower among HIV infected preeclamptic women with the GC/CC genotypes. (0.11; 0.02-0.68, $p= 0.018$), suggesting a differential dysregulation in the presence of HIV infection.

Severe PE is associated with adverse neonatal outcomes, is more likely to recur (Rey and Couturier 1994), and is also associated with increased maternal morbidity. (Sibai, Mercer et al. 1991). Moreover, women with severe PE are at high risk for cardiovascular disease later in life. (Smith, Pell et al. 2001). Eclamptic seizures, intracerebral haemorrhage, pulmonary oedema or heart failure, acute renal failure, liver dysfunction, and coagulation abnormalities are all associated with severe pre-eclampsia. (Rudra, Basak et al. 2011). Fetal complications include intrauterine growth restriction, premature delivery, and intrauterine fetal death (Rudra, Basak et al. 2011). Eclampsia, a severe form of preeclampsia characterised by seizures, is associated with a (0%-1.8%) mortality rate in developed countries rising to a high rate of 15% in developing countries (Ghulmiyyah and Sibai 2012). Furthermore, obesity is a known risk factor for preeclampsia (Stone, Lockwood et al. 1994), a finding relevant to our study (mean BMI among the HIV infected preeclamptic women was 39.4 kg/m²).

The role of miRNA 146a in HIV infection has emerged more recently. Spinello et al. (2011) reported a relationship between miR146a and CXCR4 co-receptor in HIV (Spinello, Quaranta et al. 2011). Resting CD4+T cells have high expression of miR-146a, which inhibits the expression of the co-receptor

CXCR4, and prevents the HIV entry in CD4+T cells (Spinello, Quaranta et al. 2011), (Quaranta, Olivetta et al. 2015). Furthermore, Duskova et al. (2013) demonstrated that miR-146a was significantly increased in infected patients as compared to healthy controls. (Duskova, Nagilla et al. 2013).

Our study demonstrates the relationship of miR-146a rs2910164 G>C polymorphism with preeclampsia, some of the related features, cytokines and HIV/HAART. Of note is the increased susceptibility to severe disease in local women with the ancestral genotype. However, our findings are preliminary and limited by small sample sizes. Further, the influence of other functional polymorphisms on miRNA 146a expression in co-morbidities, needs further investigation.

Conclusion:

MiR-146a rs2910164 G>C polymorphism might not be associated with PE susceptibility, cytokines or related features. However, the miR-146a GC/CC variant genotypes might reduce susceptibility to severe PE, which might be further influenced by the presence of co-morbid HIV infection among pregnant women on HAART.

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CHAPTER 6

6.1. Synthesis:

Preeclampsia (PE) and HIV/AIDS contribute significantly to morbidity and mortality worldwide, more especially in poorly resourced countries (Backes, Markham et al. 2011), (Calvert and Ronsmans 2013),(Duley 2009). Whilst the treatment of PE remains empiric, HAART is used in the management of HIV infection both for maternal health and for the prevention of vertical transmission during pregnancy. In South Africa, an upper middle income developing country, PE and HIV/AIDS remain among the leading causes of maternal mortality, and have become a National health priority. (Moodley, Pattinson et al. 2014), (Gebhardt, Fawcus et al. 2015). Moreover ,South Africa has the added challenge of a high prevalence of both these conditions (Moodley and Kalane 2006),(Nel, Mabude et al. 2012).

The exact relationship between PE and HIV remains controversial and consensus is lacking on the clinical and scientific impact of HIV/HAAART on PE (Kalumba, Moodley et al. 2013), (Frank, Buchmann et al. 2004), (Mattar, Amed et al. 2004) ,(Cassidy-Matthews 2015). Furthermore, the pathophysiology HIV/AIDS, and PE is not completely understood, despite extensive research. Contemporary studies acknowledge the role of immunological and inflammatory pathways, involving mediators such as cytokines (Shaikh, Sharma et al. 2011), as well as genetic pathways (Williams and Pipkin 2011), and epigenetic regulation.

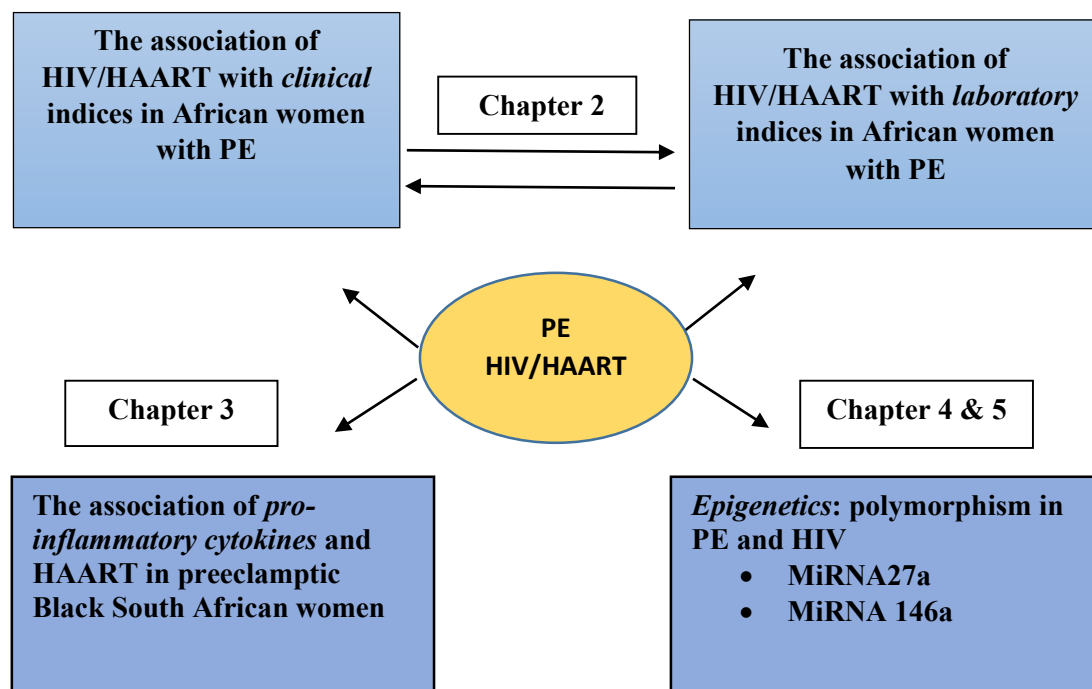


Figure 6.1. Outline of the 4 areas of investigation in this study

In line with the United Nations Millennium Development Goals (MDG's) (Bhutta, Chopra et al. 2010) to formulate effective care and improve outcomes, further clinical and scientific research of these conditions is necessary to improve our understanding and guide effective care.

In this study, we investigated four areas relating to the pathophysiology of PE and HIV/HAART viz. clinical, biochemical, immunological and genetic/ epigenetic aspects as shown in Figure 6.1. Our findings show that in preeclampsia, HIV / HAART is not associated with significant differences in the biochemical indices, clinical features or maternal and fetal adverse outcomes compared with uninfected preeclamptic women. However, we found a significant difference in gamma glutamyl transferase, a hepatic enzyme, in preeclamptic HIV infected women on HAART compared with uninfected women with preeclampsia. Because HIV has immune depressive effects, an association between HIV and preeclampsia has been suggested (Hall, Gebhardt et al. 2014). The postulated inhibitory effect of HIV on preeclampsia may be potentially reconstituted by HAART, which may explain the absence of significant differences in many of the clinical and biochemical sequelae when compared with uninfected preeclamptic women. The liver, which is a target organ both in preeclampsia and for the side effects of HAART (Obstetricians and Gynecologists 2013), (Browne, Schrier et al. 2015) may endure a dual impact, as suggested by the findings of a significant increase in gamma glutamyl transferase (GGT) between the groups.

Our findings also show that in uncomplicated/normotensive pregnancies, HAART is associated with significant decreases in IL-2, TNF- α and IL-6, and in preeclamptic women, it is associated with significant decreases in IL-2 and TNF- α , compared with uninfected women. These findings suggest that HIV/HAART impacts on pro-inflammatory cytokines in women with preeclampsia and provides a platform for further research on the immune reconstitution effects of HAART during pregnancy, and the development of potential immune modulation therapies for the management of preeclampsia. These findings may correlate with clinical data however further studies are necessary.(Mattar, Amed et al. 2004) (Hall, Gebhardt et al. 2014),

In determining the role of genetic polymorphisms and susceptibility to preeclampsia and some of the risk factors, single nucleotide polymorphisms (SNP) miRNA 27a rs 895819T>C and miRNA 146a rs 2910164 G>C, were investigated due to their association with biological processes implicated in PE. We did not find increased susceptibility to PE with variants TC/C and GC/CC, however the data show significant associations with obesity and severe disease respectively which are further dysregulated in

the presence of HIV/ HAART. Further large explorative studies on heterogeneous populations are necessary, however the findings provide an insight into the genetic/ epigenetic mechanisms that may influence the pathophysiology PE and HIV.

6.2. Recommendations:

6.2.1. Clinical:

- The benefits of HAART for maternal health and prevention of mother to child transmission outweigh the potential clinical and biochemical risks associated with it in women with PE and continued advocacy for its usage is necessary.
- Serial monitoring of Liver Function Tests are necessary in women with PE and HIV/HAART, to detect liver dysfunction early and prevent morbidity and mortality.
- The maternal records should include detailed information on HAART usage to assess its effects more accurately.
- Strategies to prevent obesity, which is likely to further complicate the long term cardiovascular and metabolic effects associated with PE, should be developed and implemented.

6.2.2. Scientific:

- The unifying link between immunological factors and features of the maternal syndrome in preeclampsia remains unclear and requires ongoing investigation.
- The role of the placenta in preeclampsia and HIV is not fully understood, and investigation of placental immunogenic factors and epigenetic factors requires further evaluation.
- Studies on preconceptual immune priming and maternal tolerisation to paternal antigenic material, are necessary to help us further understand the immunogenic basis of preeclampsia.
- Studies on racial variation and genetic susceptibility to preeclampsia may help us explain the differences in prevalence rates observed globally.

6.3. Concluding statement:

In the final analysis, it is evident that the intriguing and elusive nature of preeclampsia still prevails, and co-morbid HIV infection and HAART may have differential effects on certain aspects of the disease. PE is heterogeneous in nature and the aetiology is complex and likely to be multifactorial, extending beyond a singular pathophysiological mechanism. The next decade presents an opportunity to conduct further studies on the molecular and genetic aspects of preeclampsia, and perhaps thereafter, the cliché ‘disease of theories’ (Zweifel 1916) may slowly start to fade.

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