THE ROLE OF PERIPHERAL NATURAL KILLER CELLS IN IMMUNOCOMPROMISED PRE-ECLAMPTIC AND NORMOTENSIVE PREGNANT BLACK SOUTH AFRICANS

by

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PREFACE

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

The research described in this dissertation was carried out in the Optics and Imaging Centre, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa under the supervision of Professors T. Naicker, J. Moodley and P. Gathiram

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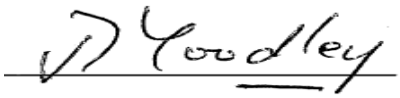

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DECLARATION

I, Anushka Ajith declare that:

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

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

(iii) This dissertation does not contain other person's data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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DEDICATION

For my daughter Azariya Rampersadh

The most sweetest, loving and greatest blessing in my life.

PEER REVIEWED PUBLICATIONS AND CONFERENCE PRESENTATIONS

PEER REVIEWED PUBLICATION

Anushka Ajith, Jagidesa Moodley, Premjith Gathiram, Niren Maharaj, Thajasvarie Naicker. Circulating Th1 and Th2 cytokine levels in HIV normotensive pregnant and early and late onset pre-eclamptic Black South Africans. European Journal of Obstretrics and Gynaecology. To be submitted 10 December 2015.

INTERNATIONAL CONFERENCE ATTENDANCE

A Ajith, J Moodley and T Naicker (2015). The role of peripheral Natural Killer cells in HIV associated pre-eclampsia in South Africa. Placenta 36(9) DOI: 10.1016/j.placenta.2015.07.357.

Anushka Ajith, Jagidesa Moodley, Premjith Gathiram, N Maharaj, Thajasvarie Naicker (2015). The role of peripheral Natural Killer cells in pre-eclampsia. Malaysian Society of Hypertension, Kuala-Lumpur, Malaysia, 23-25 January 2015. P1. p39 Poster Presentation First Prize Winner.

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- To God for His boundless love, guidance and blessings for making this journey possible.

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LIST OF ABBREVIATIONS

≥	greater than, equal to
≤	less than, equal to
ALT	alanine aminotransferase
ANOVA	analysis of variance
APC	allophycocyanin
ARV	anti retro viral
AST	aspartate aminotransferase
BMI	Body mass index
CD4	cluster of differentiation 4
CV	coefficient of variation
dNK	decidual Natural Killer
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
EOPE	early onset pre-eclampsia
FITC	fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
h	hours
HAART	highly active anti-retroviral therapy
HIV	human immunodeficiency virus
HLA	Human Leukocyte Antigen
IL	interleukin
IUGR	intrauterine growth gestation

kg/m^2	kilogram per meter squared
LDH	lactate dehydrogenase
LOPE	late onset pre-eclampsia
mg	milligram
ml	millilitres
mmHg	millimetres mercury
NK	Natural Killer
NPRI	non-pregnancy related infections
PE	phycoerythrin
pg/ml	pico gram per millilitre
PMTCT	preventing mother to child transmission
pNK	peripheral Natural Killer
SAPE	streptavidin phycoerythrin
sEng	soluble endoglin
sFlt	soluble fms like tyrosine kinase receptor
SPSS	Statistical Package for the Social Sciences
TGF-β	transforming growth factor beta
Th	T-helper (h)
TNF-α	tumour necrosis factor alpha
µl	micro litre
uNK	uterine Natural Killer
VEGF	vascular endothelial growth factor

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ABSTRACT

Introduction and Aims

HIV and pre-eclampsia are the leading causes of maternal morbidity and mortality in South Africa. Despite active research, the pathophysiology of pre-eclampsia is not fully understood. The role of peripheral NK cells in pre-eclampsia is limited. Also, the effect of HIV on peripheral NK cells in pre-eclampsia is not well documented. Additionally a dysregulation of Th1 and Th2 cytokine response is also known to occur both in pre-eclampsia and HIV. This novel study thus attempts to elucidate the role of peripheral NK cells and the Th1 and Th2 maternal immune response in HIV associated pre-eclampsia. The correlation of NK cells and their receptors with a panel of Th1 and Th2 cytokines were also determined.

Methods

Following institutional ethical approval and written informed consent, blood samples were obtained from 101 normotensive pregnant (51 HIV positive and 50 HIV negative), 81 early onset pre-eclamptic (EOPE; 37 HIV positive and 44 HIV negative) and 73 late-onset pre-eclamptic (LOPE; 29 HIV positive and 44 HIV negative) Black South Africans (>18 years) at Prince Mshiyeni Memorial Hospital, KwaZulu-Natal a regional hospital, in Durban, South Africa. These patients were either recruited directly on admission to the hospital or from the ante-natal clinic. Clinical data and demographics, laboratory blood test results and neonatal data were recorded.

Peripheral NK cell markers ($CD3^-$ BD Horizon 500, $CD56^{dim}$ PECy7 and $CD16^+$ FITC) and their receptor expression (CD69 Brilliant Violet™ 421, CD94 PerCP-Cy5, NKG2A Alexa Fluor® 500, NKG2C PE and NKG2D APC) were analysed on the BDLSRFortessa™ flow cytometer and the data further analysed using FloJo V10. Th1 (IL-2, IL-12(p70), IFN-γ, TNF-α and GM-CSF) and Th2 (IL-4, IL-5, IL-10 and IL-13) maternal serum cytokine levels were determined using the Bio-Plex Pro™ Human Cytokine Th1/Th2 9-plex panel. Cytokine concentrations (pg/ml) were obtained with the Bio-Plex®MAGPIX™ Multiplex Reader. SPSS Statistics Version 23 was used to analyze the clinical and experimental data. Differences of $p<0.05$ were considered statistically significant.

Results and Discussion

This novel study found that peripheral NK ($CD56^{dim}CD16^+$) cells were significantly higher in the HIV negative (mean rank=114.41) compared to HIV positive group (mean rank=95.13; $p=0.021$). This is surprising as peripheral NK cells possess more cytotoxic properties and would be expected to be present in larger amounts in the HIV positive compared to the negative group. However, HIV infection is known to suppress the cytotoxic properties of NK cells which may explain these results. Pre-eclampsia is associated with exaggerated inflammation and increased production of cytokines (Schumacher, 2014). This study confirms this as increased accumulation of peripheral NK cells were observed in pre-eclamptic compared to the normotensive pregnancies ($p=0.012$).

There were no significant differences in maternal serum Th1 and Th2 between the HIV positive and negative groups, pregnancy groups (normotensive, EOPE and LOPE) and across all six

study groups. In HIV associated pre-eclampsia there could be a counteraction in the Th1 and Th2 cytokine levels hence a neutralization of Th1 and Th2 response.

Conclusion

To our knowledge, the quantification of peripheral NK cells as well as Th1 and Th2 maternal serum cytokines in HIV associated pre-eclampsia is novel. Our study supports the hypothesis that the levels of peripheral NK cells are higher in the HIV positive pre-eclamptic group compared to the HIV positive normotensive group. Our study also suggests a neutralisation effect of Th1 and Th2 cytokines in HIV associated pre-eclampsia.

CHAPTER 1

INTRODUCTION

1.1 MATERNAL MORTALITY

In the last decade, global maternal mortality declined by 47%. The Millennium Development Goal 5 which recommended a 75% reduction in maternal death by 2015 has not been attained (Oyston et al., 2014; Burton, 2013). However, the World Health Organisation (WHO) estimates 800 maternal deaths per day or 210 maternal deaths per 100 000 live births. These pregnancy-related deaths are attributed to haemorrhage (35%), pre-eclampsia/eclampsia (18%), sepsis (8%), unsafe abortion (9%) and indirect causes including AIDS and malaria (18%) (Oyston et al., 2014). Notably, resource poor countries account for 99% of maternal deaths with 85% occurring in Sub-Saharan Africa and Southern Asia (Oyston et al., 2014).

In contrast, the three major causes of maternal deaths in South Africa are non-pregnancy related infections (35.8%, mostly HIV-related), obstetric haemorrhage (15.8%) and hypertensive disorders of pregnancy (14.8%) (Department of Health, Saving Mothers Reported, 2015). About 54.2% of maternal deaths from hypertensive disorders of pregnancy are due to pre-eclampsia (Department of Health, Savings Mothers Reported, 2015). Furthermore, KwaZulu-Natal accounts for the highest maternal deaths (22%) compared to the other provinces in South Africa with 42.5% due to non-pregnancy related infections (NPRI), mostly HIV-related and 8.3% to pre-eclampsia (Figure 1.1; Department of Health, Saving Mothers Reported, 2015).

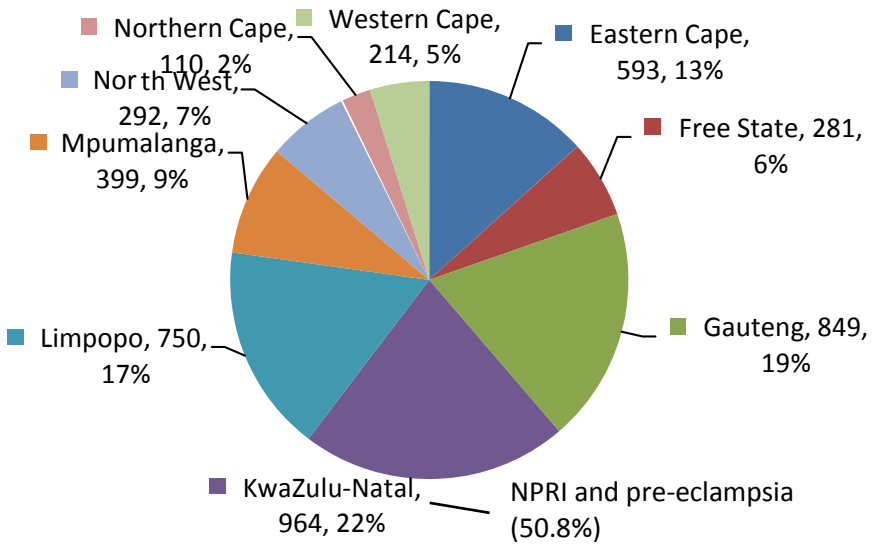


Figure 1.1: Distribution of maternal deaths per province in South Africa, 2011-2013. KwaZulu-Natal accounts for the highest maternal deaths (22%) compared to the other provinces in South Africa with 42.5% due to NPRI and 8.3% to pre-eclampsia (Department of Health, Saving Mothers Reported, 2015).

According to the Ninth Interim Saving Mothers Reported there has been a significant reduction in maternal deaths from NPRI (mostly HIV-related), however deaths due to obstetric haemorrhage and hypertensive disorders of pregnancy still remain high (Department of Health, Saving Mothers Reported, 2015). The reduction in maternal deaths due to complications of HIV infection is largely due to antiretroviral therapy. Maternal deaths from obstetric haemorrhage and hypertensive disorders of pregnancy may be due to the lack of skills in performing assisted delivery and/or caesarean section and the lack of knowledge in predicting pre-eclampsia/eclampsia in rural communities. Once diagnosed, poor infrastructure, lack of transport, poor road access and distance from rural areas to healthcare facilities place the mother and baby at risk. Advanced midwife training, adequate emergency transport service and well-

resourced healthcare facilities will aid in ameliorating these deaths (Pattinson, 2013; Oyston et al., 2014). Exacerbating this data is the fact that most pregnant women in developing countries present late for antenatal care and some never do seek postpartum care and follow-up. Therefore, education on the risks/dangers associated with pregnancy-related complications that contribute to maternal deaths are urgently warranted (Oyston et al., 2014).

1.2 NORMAL PLACENTATION

In the first trimester of normal pregnancy, maternal spiral arteries undergo remodeling by invasive foetal cytotrophoblast cells. These cells migrate across two pathways, (1) the interstitial pathway where they cross the decidua into the myometrium and (2) the endovascular pathway where they migrate into the lumen of the spiral artery (Wang et al., 2009). In the decidua, extravillous trophoblast cells replace the endothelial cells of the spiral arterioles hence enabling its physiological conversion into a large bore conduit (Figure 1.2). This allows for the nourishment of the growing fetus (Wang et al., 2009; Bachmayer et al., 2006). In pathological pregnancies such as pre-eclampsia, this transformation is incomplete (Young et al., 2010).

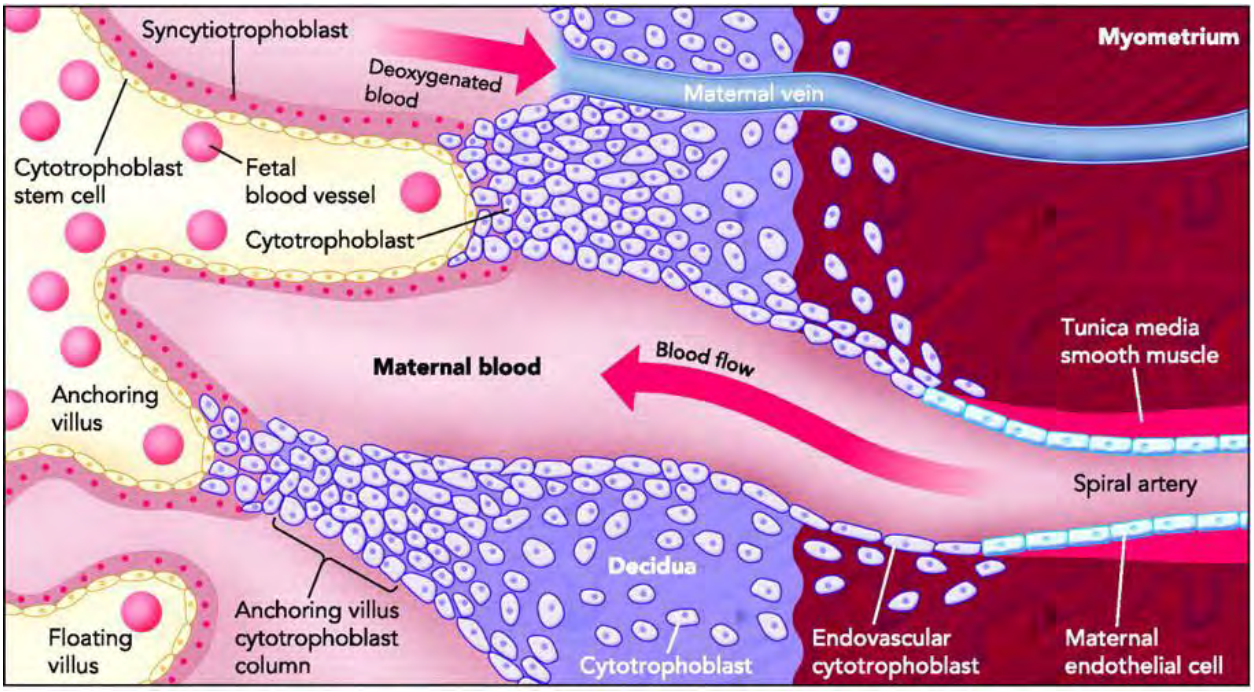


Figure 1.2: Normal placental development. Foetal cytotrophoblast cells invade the maternal uterine wall and remodel the maternal vessels into high capacitance and low resistance vessels that provide access to maternal oxygen and nutrients for the placenta and growing fetus (Wang, Rana and Karumanchi, 2009).

1.3 HYPERTENSIVE DISORDERS OF PREGNANCY

In recent years the diagnosis, classification and management of hypertensive disorders of pregnancy have been up-dated. In view of these developments, the International Society for the Study of Hypertension in Pregnancy (ISSHP) has recently revised the classification of hypertensive disorders of pregnancy into four categories (Tranquilli et al., 2014):

1. Chronic hypertension refers to high blood pressure before pregnancy or in the first trimester of pregnancy.

2. Gestational hypertension refers to new onset high blood pressure after 20 weeks of gestation without any other symptoms which define pre-eclampsia.

3. Pre-eclampsia – *de novo* (defined in 1.4 below) or superimposed on chronic hypertension. Superimposed pre-eclampsia refers to the development of any other symptom of pre-eclampsia after 20 weeks of gestation including chronic hypertension.

4. White coat hypertension refers to presentation of high blood pressure up to 160-170/110 mmHg only in a clinical setting (Tranquilli et al., 2014).

1.4 PRE-ECLAMPSIA

Pre-eclampsia is characterized by new onset high blood pressure (>140/90 mmHg) and proteinuria (≥300 mg in a 24 h urine sample or a dipstick result of ≥1+) in pregnant women in the second half of pregnancy (Magee et al., 2014) or in the absence of proteinuria, the new onset of platelet count less than 100 000/µl called thrombocytopenia, serum creatinine concentrations greater than 1.1 mg/dL or a doubling of the serum creatinine concentration in the absence of other renal disease, impaired liver function, pulmonary oedema or cerebral or visual symptoms (Tranquilli et al., 2014; ACOG Practice Bulletin., 2002).

Pre-eclampsia may progress to eclampsia which is characterized by new-onset of grand mal seizures and affects 2.7-8.2 women per 10 000 deliveries (Chaiworapongsa et al., 2014). In South Africa, 2% of maternal deaths are attributed to eclampsia (Department of Health, Saving Mothers Reported, 2012). Maternal outcomes from pre-eclampsia and eclampsia may include cerebrovascular accidents, liver rupture and pulmonary oedema or acute renal failure that can result in maternal deaths (Chaiworapongsaet et al., 2014). Perinatal outcomes include preterm delivery, intrauterine growth restriction and (IUGR) and foetal death as well as haematologic

effects and bronchopulmonary dysplasia (Backes et al., 2011). The only effective treatment of pre-eclampsia is delivery of the placenta (Naljayan and Karumanchi, 2013).

1.4.1 Pre-eclampsia classification

Pre-eclampsia can be classified as early (<34 weeks) or late (>34 weeks) onset (Tranquilli et al., 2014). Early onset pre-eclampsia (EOPE) is uncommon with a prevalence of 0.3-12% of all pre-eclampsia. It is associated with extensive villous and vascular lesions of the placenta and has a higher risk of maternal and foetal complications than late onset pre-eclampsia (LOPE) (Chaiworapongsaet et al., 2014). LOPE has a prevalence of 2.72% or 88%, minimal placental lesions, maternal factors such as metabolic syndrome and hypertension and most cases of eclampsia and maternal deaths (Wang et al., 2009; Lisonkova and Joseph 2013).

1.5 PATHOGENESIS OF PRE-ECLAMPSIA

The etiology and pathology of pre-eclampsia is not fully understood. This disease occurs only in pregnant women and remits after delivery of the placenta (Wang et al., 2009). The placenta is therefore a vital organ in understanding the pathogenesis of pre-eclampsia (Wang et al., 2009). A number of factors have been implicated in the pathogenesis of pre-eclampsia which elicit an exaggerated inflammatory response that results in defective placentation and consequential failure of the physiological transformation of maternal spiral arteries. These factors include abnormal foetal cytotrophoblast and decidual interaction, renin-angiotensin-aldosterone system II (increased AT1 autoantibodies), angiogenic and anti-angiogenic factors, immunological intolerance (genetic factors), placental oxidative stress and syncytiotrophoblast debris (Figure 1.3, (Chaiworapongsa et al., 2014; Wang et al., 2009).

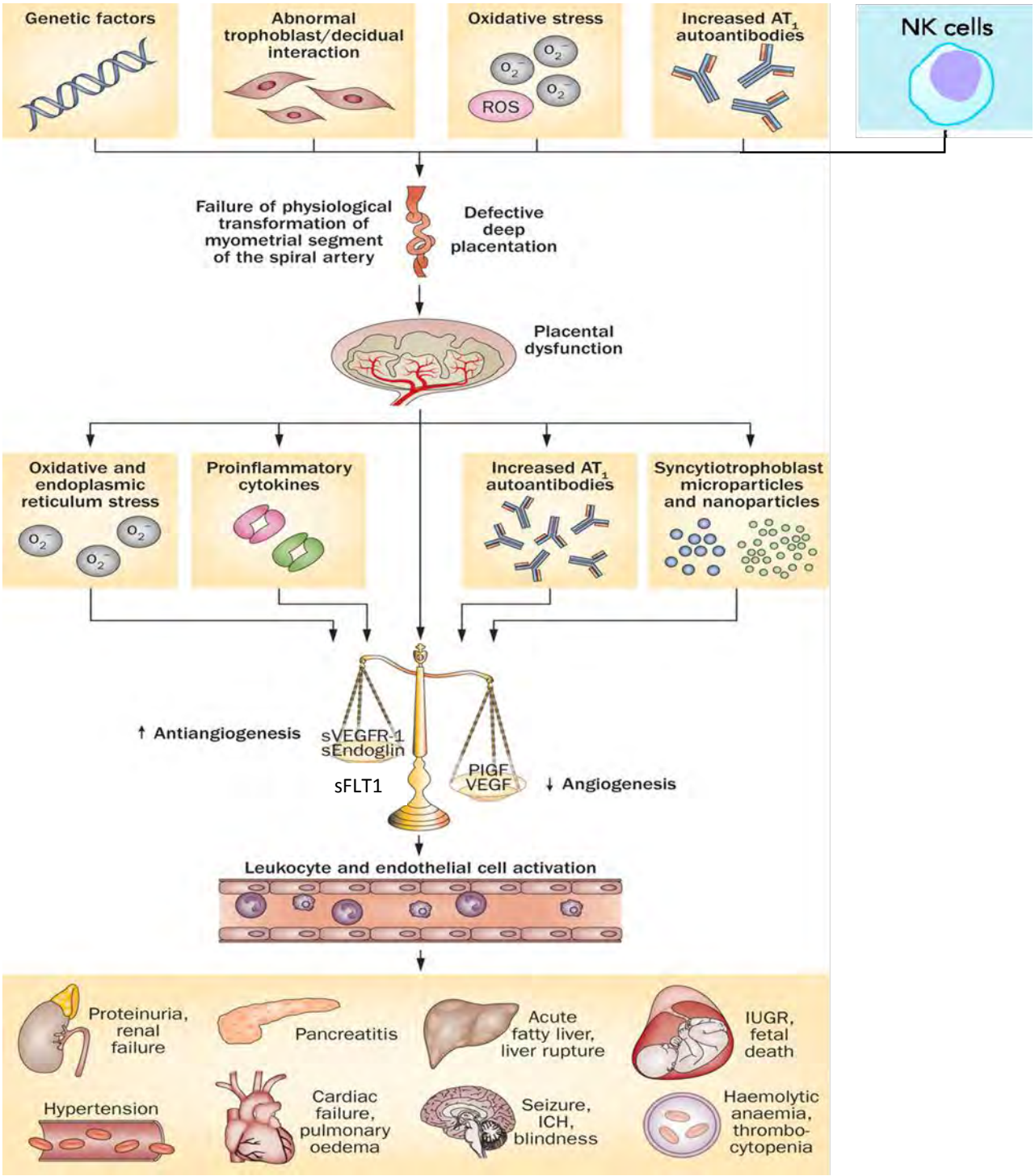


Figure 1.3: A model depicting the complex pathophysiology of pre-eclampsia. Genetic factors, oxidative stress, NK cell abnormalities and other factors result in growth restriction of the fetus. This leads to the release of antiangiogenic and angiogenic factors, as well as other inflammatory factors which induces pre-eclampsia and other diseases. (Adapted from Chaiworapongsa et al., 2014; Wang et al., 2009).

1.5.1. Abnormal placentation in pre-eclampsia

Pre-eclampsia occurs in two stages, placental and maternal. In the placental stage, the migration of invasive cytotrophoblast into the myometrium is deficient in the first trimester. Physiological conversion of the spiral arteries is limited to the decidua and their luminal diameter within the myometrium is considerably reduced (Naicker et al., 2013; Walker, 2011). Blood flow to the placenta is reduced; hence nutrient and oxygen transfer across the placenta limits foetal growth (Figure 1.4).

Furthermore, in the maternal stage after 20 weeks of gestation, trophoblast cell debris is released into the maternal circulation and induces an inflammatory response in the second trimester of pregnancy (Bachmayer et al., 2006). Both the vasocontriction and the elevated inflammatory response contribute to the hypertension, oedema and proteinuria that characterises pre-eclampsia. The invading trophoblast, maternal immunological cells such as Natural Killer cells play an important role in normal pregnancy development (Walker, 2011).

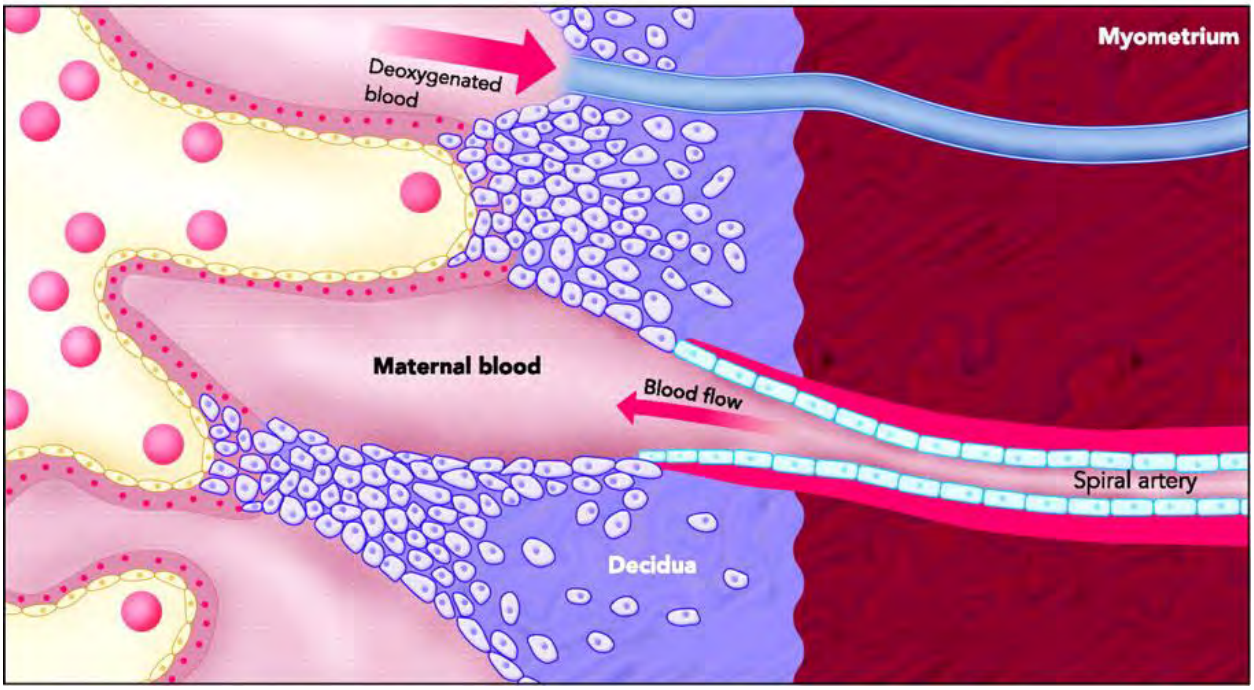


Figure 1.4: Abnormal placental development in pre-eclampsia. Depth of trophoblast invasion is less in pre-eclampsia compared to normal pregnancy which results in inadequate transformation of maternal spiral arteries. The blood flow to the feto-placental unit is reduced and this leads to poor foetal growth (Wang et al., 2009).

1.5.2 Renin--angiotensin-aldosterone system

The renin–angiotensin system (RAS) or the renin–angiotensin–aldosterone system (RAAS) is a hormone system responsible for regulating blood pressure, sodium and fluid balance (Uddin, 2014). A reduced blood flow to the kidneys causes the juxtaglomerular cells to release the enzyme called renin which cleaves angiotensinogen, the only renin substrate, into angiotensin I. Angiotensin I is then converted to angiotensin II by angiotensin-converting enzyme (ACE). Angiotensin II binds to its receptors, AT1R and AT2R leading to vasoconstriction and the release of the hormone aldosterone (Mistry and Kurlak, 2013).

1.5.3 Anti-angiogenic and angiogenic factors

Angiogenesis is the formation of new blood vessels which involves the migration, growth and differentiation of endothelial cells. Angiogenic growth factors are necessary for the formation of blood vessels. Pre-eclampsia is associated with an imbalance between anti-angiogenic and angiogenic factors. Anti-angiogenic factors such as soluble fms-like tyrosine kinase 1 (sFlt1) and soluble endoglin (sEng) are upregulated and angiogenic factors such as placental growth factor (PIGF) and vascular endothelial growth factor (VEGF) are downregulated which result in disruption of the maternal endothelium and ultimately in hypertension and proteinuria (pre-eclampsia) (Govender et al., 2013; Wang et al., 2009).

Decreased levels of sFlt1 in early pregnancy have been associated with decreased foetal cytotrophoblast invasion into maternal spiral arteries. Endoglin is a surface receptor for TGF-beta. TGF-β 1 and/or TGF-β 3 inhibits trophoblast migration and invasion (Ajith et al., 2014). The placenta may produce sEng to compensate for the increased surface endoglin which results in increased sEng in the maternal circulation in pre-eclamptic women (Govender et al., 2015; Wang et al., 2009).

1.5.4 Genetic factors contributing to pre-eclampsia

Pre-eclampsia often occurs in young women in their first pregnancy. The maternal immune system develops tolerance to the paternal alloantigens when exposed to seminal fluid and/or semen (Chelbi and Vaiman, 2008). Prolonged exposure to semen reduces the risk of pre-eclampsia, in contrast primigravid women and women undergoing artificial insemination have a higher risk of developing pre-eclampsia (Chaiworapongsa et al., 2014).

Anyikam et al. (2004) reporteded that women with ≥3 genderual partners are at a higher risk of developing pre-eclampsia than women with ≤2 genderual partners. Also, women experiencing menarche at ≤12 years are at a higher risk of pre-eclampsia development than women at later ages. Moodley (2008) reporteds that teenage pregnancies are at risk of developing pre-eclampsia.

Other risk factors of pre-eclampsia include extreme maternal ages (<20 years or >35 years), history of pre-eclampsia in previous pregnancy, multi foetal gestation, obesity, family history of pre-eclampsia, pre-existing medical conditions such as chronic hypertension, diabetes mellitus, antiphospholipid syndrome, thrombophilia, autoimmune disease, renal disease and infertility and urinary tract infection (Chaiworapongsa et al., 2014; Sanchez-Aranguren, 2014).

1.5.5 Oxidative stress

The traditional understanding of the pathophysiology of pre-eclampsia is that the ischemic placenta produces soluble toxins into the maternal circulation which are responsible for the clinical presentation of the disease (Siddiqui et al., 2010). These soluble factors cause endothelial dysfunction, intravascular inflammation and an activation of the haemostatic system. Pre-eclampsia is therefore considered primarily to be a vascular disorder (Sanchez-Aranguren, 2014). The clinical manifestations result from the involvement of multiple organs such as the kidneys, liver, brain, heart, lung, pancreas and the vasculature (Chaiworapongsa et al., 2014).

1.5.6 Immune maladaptation

1.5.6.1 Cytokine production in pre-eclampsia

Pre-eclampsia is associated with exaggerated inflammation and increased production of cytokines (Bachmayer et al., 2009), the role of which remains controversial (Mansouri et al., 2007). T-helper (h)1 and Th2 cytokines play a role in cell-mediated and humoral immunity respectively. Th1 immune response inhibits Th2 response and *vice versa* through the production of cytokines therefore many immune responses tend to be dominated by one or the other cell type (Mikovits et al., 1994).

In healthy non-pregnant women there is a balance between the Th1 and Th2 response (Laresgoiti-Servitje et al., 2010). However, in successful normal pregnancy, with the presence of the placenta there is a shift from the Th1 to Th2 immune response (Figure 1.5, Laresgoiti-Servitje et al., 2010). In pathological pregnancies such as pre-eclampsia, spontaneous abortions and IUGR, the regulation of the maternal immune system is further altered and the shift to the Th2 immune response does not occur (Figure 1.5) (Hu et al., 2007). Wegmann et al. (1993) proposed that the placenta is a Th2 organ that stimulates the production of Th2 cytokines in the maternal immune system. When the placenta is abnormal, the maternal immune system responds by producing Th1 cytokines. Additionally, transforming growth factor-β cytokines surge thereby retarding cytotrohophoblast migration (Fukui et al., 2011; Laresgoiti-Servitje et al., 2010). Moreover, syncytiotrophoblast debris is increased in pre-eclamptic maternal circulation due to the ischemic placenta which is possibly responsible for triggering the Th1 response (Sargent, et al., 2007).

Th2 cytokines respond to extracellular infection and are known to suppress the immune system (Becker, 2004). In HIV infected patients there is a decrease in the Th1 and an increase in Th2 cytokines (Clerichi and Shearer, 1993). There are however, conflicting reporteds on the circulating levels of cytokines in pre-eclampsia (Kumar et al., 2013).

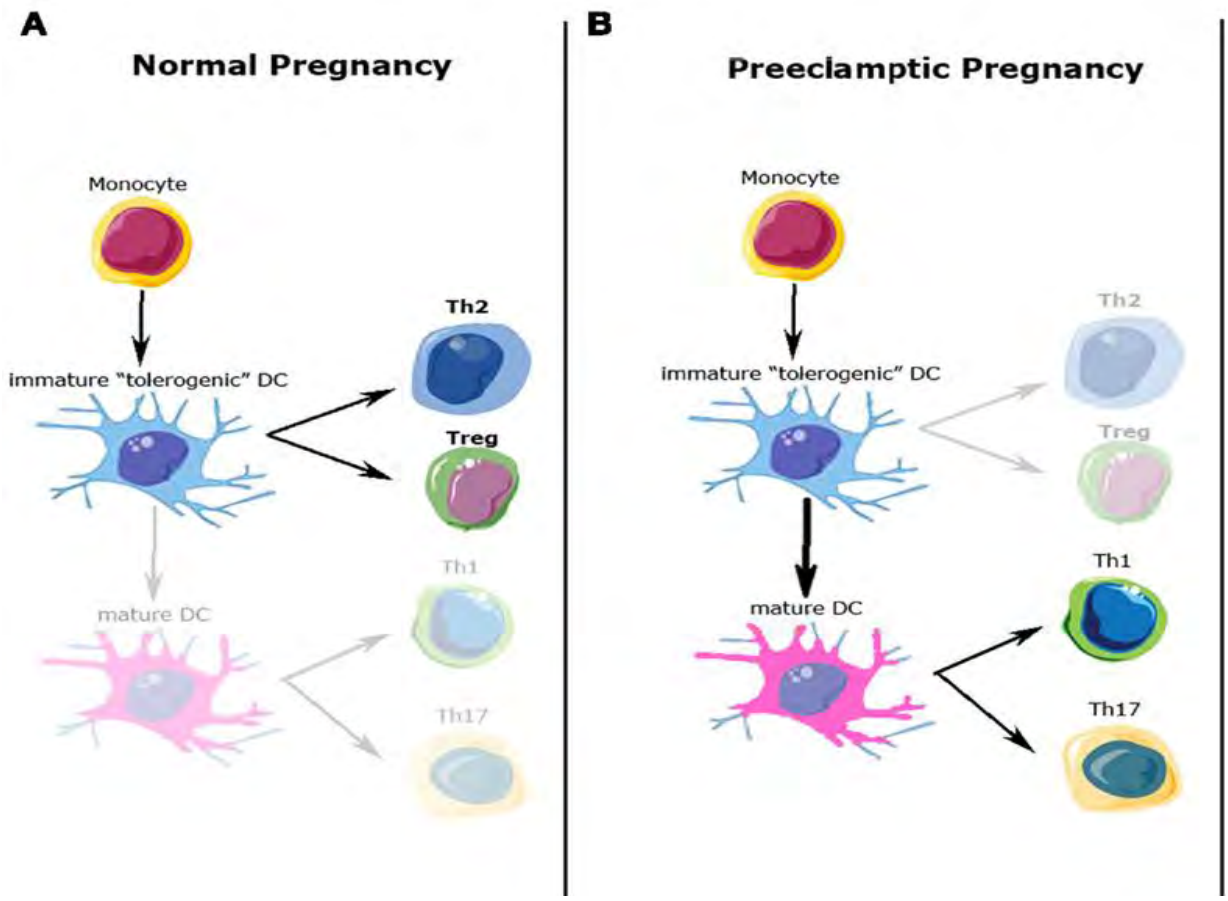


Figure 1.5: Th1 and Th2 response in normal and pathological pregnancy. In successful normal pregnancy there is a shift from the Th1 to a Th2 immune response with an increased production of Th2 cytokines. In pre-eclampsia the shift to a Th2 immune response does not occur resulting in an increase in Th1 cytokines (Perez-Sepulveda et al., 2014).

1.5.6.2 Natural Killer cell production in pre-eclampsia

The two interfaces of pregnancy are the invasive extravillous foetal cytotrophoblast and the maternal immune cells at the decidua (Sargent et al., 2007). The most abundant lymphoid cell population at the decidua is the decidual Natural Killer (NK) cells which interact with the major histocompatibility complex (MHC) antigens through receptors such as Human Leukocyte Antigen (HLA)-E and CD94/NKG2A (Sargent et al., 2007).

Natural Killer cells are triggered by cytokines, interleukin (IL)-2, IL-12, IL-15 and IL-18 (Vacca et al., 2011). Once activated NK cells express CD69 which activate interferon (IFN)-γ production that contribute to a Th1 immune response in circulation in pre-eclampsia (Bachmayer et al., 2009). IFN-γ expression was also found to be higher at the decidua in pre-eclampsia (Darmochwal-Kolarz and Kludka-Sternik, 2010). NK cells play a crucial role at the decidual and syncytiotrophoblast interface and abnormal activation of NK cells results in pre-eclampsia development (Sargent et al., 2007).

1.6 NATURAL KILLER CELLS

Natural Killer (NK) cells are large granular lymphocytes that comprise 10% of total blood lymphocytes, 70% T cells and 20% B cells (Acar et al., 2011). NK cells are the first line of defense against infection. Natural Killer cells that target viral and tumour cells, are recruited to the site of infection or to the transformed cells where they recruit and activate other immune cells (Vacca et al., 2013). These cells are cytotoxic and function by releasing proteins from cytoplasmic granules such as perforin and granzyme which lyse target cells (Acar et al., 2011).

1.6.1 Mechanism of Natural Killer cell recognition

Natural Killer (NK) cells are an important part of the innate immune system which provide an immediate defense against infection (Figure 1.6). In contrast the adaptive immune system provides long lasting immunity to the host. NK cells do not require activation to kill cells that are missing self-markers of MHC class I. This is important because harmful cells missing MHC I cannot be detected and destroyed by other immune cells such as T lymphocytes. There are two distinct mechanisms by which NK cells kill their target viz., antibody-dependent cell mediated cytotoxicity (ADCC) and their "Natural Killer" activity (Acar et al., 2011).

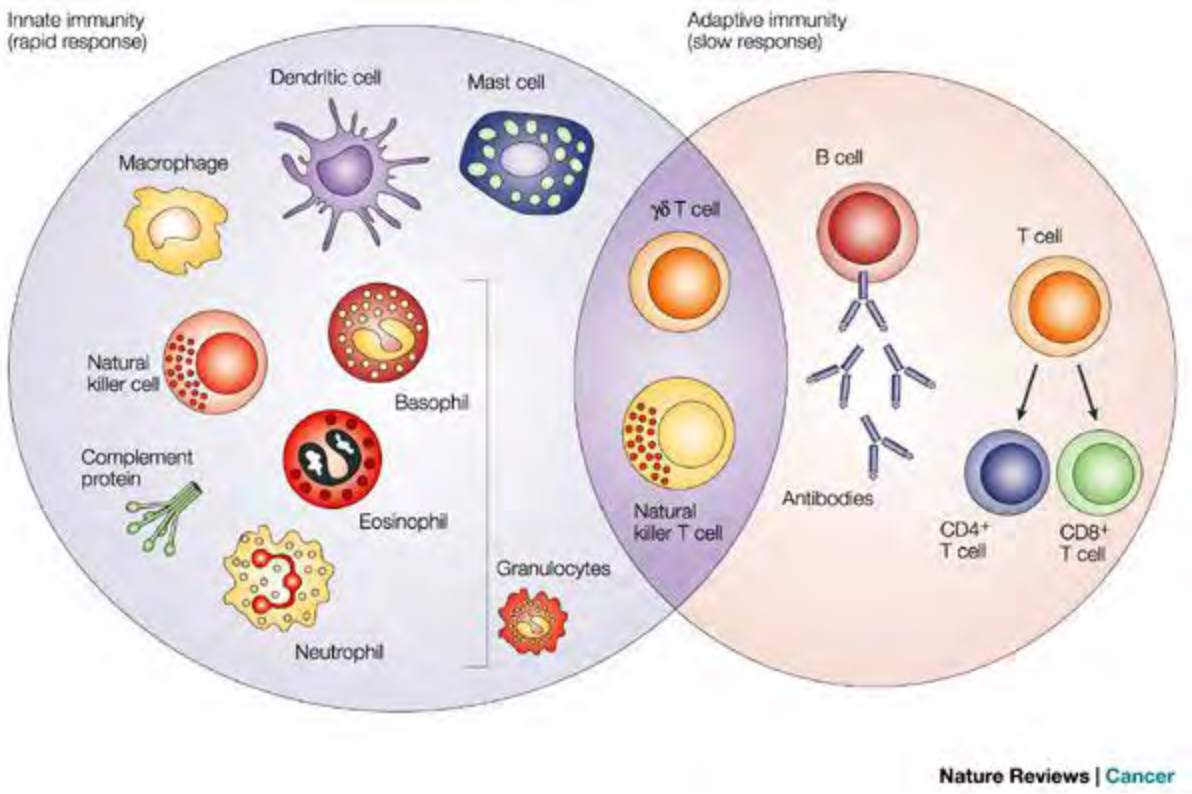


Figure 1.6: Natural Killer cells part of innate immunity (Dranhoff, 2004).

1.6.2 Classification of Natural Killer cells

Natural Killer cells are characterized by the absence of CD3 and presence of the CD56 surface antigen. CD16 are present on the surface of NK cells and are involved in antibody-dependent cellular cytotoxicity (Acar et al., 2011). There are two types of NK cells namely, uterine and peripheral NK cells. Uterine NK cells (uNK) constitute 70-90% of all blood lymphocytes whilst peripheral NK cells (pNK) constitute 5-10% (Fukui et al., 2011). NK cells occur either as $CD56^{bright}$ or $CD56^{dim}$ (Figure 1.7). The former are the major pro-inflammatory cytokine producers, are less cytotoxic and found mainly in the decidua whilst the latter are cytotoxic, produce less cytokines and found mainly in peripheral blood (Bachmayer et al., 2009; Fukui et al., 2011).

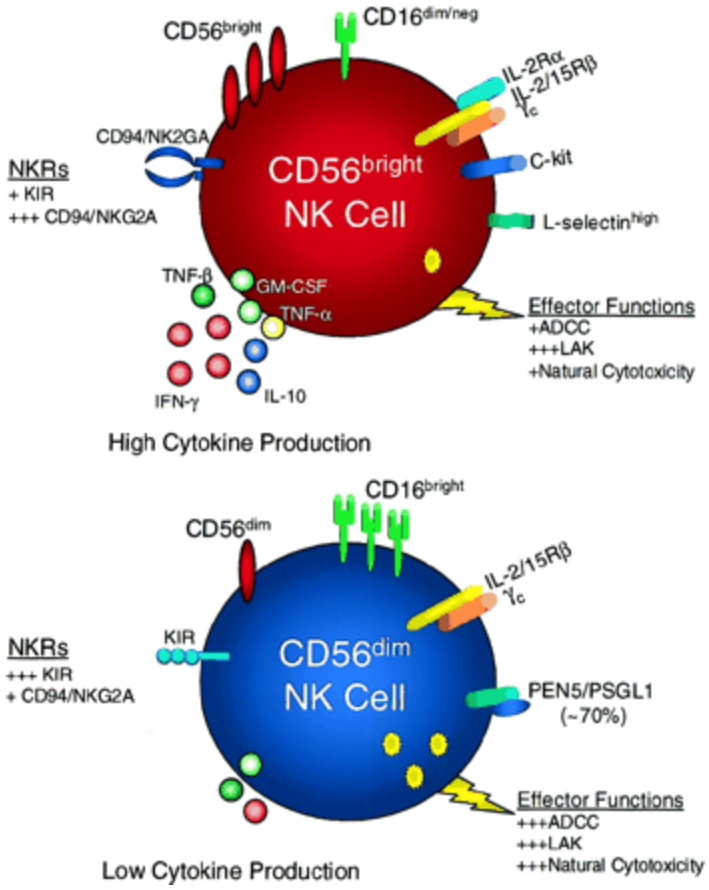


Figure 1.7: Natural Killer cells and receptors. $CD56^{bright}$ NK cells produce more cytokines compared to the $CD56^{dim}$ NK cells which are cytotoxic. Each NK cell has its own inhibiting and activating receptors (Cooper et al., 2001).

1.6.3 Natural Killer cell receptors

The activity of NK cells are regulated by inhibitory and activating receptors. Each NK cell expresses its own inhibitory and activating receptors (Cooper et al., 2001). NK cell activating receptors are the natural cytotoxicity receptors viz., NKp46, NKp30, NKp44, NKG2D, CD16, 2B4, NTBA, CD69, NKp80 and DNAM-1. Inhibitory receptors include HLA class I molecules, p75/AIRM1 (Siglec-7) and IRP60 (Vacca et al., 2011). There are three families of NK cell receptors, namely killer-cell immunoglobulin-like receptors (KIRs), C-type lectin receptors,

CD94/NKG2 (NKG2A-F, Figure 1.7) and immunoglobulin-like transcripts (ILTs) (Acar et al., 2011) and natural cytotoxicity receptors (NCRs) which regulate NK cell cytotoxicity and cytokine production (Fukui et al., 2012).

Killer-cell Ig-like receptors (KIRs) are type I transmembrane glycoproteins and are classified by the number of Ig-like domains, two (KIR2D) or three (KIR3D) and by the presence of long (KIR2DL and KIR3DL) or short (KIR2DS and KIR3DS) cytoplasmic tails. KIRs with long tails are associated with inhibition of NK cell mediated cytotoxicity and cytokine secretion whilst the short tails are associated with NK cytolysis. KIR2D receptors recognize HLA-C alleles and KIR3D receptors recognize HLA-A and HLA-B alleles (Sánchez-Rodríguez et al., 2011). The interaction between maternal KIRs and trophoblast cells play a physiological role in placental development. In mothers lacking most or all the activating KIRs, their fetuses possess HLA-C and show greater risk of pre-eclampsia development (Acar et al., 2011). Yan et al. (2007) showed higher KIR2DL4 protein levels in normal pregnancies compared to recurrent spontaneous abortion patients whilst Witt et al. (2004) showed no differences.

CD94/NKG2 receptor is a member of the C-type lectin superfamily. The NKG2 family comprises five genes, NKG2A, NKG2C, NKG2D, NKG2E and NKG2F which determine whether the receptor is inhibitory or activating. CD94/NKG2 receptors include NKG2A/B which provides an inhibitory signal and inhibits NK function whilst NKG2C and NKG2D are NK cell activating receptors (Bachmayer et al., 2009). $CD56^{dim}$ NK cells express the inhibitory killer cell immunoglobulin-like receptors (KIR) which are found predominately in the peripheral blood. $CD56^{bright}$ lack KIR expression but express high levels of the activating CD94/NKG2 receptors

which are found in the peripheral tissues. These receptors play an important role in the formation of the decidua and placenta (Bachmayer et al., 2006).

ILT genes are also known as leucocyte Ig-like receptors (LIRs). They are expressed on B cells, T cells, NK cells, dendritic cells, monocytes and macrophages. They consist of eight members, LIR-1-8 and only IL2 and 4 bind to HLA class I molecules including HLA-G (Acar et al., 2011). In normal pregnancy, HLA-G with the aid of ILT4 receptor modulates the behavior of dendritic cells, monocytes and macrophages. The role of ILT receptors in pre-eclampsia is yet to be determined (Laresgoiti-Servitje et al., 2010).

1.6.4 The role of NK cell receptors in HIV

NK cells may recognize cells under stress by expression of their ligands by means of NK cell receptors such as KIRs (NKRs) (Naranbhai et al., 2013). Viruses have evolved specific mechanisms to reduce the expression of HLA which is a KIR ligand (Naranbhai et al., 2013). The mechanism by which HIV suppresses NK cell activity is not known. IFN-γ NK cell responses may protect against HIV infection (Tiemessen et al., 2001).

1.6.5 Functions of Natural Killer cells in pregnancy

In early pregnancy, the major subset of uNK cells is $CD16^-$ and $CD160^-$ whilst that of pNK cells is $CD16^+$ and $CD160^+$ (Le Bouteiller et al., 2011). In normal pregnancies, despite the fetus being semi-allogeneic, it escapes rejection from the maternal immune system (Vacca et al., 2011). Leukocytes such as Natural Killer cells, $CD14^+$ myelomonocytic ($dCD14^+$) cells and T

lymphocytes play a role in establishing and maintaining maternal immuno-tolerance (Vacca et al., 2011).

NK cells also play an important role in ensuring reproductive success (Fukui et al., 2011). In early pregnancy NK cells within the decidua are known to play a role in placentation, angiogenesis and in the regulation of trophoblast invasion (Bachmayer et al., 2009). $CD56^{+}$ aggregates have been observed around spiral arteries and glands. The number of uNK cells decline after 20 weeks of gestation when trophoblast invasion is complete (Acar et al., 2011). Although studies have indicated a beneficial role of uNK cells in ensuring pregnancy success, the function of pNK cells is not well known (Fukui et al., 2011).

1.7 UTERINE NATURAL KILLER CELLS

Uterine Natural Killer cells located within the non-pregnant endometrium are called endometrial NK cells whilst in the decidua are called decidual NK (dNK) cells. Uterine Natural Killer cells constitute the major lymphocyte population in early gestation in the uterus which decreases after 20 weeks of gestation when trophoblast invasion is complete (Acar et al., 2011).

Uterine Natural Killer cells are characterized as $CD56^{bright}$, $CD16^{-}$ and $CD3^{-}$. They are less cytotoxic and produce large amounts of cytokines compared to peripheral Natural Killer cells. Many studies have shown that $CD56^{bright}$ $CD16^{-}$ uNK cells originate in the blood from $CD56^{bright}$ $CD16^{-}$ NK cells (Acar et al., 2011) whilst Keskin et al. (2007) suggest that uNK cells may originate from $CD56^{dim}$ $CD16^{+}$ pNK cells in the blood and differentiate in tissue by TGF-β and other factors. Uterine NK cells may differentiate from precursor cells in the uterus (Acar et al.,

2011). $CD56^{bright}$ cells in peripheral blood are agranular compared to uterine tissue where they are granular (Fukui et al., 2011).

1.7.1 Receptors of uterine Natural Killer cells

The trophoblast is the outermost layer of the placenta and is in direct contact with the maternal immune system (Vacca et al., 2011). The human trophoblast lacks HLA-A, -B but express HLA-C, -E and –G. Both maternal KIR genotype and foetal HLA molecules can result in NK cell inhibition and may be associated with high risk of pre-eclampsia due to inadequate trophoblast invasion and spiral artery transformation (Koopman et al., 2003).

Trophoblast cells express ligands for activating NK receptors such as NCRs, DNAM-1 and NKG2D. When dNK cells interact with the trophoblast they release IL-8, VEGF, SDF-1 and IP10. This interaction facilitates trophoblast migration, angiogenesis and the remodeling of the decidual and myometrial spiral arteries (Figure 1.8) (Vacca et al., 2011).

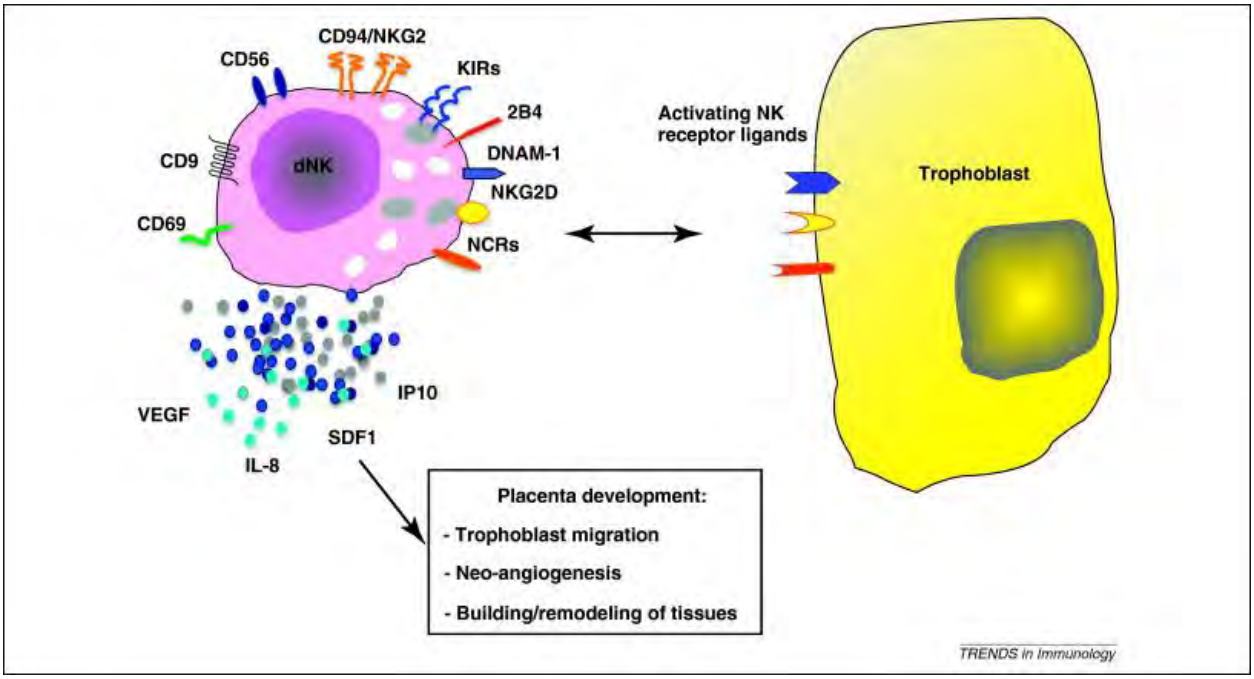


Figure 1.8: The interaction between uterine Natural Killer cell receptors and trophoblast cells in ensuring successful placental development (Vacca et al., 2011).

Despite having cytotoxic properties, uNK cells appear to be important regulators of implantation and successful pregnancies (Acar et al., 2011). The number and range of uNK receptors differ between normal and pathological pregnancies, such as pre-eclampsia or intrauterine growth (Acar et al., 2011). Le Bouteiller et al. (2011) have shown that the interaction between the cytotoxic uNK, NKp46 activating receptor and CD94/NKG2A with its HLA-E ligand inhibits the cytolytic action of uNK cells in a non-infected pregnant uterus. NKG2A inhibitory receptor and its HLA-E ligand expressed by trophoblast and other decidual cells inhibit the cytotoxic activity of the NKp46 receptor of uNK cells. uNK cells produce cytokines and chemokines by interacting with ligands of receptors expressed by trophoblast and decidual stromal cells (Le Bouteiller et al., 2011).

1.7.2 Functions of uterine Natural Killer cells

Uterine Natural Killer cells are the major lymphocyte population found during early gestation in the uterus and decreases as pregnancy progresses (Fukui et al., 2012). These cells occur in the endometrium, however they have shown to play a role in trophoblast invasion and spiral remodeling which occurs in the inner myometrium where uNK cells have not been found (Acar et al., 2011). They occur in abundance at the maternal-foetal interface in close proximity to extravillous trophoblast cells and play an important role in implantation and in placental development (Lash et al., 2010).

Uterine Natural Killer cells, increase during the differentiation of the human endometrial epithelium and produce angiogenic factors such as angiopoietin (Ang) 1, Ang 2, vascular endothelial growth factor (VEGF), placental growth factor (PIGF) and TGF-β and cytokines such as IL-8 and IFN-inducible protein 10 which play an important role in angiogenesis (Acar et al., 2011; Fukui et al., 2012).

Uterine Natural Killer cells may contribute to pre-eclampsia by inducing the lysis of trophoblast cells lacking HLA-G (Vacca et al., 2011). Fewer trophoblast cells will then invade the maternal spiral arteries which results in lack of oxygen and nutrients to the fetus (Vacca et al., 2011).

1.8 PERIPHERAL NATURAL KILLER CELLS

Peripheral Natural Killer (pNK) cells are characterized as $CD56^{dim}$ and $CD16^{+}$ cells and are mostly cytotoxic and release IFN-α rapidly on activation. Although NK cells are abundantly

found in peripheral blood, they primarily exert their function in tissues and secondary lymphoid organs in response to inflammation (Vacca et al., 2011).

Eriksson et al. (2006) showed that $CD56^{bright}$ pNK and uNK cells shared positive expression of surface markers CD56 and CD94, negative expression of CD57 and negative or low expression of CD16 (Acar et al., 2011). Uterine NK cells are differentiated from pNK cells by the expression of CD9 (Acar et al., 2011).

1.8.1 Receptors of peripheral Natural Killer cells

$CD56^{dim}$ NK cells express high levels of killer-immunoglobulin-like receptors (KIRs) and low levels of NKG2A. Bachmayer et al. (2009) reporteded that levels of inhibitory NKG2A and activating NKG2C were higher in pre-eclamptic compared to normal pregnant women (Vacca et al., 2011). Mela et al. (2007) have shown decreased expression of NKG2A on pNK cells of HIV-1 infected individuals (Le Bouteiller et al., 2011).

1.8.2 Functions of peripheral Natural Killer cells

Natural Killer cell expression is important for successful pregnancy (Mosimann et al., 2013) and have shown that $CD56^{dim}CD16^{+}$ cells were significantly higher than $CD56^{dim}CD16^{-}$ cells in peripheral blood in reproductive failures compared to successful pregnancies (Vacca et al., 2011).

The interaction between peripheral blood NK cells and trophoblasts is not well understood. NK cells preferentially kill targets with lower expression of major histocompatibility complex

(MHC) class I proteins, because fewer inhibitory receptors are engaging ligands. As a consequence, syncytiotrophoblasts are vulnerable to cytotoxic effect of peripheral blood NK cells (Fukui et al., 2011). Moreover, Moffett et al. (2004) reporteded that examination of peripheral blood NK cells is not informative of what is actually happening within the uterus.

Abnormalities in NK cell response amongst other immune responses may cause hypertension and high levels of proteinuria with consequential placental dysfunction and resultant pre-eclampsia (Cerdeira and Karumanchi, 2012). Some studies have also shown the abnormal production of angiogenic factors by peripheral NK cells (Fukui et al., 2012). Molvarec et al. (2010) has shown that pNK cells produce lower levels of VEGF in pre-eclamptic women compared to healthy pregnant women (Vacca et al., 2011). Peripheral Natural Killer cells secrete MIP-1α and MIP-1β ligands of CCR5 which inhibit viral entry and GM-CSF which suppress HIV replication (Le Bouteiller et al., 2011).

Table 1.1 Phenotype and functions of uNK and pNK cells (Vacca et al., 2011)

Phenotype	**uNK**	**pNK CD56^{bright}**	**pNK CD56^{dim}**
CD16	−	−/+	++
CD94/NKG2	++	++	−
KIRsa	+	−	+
KIR2DL4	+	−	−
CD9	+	−	−
CD151	+	−	−
CD69	+	−	−
CD49a	+	−	−
CD49f	−	+	+
CD62L (L-Selectin)	−	+	−/+
Granzymes and Perforin	++	−/+	++
Functions			
Cytotoxicity	−/+	−/+	++
IFN-γ	+	++	
GM-CSF	++	+	+
IL-8	+++	−	−
IP10	+	−	−
VEGF	++	−	−

1.9 NATURAL KILLER CELLS AND HIV ASSOCIATED PRE-ECLAMPSIA

Although the role of Natural Killer cells is to destroy viral cells, HIV has the ability to suppress the function of NK cells. NK cells function by the interaction of their activating receptors with cell surface ligands either of the infected host cells or by the virus itself (Figure 1.9, Le Bouteiller et al., 2011). The integration of inhibiting and activating receptor signals determines NK cell anti-viral response (Le Bouteiller et al., 2011).

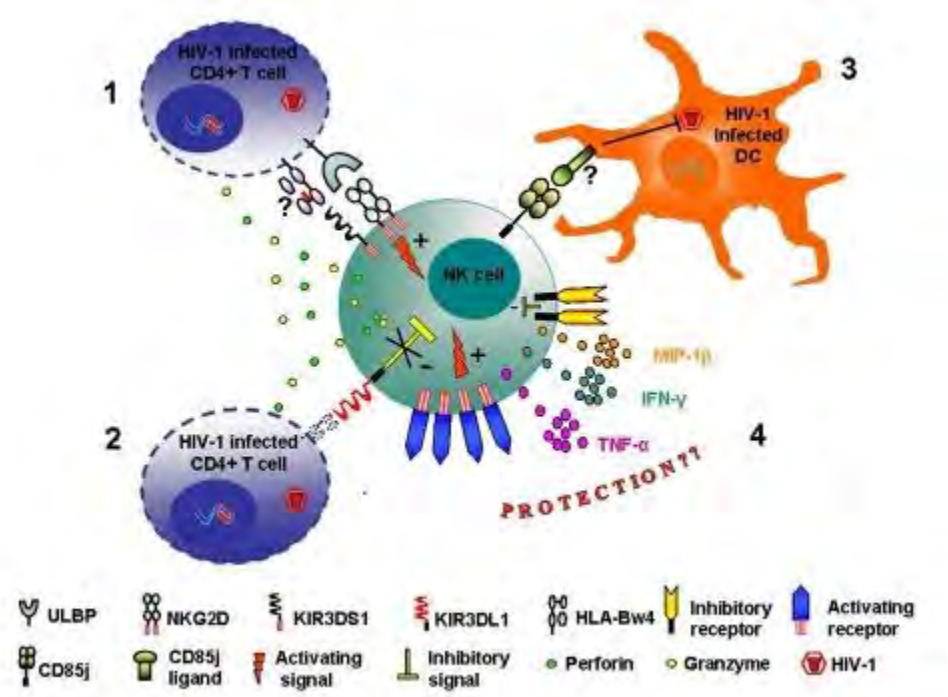


Figure 1.9: NK cell receptor mediated protection in HIV-infection (Le Bouteiller et al., 2011).

Although the role of NK cells in HIV infection is well documented, its function in HIV associated pre-eclampsia has produced conflicting results (Conde-Agudelo et al., 2008). Antiretroviral therapy has been implicated in the rate of pre-eclampsia development (Kalumba, 2012). Other studies reporteded that there is no difference in treated and untreated HIV infection

in pre-eclampsia (Mattar et al., 2004). Kalumba, 2012 showed that HIV positive women were less likely than HIV negative women to develop pre-eclampsia.

1.10 HYPOTHESIS

The population of peripheral NK cells are higher in HIV positive pre-eclamptic than HIV positive normotensive pregnant women.

1.11 NULL HYPOTHESIS

There is no correlation between Th1 and Th2 cytokines with NK cells ($CD3^-$, $CD56^{dim}$ and $CD16^+$) and NK receptor expression (CD69, CD94, NKG2A, NKG2C and NKG2D) across all study groups.

1.12 AIM OF THE STUDY

The aim of the study is to determine the correlation between the phenotypic properties of peripheral Natural Killer (NK) cells ($CD3^-$, $CD56^{dim}$ and $CD16^+$) and their receptors (NKG2A and NKG2C) with Th1 and Th2 cytokine expression in pre-eclamptic (early and late onset) and normotensive pregnant Black South Africans infected and uninfected with HIV.

1.13 SPECIFIC OBJECTIVES

- To quantitate peripheral NK cell ($CD3^-$, $CD56^{dim}$ and $CD16^+$) and their receptor (CD69, CD94, NKG2A, NKG2C, NKG2D) expression in immunocompromised normotensive pregnant *vs* pre-eclamptic (early and late onset) Black South Africans.
- To compare the maternal serum levels of Th1 (IL-2, IL-12(70), IFN-γ, TNF-α, and GM-CSF,) and Th2 (IL-4, IL-5, IL-10, p=0.795 and IL-13) cytokines across all six study groups.

- To correlate the expression of the Th1 (IL-2, IL-12(70), IFN-γ, TNF-α, and GM-CSF,) and Th2 (IL-4, IL-5, IL-10, p=0.795 and IL-13) cytokines with NK cell ($CD3^-$, $CD56^{dim}$ and $CD16^+$) and their receptor (CD69, CD94, NKG2A, NKG2C, NKG2D) expression across all six study groups.

CHAPTER TWO

METHODS

2.1 ETHICAL APPROVAL

This study received postgraduate approval from the School of Laboratory Medicine and Medical Sciences, College of Health Sciences at the University of KwaZulu-Natal (UKZN; Addendum I). Ethical approval was obtained from UKZN Biomedical Research Ethics Committee (BREC reference no: BE176/13; Addendum II). Additionally, Department of Health (Addendum III) and Hospital Manager approval from Prince Mshiyeni Memorial Hospital (Addendum IV) were obtained before recruitment of patients. Study information was given and informed consent (Addendum V) was obtained for participation in the study.

2.2 RECRUITMENT OF PATIENTS

Primigravid and multigravid pre-eclamptic and normotensive pregnant HIV positive and negative Black South Africans were recruited from Prince Mshiyeni Memorial Hospital, Umlazi, Durban. Prince Mshiyeni Memorial Hospital is a district and regional hospital in eThekwini which serves the catchment area and part of the Eastern Cape. Purposeful sampling was used to recruit patients based on the inclusion and exclusion criteria outlined in 2.2.1. The research assistant identified potential study patients. Only those patients that fulfilled the criteria were recruited to the study. These patients were either recruited directly from the labour ward or from the ante natal clinic. Both pre-eclamptic and normotensive groups consisted of HIV positive and negative patients.

2.2.1 Inclusion criteria and study population

Primigravid and multigravid patients with a diagnosis of pre-eclampsia (new onset blood pressure of ≥140/90 mmHg and at least 1+ proteinuria) formed the study group. For analysis, participants were grouped into early or late-onset pre-eclampsia that is ≤ 33 weeks and ≥ 34 weeks of gestation respectively. The control group consisted of healthy, primigravid and multigravid normotensive patients with a blood pressure of ≤120/80 mmHg. Only Black patients' ≥18 years were included in the study. The rationale for inclusion of Black African patients was that a higher percentage of Black African women compared to other ethnic groups attend Prince Mshiyeni Memorial Hospital. Thus, this site selection enabled us to maintain ethnic homogeneity of our study population. Infection with human immunodeficiency virus was based on CD4+ cell count.

The study groups are outlined below:

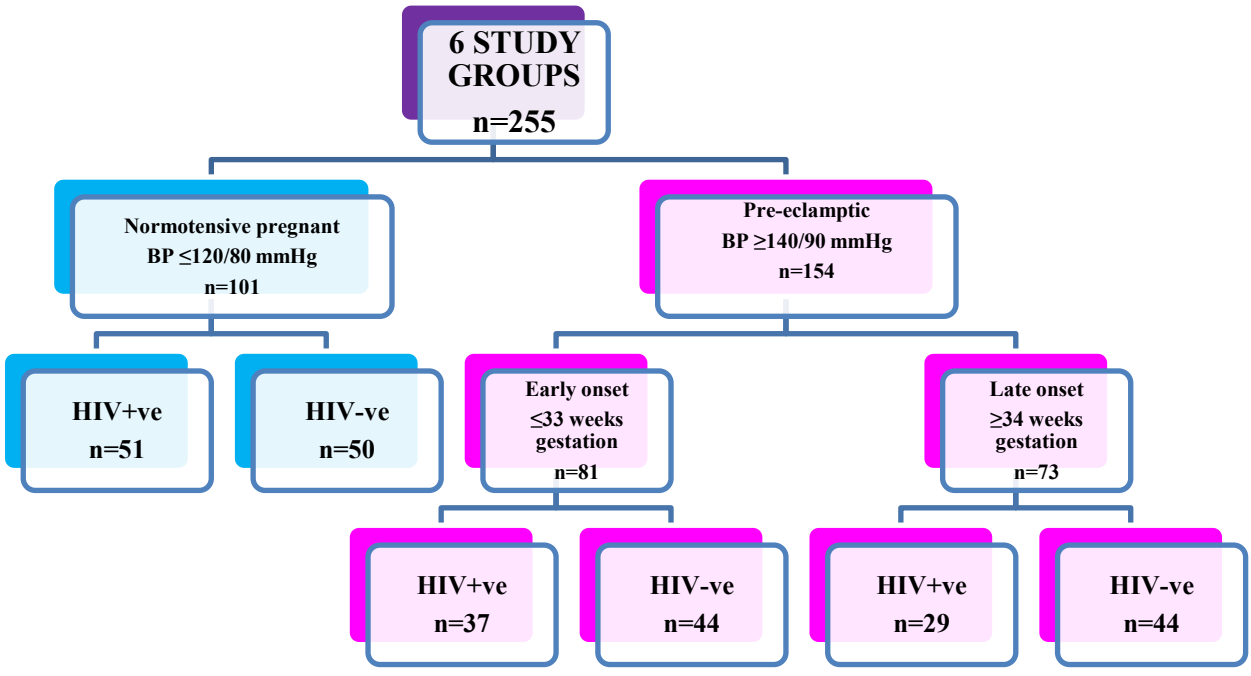


Figure 2.1: Study groups and sample size of total population.

2.2.2 Exclusion criteria

Non-Black African patients, <18 years of age and those who declined entry into the study were excluded. Exclusion criteria for the pre-eclamptic group included polycystic ovarian syndrome patients, chorioamnionitis, chronic hypertension, eclampsia, abruption placentae, intrauterine death, pre-gestational diabetes, gestational diabetes, chronic diabetics, chronic renal disease, systemic lupus erythematous, sickle cell disease, antiphospholipid antibody syndrome, thyroid disease, cardiac disease, active asthma requiring medication during pregnancy, pre-existing seizure disorder, HIV status unknown and unbooked patients.

2.3 SAMPLE COLLECTION

Patient demographics were recorded on the patient data form by the clinical research assistant. The following information was recorded: maternal age, parity, gravidity, gestational age, blood pressure, proteinuria, height, weight, BMI (body mass index), HIV status and CD4+ cell counts, obstetric and neonatal outcomes and maternal and neonatal complications (Addendum VI).

Blood samples were collected by the clinical research assistants. During routine blood tests, an additional 9 ml of blood was drawn aseptically. Four ml of blood was collected in a sterile tripotassium ethylenediamine tetra-acetic acid (K_3 EDTA) vacutainer blood collection tube (Greiner Bio-One, USA) for multicolour flow cytometry analysis and 5 ml in a sterile serum separating tube (SST; Greiner Bio-One, USA) without anticoagulant for multiplex enzyme-linked immunosorbent assay (ELISA) analysis.

2.4 QUANTIFICATION OF PERIPHERAL NATURAL KILLER CELLS USING MULTICOLOUR FLOW CYTOMETRY

2.4.1 Principle of multicolour flow cytometry

The BDLSRFortessa™ flow cytometer (Becton, Dickinson and Company, San Jose, CA 95131 USA) was used for the multicolour flow cytometry analysis. Fluorochromes such as allophycocyanin (APC), fluorescein isothiocyanate (FITC) or phycoerythrin (PE) are covalently conjugated to a monoclonal antibody that binds onto a specific antigen. Fluorochrome stained cells as sample pass through the instrument in a single file. A laser light source passes through and activates the stained cells and the forward and side scattered light (fluorescence) from all cells is detected and emitted at a particular wavelength. The forward scatter determines the relative cell size whilst the side scatter determines the granularity of a cell. The fluorescence emitted from the stained cells is guided through a series of filters and mirrors to a photomultiplier tube, which detects the photons, amplifies the signal and converts it to an electrical signal. This is converted to a digital signal that is analysed by the FACSDiva™ Software (Becton, Dickinson and Company, San Jose, CA 95131 USA; Brown and Whittwer, 2000; Figure 2.2; http://www.rudbeck.uu.se/node47).

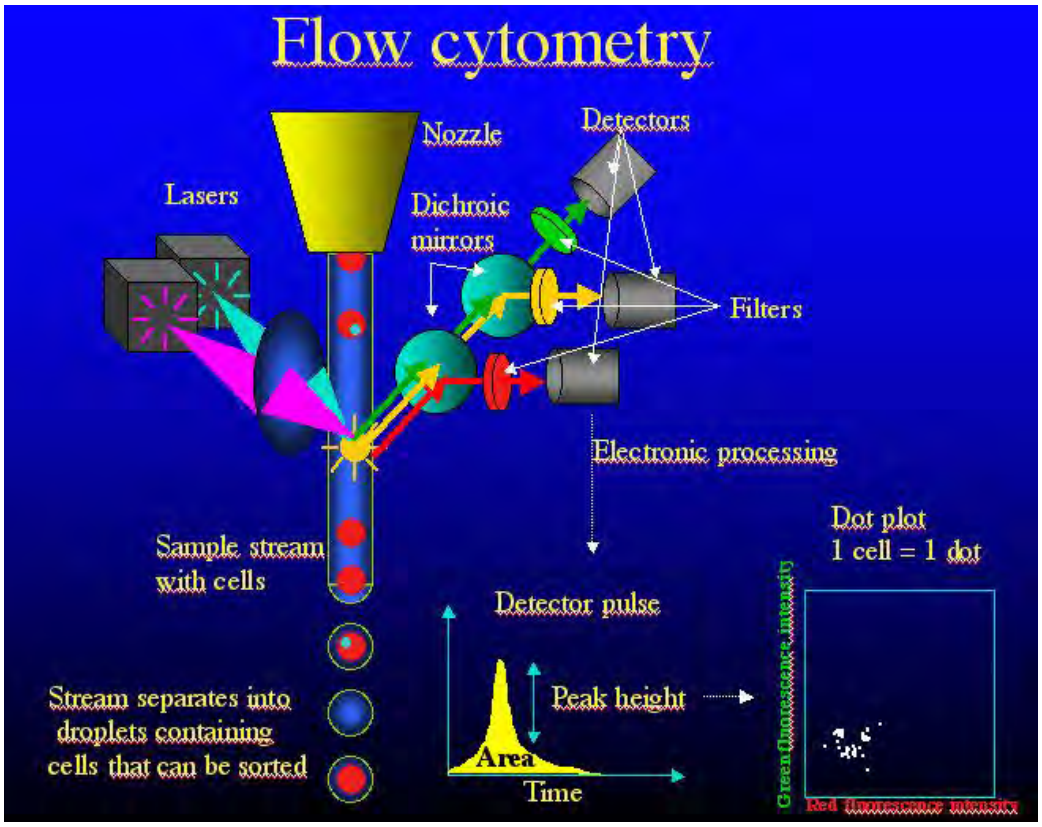


Figure 2.2: Principle of multicolour flow cytometry. The laser excites the fluorochrome conjugated to the antibodies as the cells pass through the instrument in a single file. The emitted light is detected by a series of filters and dichroic filters and detected by photomultiplier tubes as an electronic impulse is captured for data analysis (Brown and Wittwer, 2000); http://www.rudbeck.uu.se/node47).

2.4.2 Estimation of peripheral Natural Killer cells ($CD3^-$, $CD56^{dim}$, $CD16^+$) and its receptors (CD69, CD94, NKG2A, NKG2C and NKG2D)

2.4.2.1 Optimising antibody dilutions

Doubling dilutions were used to determine the optimal antibody concentrations for the determination of peripheral NK cells using multicolour flow cytometry analysis. Five Falcon tubes (5 ml) (Becton, Dickinson and Company, Franklin Lakes, NJ 07417 USA) were labeled

and 20 µl of staining buffer (1x phosphate-buffered saline, LONZA BioWhittaker®, Belgium + 0.1% (w/v) sodium azide, Sigma-Aldrich®, Saint Louis, MO 63103, USA) was added to each tube. To tube one, 10 µl of antibody solution and 10 µl of staining buffer were added, to make a final volume of 40 µl. The tube was vortexed gently using the Vortex-Genie® 2 (Scientific Industries, INC, USA) and 20 µl of solution was transferred from tube 1 to tube 2. Tube 2 was mixed by vortexing and 20 µl of solution was transferred from tube 2 to tube 3. This was repeated for tube 4 and 5 and 20 µl from tube 5 was discarded to maintain a total volume of 40 µl per tube. Staining and lysis of blood samples were as outlined below.

2.4.2.2 Procedure for setting compensation

Compensation is the electronic subtraction of spillover from the different fluorochromes utilized in the multicolour flow cytometry analysis. The BD™ CompBeads (Becton, Dickinson and Company, San Diego, CA 92121, USA) were vortexed thoroughly before use. A Falcon tube for each fluorochrome conjugated antibody (Table 2.1) was labelled. To each tube, 100 µl of staining buffer and one drop of the BD™CompBead Negative control and one drop BD™ CompBeads Anti-Mouse Ig, κ were added. Each antibody was added to the appropriate tubes containing the beads, vortexed and incubated for 30 minutes at room temperature. During incubation the BD™ CompBead Negative control was used to set the flow cytometer instrument photomultiplier tube voltage settings. Following incubation, 2 ml of staining buffer was added to each tube and centrifuged at 200 x g (1092.06 rpm) for 10 minutes (Eppendorf centrifuge 5804R, Hamburg, Germany). The supernatant from each tube was carefully removed with a 3 ml Pasteur pipette (Lasec SA (Pty) Ltd, SA) and each pellet was suspended in 0.5 ml staining buffer and

vortexed thoroughly. Based on the forward and side scatter a singlet bead population was gated from which a negative and positive bead population were determined.

Table 2.1: Fluorochrome conjugated antibodies and dilutions used for multicolour flow cytometry analysis

Antibody	**Fluorochrome***	**Dilution (µl)**
CD3	BD Horizon V500#	3.0
CD16	FITC#	5.0
CD56	Pe-Cy7#	1.5
CD69	Brilliant Violet™ 421#	3.0
CD94	PerCP-Cy5.5#	2.5
NKG2A	Alexa Fluor® 500^	2.5
NKG2C	PE^	5.0
NKG2D	APC#	10.0

*The brighter fluorochromes were conjugated to the smaller expected antibody population and *vice versa*.

Becton, Dickinson and Company, San Jose, CA 95131 USA

^R&D Systems®, Minneapolis, MN 55413, USA

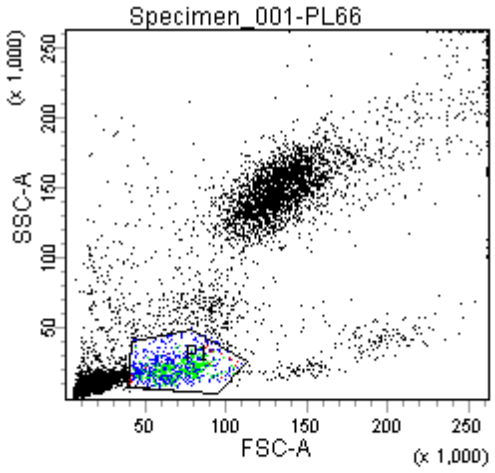
2.4.2.3 Staining and lysis of maternal whole blood

Blood samples were collected in 4 ml K_3 EDTA vacutainer sterile blood collection tubes. Conjugated antibodies were titrated (1.5-10 µl) (Table 2.1) before staining 300 µl of blood sample. The samples were vortexed and incubated for 30 minutes in the dark at room temperature. BDFACS™ lysing solution (2 ml) (Becton, Dickinson and Company, San Jose, CA 95131 USA) was added to the samples, vortexed and incubated for 10 minutes in the dark at

room temperature. Samples were then centrifuged at 500 x g for 5 minutes and the supernatant was removed. Samples were washed with staining buffer, centrifuged at 500 x g for 5 minutes and the supernatant removed for analysis on the BDLSRFortessa™ flow cytometer. Fluoresence minus one (FMO) controls were used to ensure correct interpretation of the data. These controls contain all the fluorochromes to be used in the experiment except one that is measured. This is used to identify the spread of a flurochrome into a channel of interest. Sphero™ Rainbow Fluorescent Particles, 3.0-3.4 μm (mid-range F1 fluoresence) (Becton, Dickinson and Company, San Diego, CA 92121, USA), three drops were used for each multicolour flow cytometry analysis. These beads are single bead population particles dyed with several fluorescence intensities to ensure calibration of the flow cytometer.

2.4.3 Analyses

The data was captured from the BDLSRFortessa™ flow cytometer with the FACSDiva™ software and analysed using FloJo, LLC Data Analysis Software Version X (Oregon, USA). Lymphocytes were identified using forward scatter/side scatter (FSC/SSC) and the $CD3^-$ population was identified (Mosimann *et al.*, 2013). Peripheral NK cells were further classified as $CD16^+$ and $CD56^{dim}$. From this population the CD69, CD94, NKG2A, NKG2C and NKG2D receptors were determined. This data was entered into an Excel spreadsheet and analysed using IBM SPSS Statistics Version 23 (Figure 2.3).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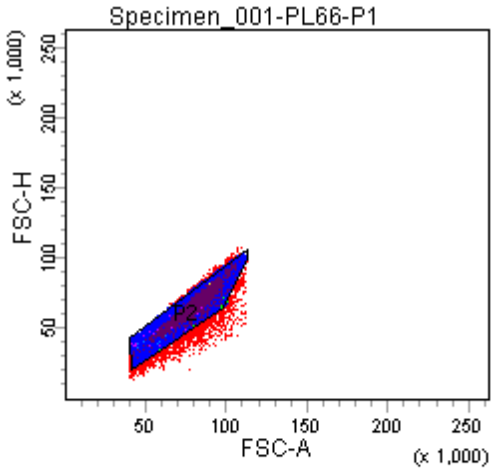
(x 1,000)
FSC-A
(x 1,000)
P1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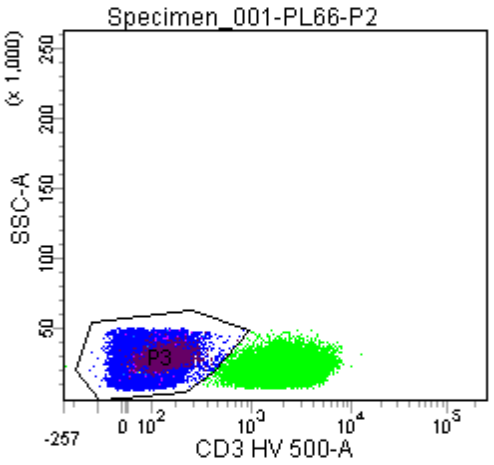
(x 1,000)
FSC-A
(x 1,000)
P2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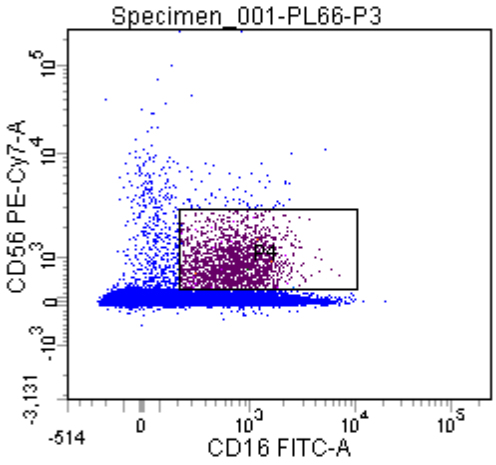
(x 1,000)
CD3 HV 500-A
P3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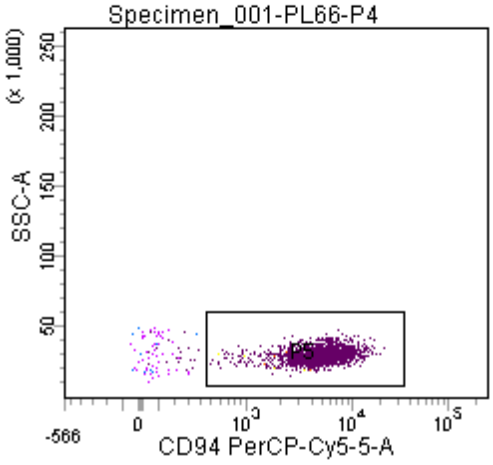
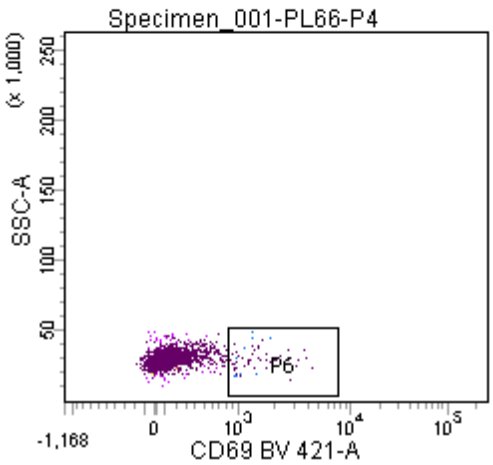
CD16 FITC-A
P4


Specimen_001-PL66-P4
SSC-A
(x 1,000)
CD94 PerCP-Cy5-5-A
P5


Specimen_001-PL66-P4
SSC-A
(x 1,000)
CD69 BV 421-A
P6

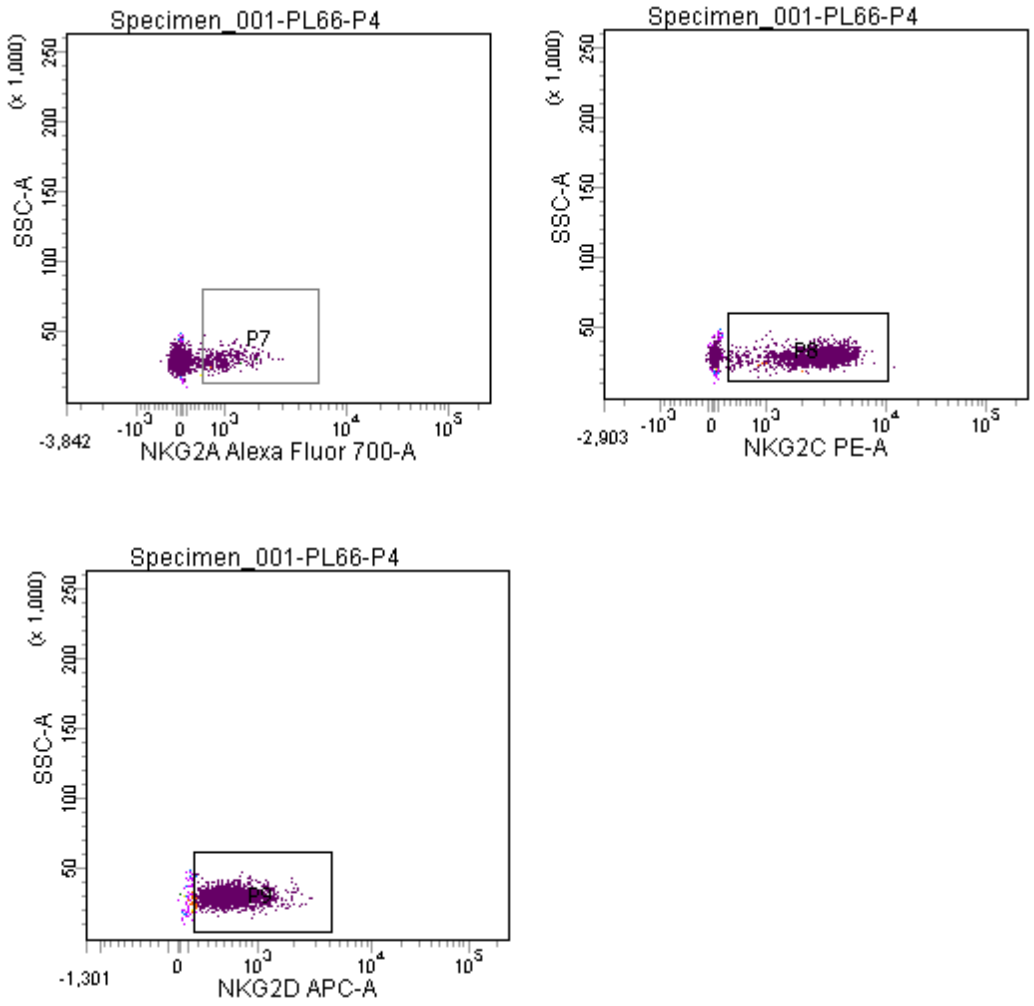


Figure 2.3: Gating strategy used to analyse Natural Killer cells, peripheral Natural Killer cells and its receptors. P1 gate represents lymphocytes, P2 single cells, P3 Natural Killer ($CD3^-$) cells, P4 peripheral Natural Killer ($CD56^{dim}CD16^+$) cells, P4 CD94 inhibiting receptor, P5 CD69 activating receptor, P6 NKG2A inhibiting receptor, P7 NKG2C stimulatory receptor and P8 NKG2D stimulatory receptor.

2.5 QUANTIFICATION OF MATERNAL SERUM TH1 AND TH2 CYTOKINES USING MULTIPLEX ELISA

2.5.1 Principle of multiplex ELISA

The Bio-Plex® suspension array system uses Luminex xMAP technology that allows 100 different analytes which are uniquely bound by 100 different fluorescently dyed beads to be simultaneously detected in one well of a 96 well plate. The system has two lasers and associated optics to detect the molecules bound to the beads and a signal processor to manage the fluorescent output. The principle of multiplex ELISA is similar to a sandwich immunoassay where an antibody covalently coupled to a dyed bead reacts with a sample containing the desired cytokine. A sandwich of antibodies is formed when a biotinylated detection antibody specific for a different epitope is added to the reaction. Streptavidin-phycoerythrin (streptavidin-PE) is added and binds to the biotinylated detection antibodies on the beads surface to increase sensitivity and increase signal output. The data was captured on the Bio-Plex®MAGPIX™ Multiplex Reader (Luminex system; Bio-Rad Laboratories Inc., Hercules, CA 94547, USA) where the lasers and optics detect the fluorescently dyed beads and the signal from the beads represents each target protein in the well. The intensity of the fluorescence indicates the quantity of the target protein in the sample. The fluorescence intensity (data output) is managed by a processor and captured on the Bio-Plex Manager™ software Version 6.1 (Bio-Rad Laboratories Inc., Hercules, CA 94547, USA).

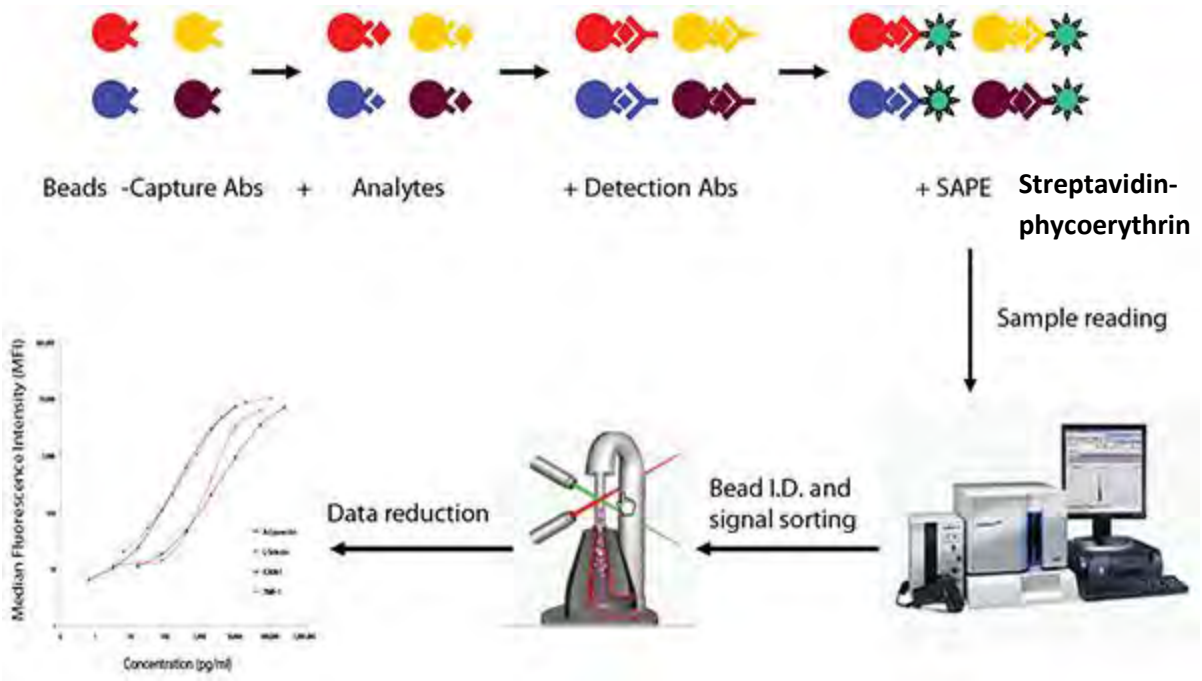


Figure 2.4: Principle of multiplex Luminex assay. Luminex xMap technology uses the same principle as simple ELISAs, however different analytes are attached to multiple fluorescent beads and detection antibodies which allows for the simultaneous quantification of multiple analytes in a single well.

2.5.2 Separation of serum from maternal whole blood

Blood collected in serum separation tubes within 4 hours were centrifuged at 3000 rpm for 10 minutes at 4°C in the Eppendorf centrifuge 5804R. The supernatant containing the serum was transferred to a clean polypropylene tube using a 3 ml Pasteur pipette and stored in 0.5 ml aliquots in 2 ml cryotubes (Lasec SA (Pty) Ltd, SA) at -80 °C.

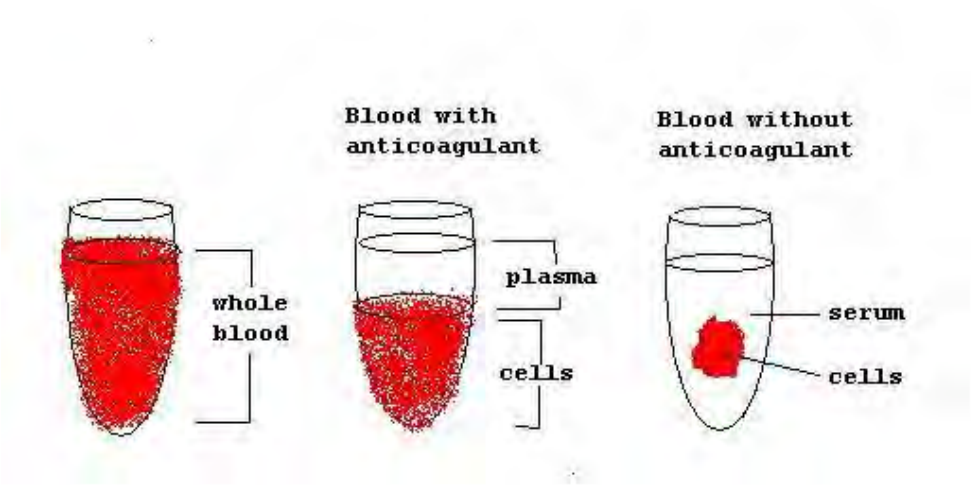
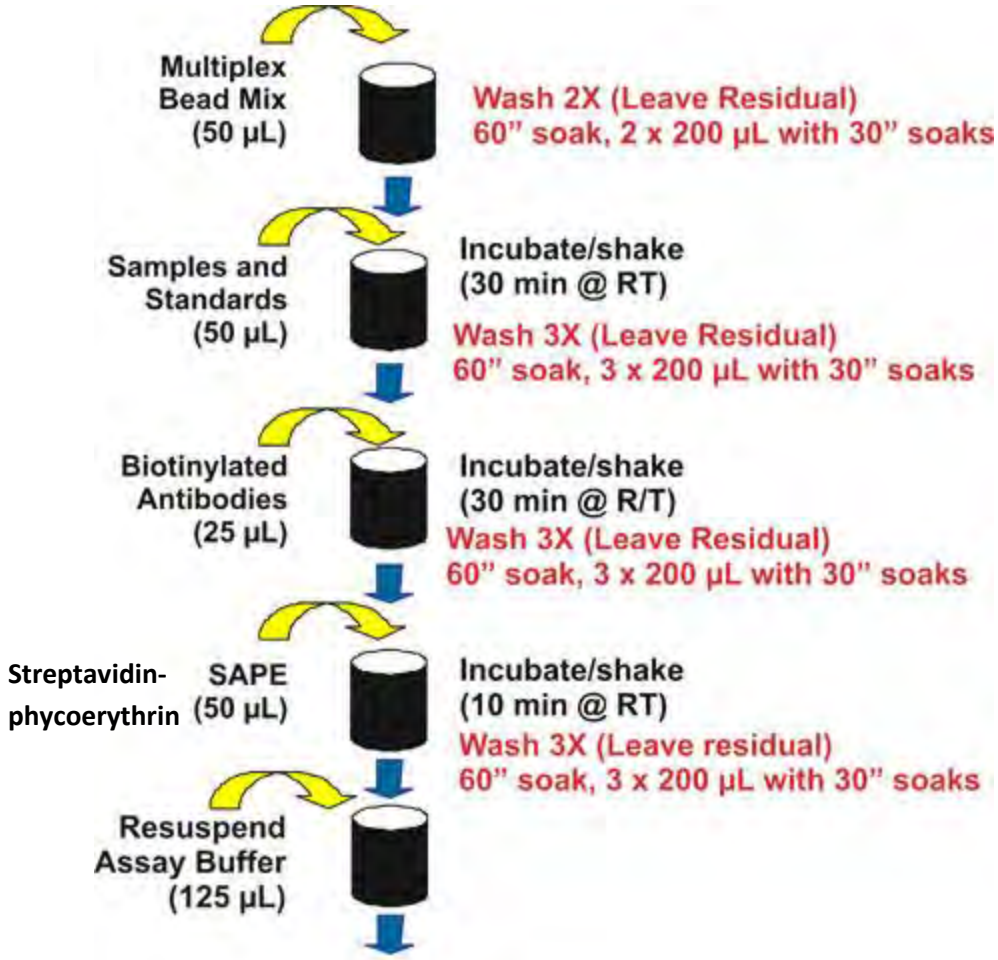


Figure 2.5: Separation of maternal serum from whole blood. Whole blood is allowed to clot and the clot is removed by centrifugation. The resulting supernatant called serum is removed with a Pasteur pipette.

2.5.3 Procedure for the estimation of the Th1 and Th2 cytokines

The Bio-Plex Pro™ Human Cytokine Th1/Th2 Panel, 9-Plex was used (Bio-Rad Laboratries, Inc, USA) to determine the quantity of the Th1 (IL-2, IL-12(70), IFN-γ, TNF-α, and GM-CSF) and Th2 (IL-4, IL-5, IL-10, and IL-13) cytokines. The 1x diluted beads (50 µl) were added to each well of the 96 well plate. The plate was washed twice with 100 µl of Bio-Plex wash buffer and covered with foil to protect from light. The standards, serum samples and a blank (standard diluent), 50 µl each were added to each well. The plate was sealed with sealing tape, covered with foil and incubated on a shaker (Stat Fax™ 2200, 220 V Incubator/Shaker, Califirnia, USA) at 850 rpm for 30 minutes at room temperature. The plate was washed three times with 100 µl of wash buffer per well with the Bio-Rad Bio-Plex Pro™ II Wash station (Bio-Rad Laboratries, Inc, USA). The 10x detection antibodies (25 µl) were added to each well and incubated for 30

minutes at room temperature. The plate was washed with 100 µl of wash buffer three times before adding 50 µl of 1x streptavidin alkaline phosphatase (SAPE) to each well and incubated for 10 minutes at room temperature. The beads were then re-suspended in 125 µl assay buffer and incubated on a shaker at 850 rpm for 2 minutes at room temperature. The plate was read using the Bio-Plex®MAGPIX™ Multiplex Reader.



Bio-Plex®MAGPIX™ Multiplex Reader and

Bio-Plex Manager™ software

Figure 2.6: Procedure for multiplex Luminex method. Diluted beads are added to each well before the standards, serum samples and blanks are plated. Plate is incubated and washed. The detection antibodies are added to each well after which Streptavidin-phycoerythrin (SAPE) is added to each well and re-suspended in assay buffer prior to analysis on the Bio-Plex®MAGPIX™ Multiplex Reader (Held, 2014).

2.5.4 Analyses

A protocol was created on the software package, Bio-Plex Manager^{TM} software version 6.1 which was used to obtain the data from the multiplex ELISA analysis. The known concentration (pg/ml) of each analyte was used to generate a standard curve for each cytokine by plotting the median fluorescent intensity (MFI) signal against concentration (Held, 2014). These standards were used to interpolate the concentrations of the unknown samples. Inter- and intra-plate variability were determined with CV <20% and (Obs/Expected)*100 between 70-130% (r=0.8, p=0.05). The data was imported into an Excel spreadsheet for statistical analysis.

2.6 STATISTICAL ANALYSIS

IBM SPSS Statistics Version 23 was used to analyze the data. Descriptive statistics for continuous data is presented either by mean±standard deviation or median (range). To determine the statistical difference between study groups, the Mann Whitney U and Kruskal-Wallis test were used for non-parametric data and Independent samples t-test and ANOVA for parametric data. For correlation between cytokines and NK cells and receptor expression, Pearson correlation coefficient for parametric data and Spearman rank correlation for non-parametric data was used. Categorical data was presented in frequency tables displaying n (%). Pearson's Chi square test was used to compare the proportions across the study groups.

CHAPTER THREE

RESULTS

3.1 STUDY POPULATION

The total study population comprised of 255 Black South African women of which 101 (39.61%) were normotensive and 154 (60.39%) pre-eclamptic. The latter group was sub-stratified into early and late onset pre-eclampsia (EOPE and LOPE) comprising 81 (52.60%) and 73 (47.40%) women respectively. The normotensive and pre-eclamptic groups were further sub-stratified according to HIV status, ie., HIV positive and HIV negative groups (Table 3.1).

Table 3.1 Sample size (percentage) of the six study groups

Study group	Sample size (N)	Percentage of the total study population (%)
Normotensive HIV negative	50	19.61
Normotensive HIV positive	51	20.00
EOPE HIV negative	44	17.25
EOPE HIV positive	37	14.51
LOPE HIV negative	44	17.25
LOPE HIV positive	29	11.37

3.2 CLINICAL DATA AND DEMOGRAPHICS

The clinical data and demographics of all six study groups are shown in Table 3.2.

3.2.1 Maternal age

The mean maternal age±SD of the total study population was 26.74±6.62 y (Table 3.2). There was a significant difference in maternal age between HIV positive and negative groups [U=6418.50, p=0.006; Mann-Whitney U test]. Maternal age in the HIV positive group was higher (mean rank=141.17) than the HIV negative group (mean rank=116.01). There was no significant difference in maternal age between the normotensive and pre-eclamptic groups [U=6959.5, p=0.180; Mann-Whitney U test]. There was a significant difference in maternal age between the primigravid and multigravid groups [U=2051.00, p<0.001; Mann-Whitney U test]. Maternal age in the multigravid group was higher (mean rank=157.44) than the primigravid group (mean rank=66.92).

The distribution of maternal age was the same between the normotensive pregnant *vs* the EOPE *vs* the LOPE groups [$\chi^2(2)$=2.415, p=0.299; Kruskal-Wallis test]. There was no significant difference in maternal age across all six study groups [$\chi^2(5)$=10.698, p=0.058; Kruskal-Wallis test].

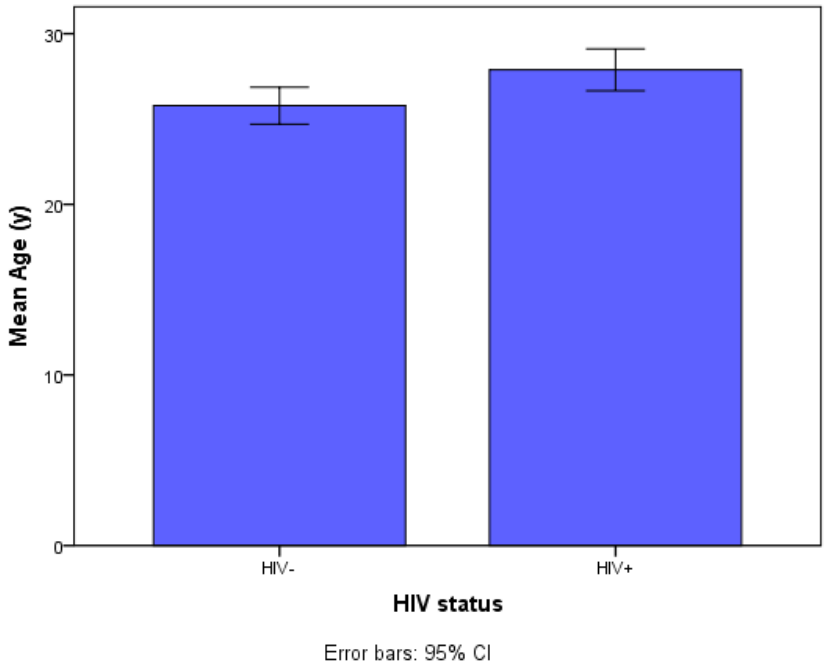


Figure 3.1: Maternal age (y) between the HIV positive (HIV+) and negative (HIV-) groups

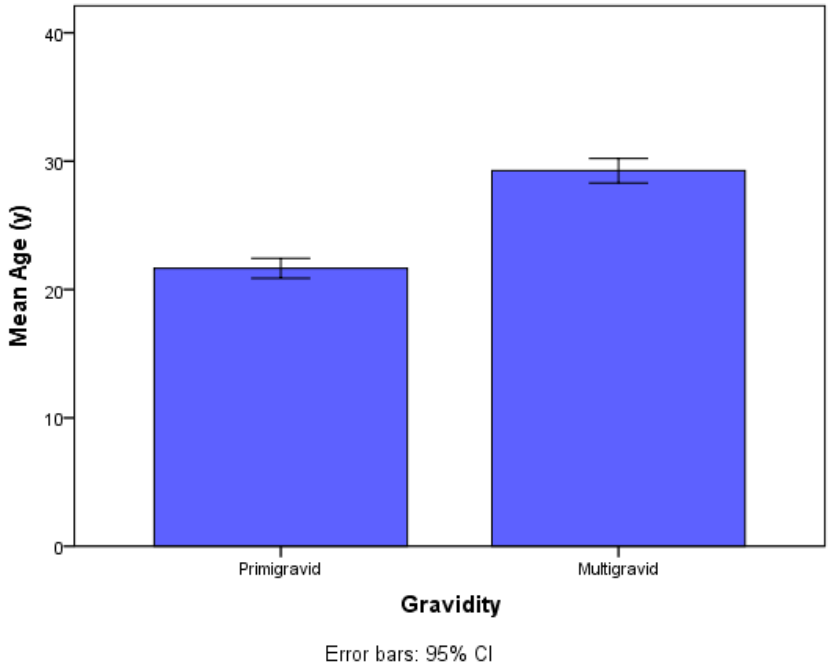


Figure 3.2: Maternal age (y) between the primigravid and multigravid groups

3.2.2 Maternal weight

The mean maternal weight±SD of the total study population was 75.95±17.61 kg (Table 3.2). There was no significant difference of maternal weight between the HIV positive and negative groups [U=7842.00, p=0.781; Mann-Whitney U test]. There was no significant difference in maternal weight between the normotensive and pre-eclamptic groups [U=6995.50p=0.202; Mann-Whitney U test]. There was a significant difference in maternal weight between the primigravid and multigravid groups [U=5025.50, p<0.001; Mann-Whitney U test]. Maternal weight in the multigravid group was higher (mean rank=140.26) than the primigravid group (mean rank=102.12).

The distribution of maternal weight was the same between the normotensive pregnant *vs* the EOPE *vs* the LOPE groups [$\chi^2(2)=2.058$, p=0.357; Kruskal-Wallis test]. There was no significant difference in maternal weight across all six study groups [$\chi^2(5)=3.166$, p=0.674; Kruskal-Wallis test].

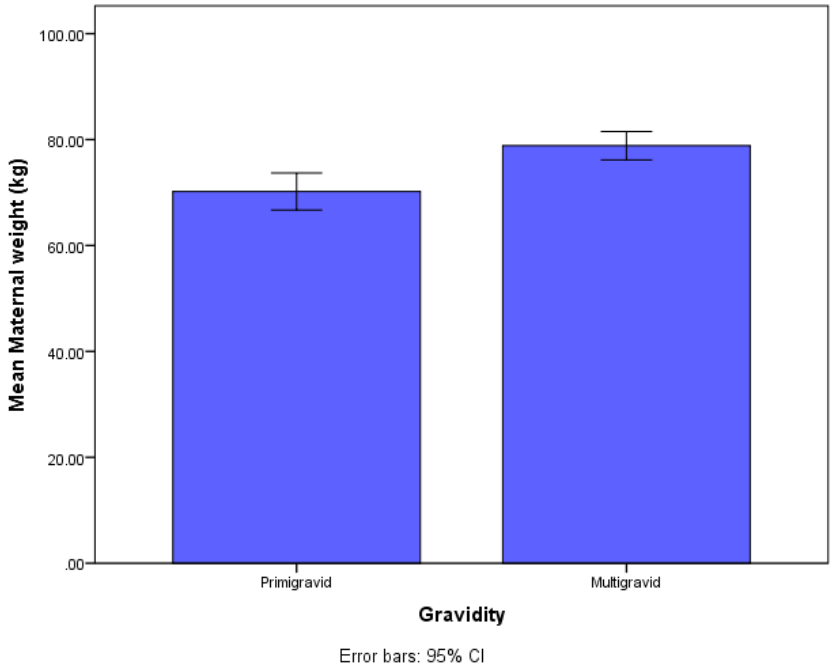


Figure 3.3: Maternal weight (kg) between the primigravid and multigravid groups

3.2.3 Maternal height

The mean maternal height±SD of the total study population was 157.65±6.49 cm (Table 3.2). There was no statistical significance of maternal height between the HIV positive and negative groups [$t(212)=2.570$, $p=0.365$; Independent samples t-test]. There was no statistical significance of maternal height between the normotensive and pre-eclamptic groups [$t(212)=-1.428$, $p=0.702$; Independent samples t-test], the normotensive pregnant *vs* the EOPE *vs* the LOPE groups [$F(2,252)=365.69$, $p=0.155$; ANOVA] and among all six study groups [$F(5,208)=1.252$, $p=0.286$; ANOVA].

3.2.4 BMI

The mean BMI±SD of the total study population was 30.80± 6.67 kg/m^2 (Table 3.2). There was no significant difference of BMI between the HIV positive and negative groups [U=5449.00,

p=0.709; Mann-Whitney U test]. There was no significant difference in BMI between the normotensive and pre-eclamptic groups [U=5084.50, *p*=0.311; Mann-Whitney U test]. There was a significant difference in BMI between the primigravid and multigravid groups [U=3511.0, *p*<0.001; Mann-Whitney U test]. BMI in the multigravid group was higher (mean rank=118.10) than the primigravid group (mean rank=85.26).

The distribution of BMI was the same between the normotensive pregnant *vs* the EOPE *vs* the LOPE groups [$\chi^2(2)=2.647$, p=0.266; Kruskal-Wallis test]. There was no significant difference in BMI across all six study groups [$\chi^2(5)=4.486$, p=0.482; Kruskal-Wallis test].

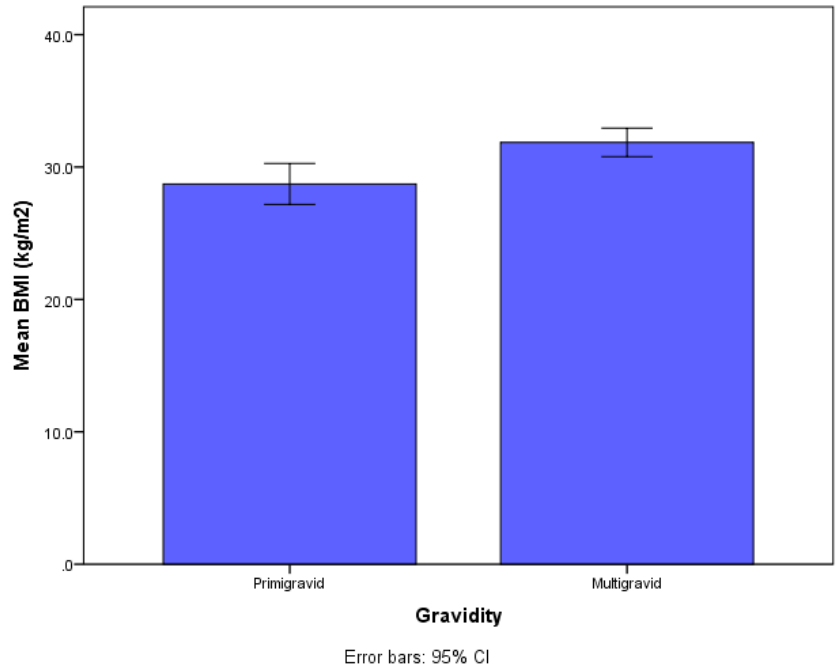


Figure 3.4: BMI (kg/m^2) between the primigravid and multigravid groups

3.2.5 Parity

The mean parity±SD of the total study population was 1.02±1.09 (Table 3.2). There was a significant difference in parity between HIV positive and negative groups [U=6816.00, p=0.023; Mann-Whitney U test]. Parity in the HIV positive group was higher (mean rank=138.74) than the HIV negative group (mean rank=118.89). There was no significant difference in parity between the normotensive and pre-eclamptic groups [U= 7700.00, p=0.888; Mann-Whitney U test] regardless of HIV status. There was a significant difference in parity between the primigravid and multigravid groups [U=425.00, p<0.001; Mann-Whitney U test]. Parity in the multigravid group was higher (mean rank=168.00) than the primigravid group (mean rank=48.00).

The distribution of parity was the same between the normotensive pregnant *vs* the EOPE *vs* the LOPE groups [$\chi^2(2)$=0.527, p=0.768; Kruskal-Wallis test]. There was no significant difference in parity across all six study groups [$\chi^2(5)$=5.966, p=0.310; Kruskal-Wallis test].

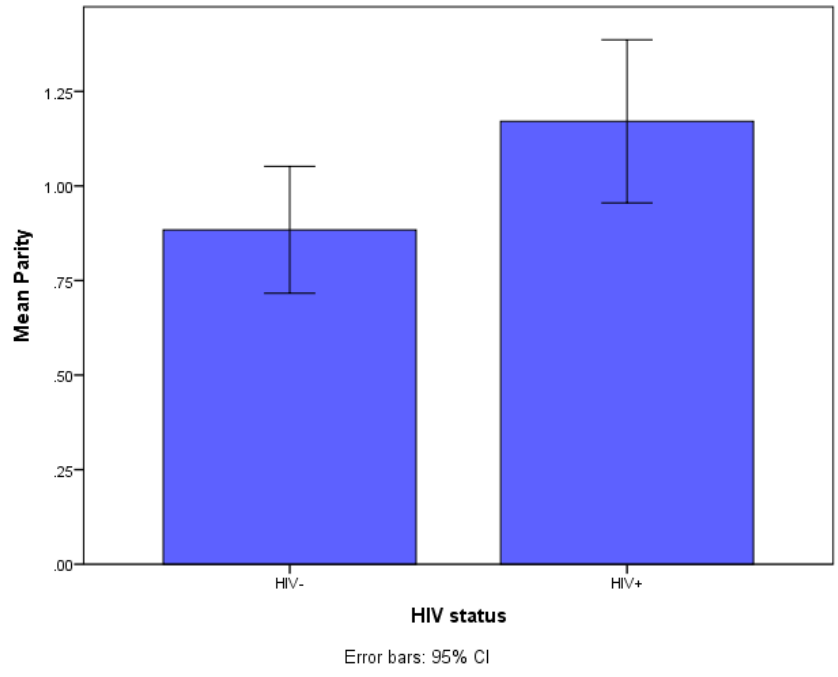


Figure 3.5: Parity between HIV positive and negative groups

3.2.6 Gravidity

The mean gravidity±SD of the total study population was 2.24±1.43 (Table 3.2). There was a significant difference in gravidity between HIV positive and negative groups [U=6841.50, p=0.028; Mann-Whitney U test]. Gravidity in the HIV positive group was higher (mean rank=138.53) than the HIV negative group (mean rank=119.08). There was no significant difference in gravidity between the normotensive and pre-eclamptic groups [U=7684.50, p=0.867; Mann-Whitney U test]. The distribution of gravidity was the same between the normotensive pregnant *vs* the EOPE *vs* the LOPE groups [$\chi^2(2)$=0.168, p=0.919; Kruskal-Wallis test]. There was no significant difference in gravidity across all six study groups [$\chi^2(5)$=4.970, p=0.420; Kruskal-Wallis test].

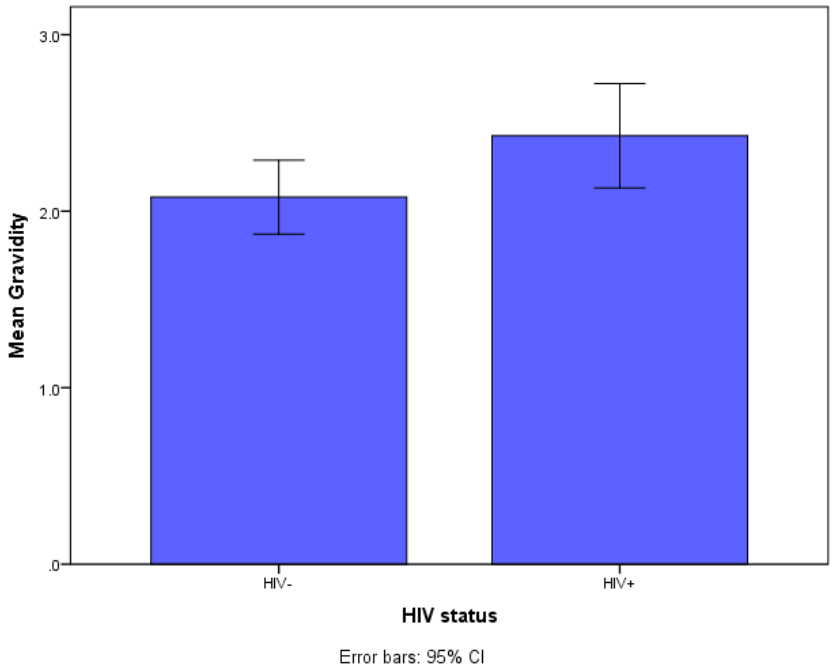


Figure 3.6: Gravidity between HIV positive (HIV+) and negative (HIV-) groups

3.2.7 Gestational age

The mean gestational age±SD of the total study population was 34.51±4.84 weeks (Table 3.2). There was no significant difference of gestational age between the HIV positive and negative groups [U=7871.00, p=0.976; Mann-Whitney U test]. There was a significant difference in gestational age between the normotensive and pre-eclamptic groups [U=2256.50, p<0.001; Mann-Whitney U test]. Gestational age in the normotensive group was higher (mean rank=180.21) than the pre-eclamptic group (mean rank=91.75).

The distribution of gestational age was significantly different between the normotensive pregnant *vs* the EOPE *vs* the LOPE groups [$\chi^2(2)$=169.86, p<0.001; Kruskal-Wallis test]. The gestational age was higher in the normotensive group (mean rank=180.21) followed by the LOPE group (mean rank=147.44) and the EOPE group (mean rank=42.24). There was a significant difference in gestational age across all six study groups [$\chi^2(5)$=170.146, p<0.001; Kruskal-Wallis test]. Gestational age was higher in the normotensive HIV negative group (mean rank=181.77) followed by the normotensive HIV positive group (mean rank=178.74), LOPE HIV negative (mean rank=150.83), LOPE HIV positive group (mean rank=142.13), EOPE group HIV negative (mean rank=42.26) and EOPE (mean rank=42.22).

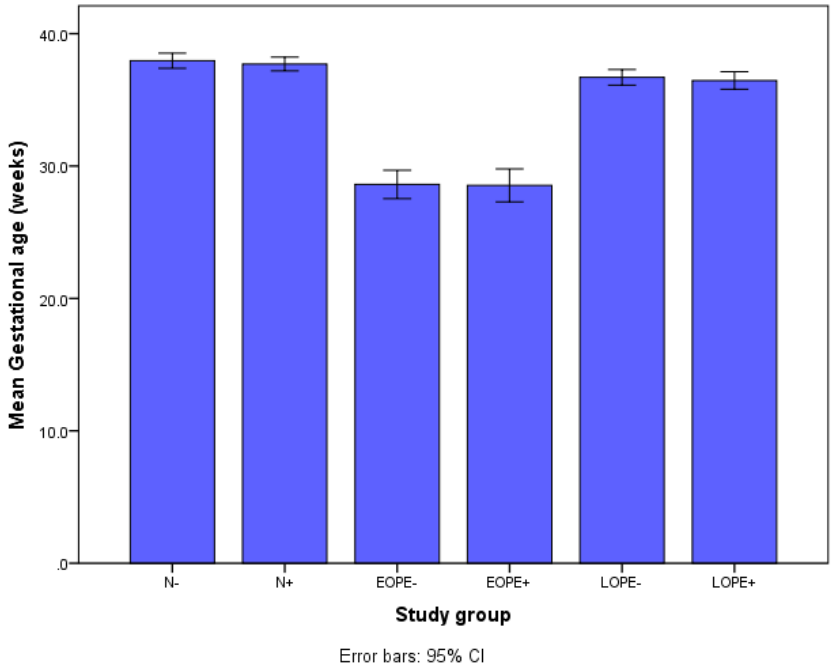


Figure 3.7: Gestational age (weeks) across six study groups

3.2.8 Systolic and diastolic pressure

The mean systolic and diastolic pressure±SD of the total study population was 138.55±26.85 and 89.04±17.53 mmHg respectively (Table 3.2). There was no statistical significance in systolic pressure between the HIV positive and negative groups [$t(255)=0.881$, $p=0.379$; Independent samples t-test] and in diastolic pressure between the HIV positive and negative groups [$t(255)=1.087$, $p=0.278$; Independent samples t-test].

However, there was a statistical significance in systolic pressure between the normotensive (110.07±10.06 mmHg) and pre-eclamptic groups [157.23±15.63 mmHg; $t(255)=-26.88$, $p<0.001$; Independent samples t-test] and the normotensive pregnant (110.07±10.06 mmHg) *vs* the EOPE (159.06±14.23 mmHg) *vs* the LOPE groups [155.19±16.93 mmHg; $F(2,252)=365.69$, $p<0.001$; ANOVA] and between all six study groups [$F(5,249)=146.51$, $p<0.001$; ANOVA] and

in diastolic pressure between the normotensive (69.97±7.79 mmHg) and pre-eclamptic groups [101.55±8.56 mmHg; $t(255)$, $p<0.001$; Independent samples t-test] and the normotensive (69.97±7.79 mmHg) *vs* the EOPE (102.7±8.62 mmHg) *vs* the LOPE groups [100.2±8.36 mmHg; $F(2,252)=451.17$, $p<0.001$; ANOVA] and across all six study groups [$F(5,249)=179.51$, $p<0.001$; ANOVA].

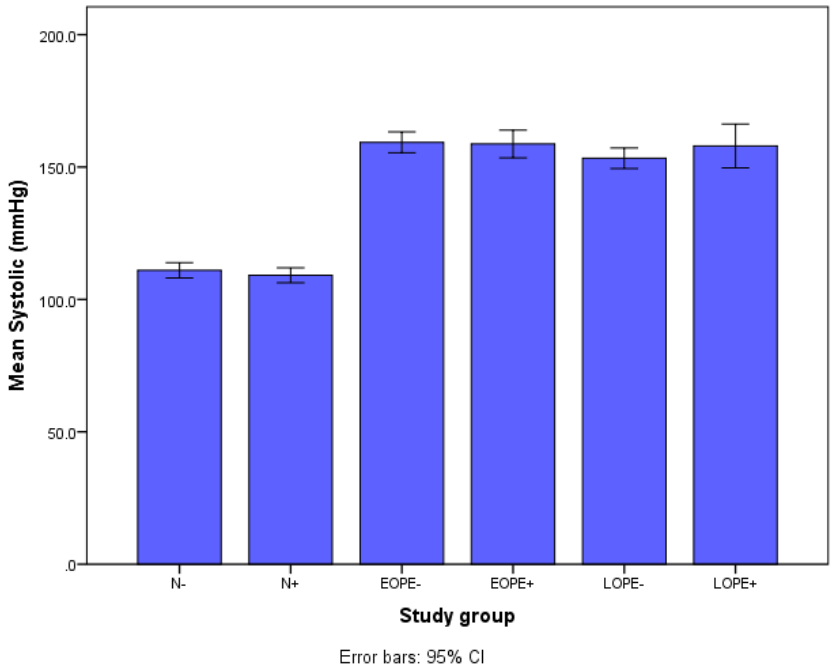


Figure 3.8: Systolic pressure (mmHg) across six study groups

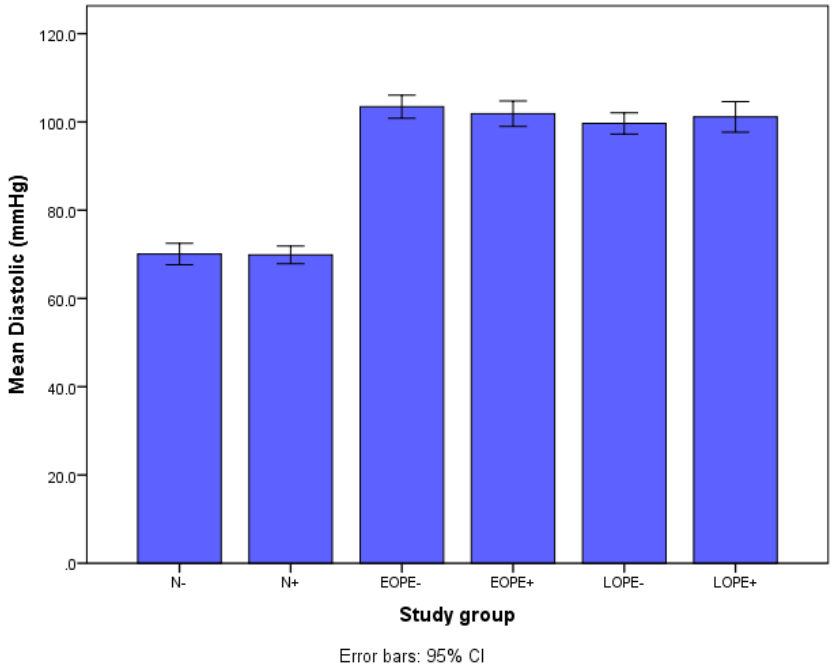


Figure 3.9: Diastolic pressure (mmHg) across six study groups

3.2.9 Proteinuria

There was no statistical significance in the level of urinary protein concentration between the HIV positive and negative groups [$\chi^2(3, N=163)=2.55$, $p=0.466$]. However, there was a statistical significance in proteinuria between the normotensive and pre-eclamptic groups [$\chi^2(3, N=163)=11.63$, $p=0.009$] regardless of HIV status, the normotensive *vs* the EOPE *vs* LOPE groups [$\chi^2(6, N=163)=14.73$, $p=0.022$] and between all six study groups, [$\chi^2(15, N=163)=27.65$, $p=0.024$]. Of the total population 8.6% of normotensive women had a dipstick reading between 1(71.4%), 2(14.3%), 3(0.0%) and 4(14.3%) whilst 47.9% of the EOPE group had a dipstick reading between 1(52.6%), 2(30.8%), 3(15.4%) and 4(1.3%) and LOPE group 1(66.2%), 2(22.5%), 3(9.9%) and 4(1.4%).

3.2.10 CD4+ cell count

The mean CD4+ cell count±SD of the total study population was 470.75±231.67 cells/mm^3 (Table 3.2). There was no significant difference in CD4+ cell count between the normotensive and pre-eclamptic groups [U=551.00, p=0.782; Mann-Whitney U test]. The distribution of CD4+ cell count was the same between the normotensive pregnant *vs* the EOPE *vs* the LOPE groups [$\chi^2(2)$=0.313, p=0.855; Kruskal-Wallis test]. There was no significant difference in CD4+ cell count across all six study groups [$\chi^2(5)$=0.313, p=0.855; Kruskal-Wallis test]. There was no significant difference in CD4+ cell count between the primigravid and multigravid groups [U=409.50, p=0.444; Mann-Whitney U test].

3.2.11 Residence (Urban or rural)

There was no significant difference in HIV status of women living either in a rural or urban area [$\chi^2(1, N=253)$=3.11, p=0.078].

There was a significant difference in a CD4+ cell count <350 and a CD4+ cell count >350 of women living in either a rural or urban area, $\chi^2(1, N=119)$=6.98, p=0.008. Of the total population, 32.8% of women had a CD4+ cell count <350 and 67.2% had a CD4+ cell count >350. A total of 45.5% of women with a CD4+ cell count <350 live in a rural area whilst 54.5% live in an urban area. A total of 15.6% of women with a CD4+ cell count >350 live in a rural area and 84.4% live in an urban area.

There was no significant difference between the normotensive and pre-eclamptic groups of women living either in a rural or urban area [$\chi^2(2, N=253)=0.352$, $p=0.839$]. There is no significant difference amongst all six study groups [$\chi^2(5, N=253)=4.836$, $p=0.436$].

Table 3.2 Clinical data and demographics across the six study groups

	Normotensive HIV negative	Normotensive HIV positive	Early onset pre-eclamptic HIV negative	Early onset pre-eclamptic HIV positive	Late onset pre-eclamptic HIV negative	Late onset pre-eclamptic HIV positive	*p* value
n	50	51	44	37	44	29	255
Maternal age (years)	23(18-37)	26(18-40)	26(18-43)	26(19-40)	23(18-40)	29(18-44)	0.058
Parity	1(0-4)	1(0-3)	1(0-4)	1(0-8)	1(0-4)	1(0-4)	0.310
Gravidity	2(1-5)	2(1-7)	2(1-7)	2(1-14)	2(1-7)	2(1-5)	0.420
Gestational age (weeks)	38(31-41)	38(30-41)	30(21-38)	30(20-38)	37(30-40)	37(34-40)	<0.001*
Maternal weight (kg)	72.9(49-143)	70.5(41-111)	78(54.5-124)	73.2(51-125)	72(46-121)	73(54-122)	0.674
Systolic pressure (mmHg)	111±10.07	109.16±10.06	159.34±13.05	158.73±15.69	153.34±12.74	158.00±21.77	<0.001*
Diastolic pressure (mmHg)	70.06±8.50	69.88±7.10	103.43±8.63	101.87±8.66	99.66±7.90	101.14±9.08	<0.001*
Maternal height (cm)	157.92±4.90	155.99±7.62	159.14±7.23	158.60±6.64	156.98±5.89	158.09±5.82	0.286
BMI (kg/m^2)	29.8(18.7-53.2)	29.2(18.2-50.1)	29.9(20.4-43.9)	29.2(20.1-45.9)	31.6(21.2-40.7)	32.0(21.4-44.8)	0.482
CD4+ cell counts	-	400(<100-950)	-	358(82-1128)	-	558(182-726)	0.855

Systolic and diastolic pressure and maternal height are expressed as mean±SD and data analysis performed by ANOVA followed by Tukey post-test.

Maternal age, parity, gravidity, gestational age, maternal weight and CD4+ cell counts are expressed as median (range) and data analysis performed by Kruskal-Wallis.

Statistical significance at $p<0.05$ are highlighted by *.

3.3 NEONATAL DATA

3.3.1 Method of delivery

Method of delivery was classified as normal vaginal, normal induced, elective caesarean section and emergency caesarean section. There was no significant difference in the method of delivery between the HIV positive and negative groups [$\chi^2(3, N=203)=01.785$, $p=0.618$], the normotensive and pre-eclamptic groups [$\chi^2(3, N=203)=5.139$, $p=0.162$], normotensive pregnant *vs* the EOPE *vs* the LOPE groups [$\chi^2(6, N=203)=7.142$, $p=0.308$] and all six study groups [$\chi^2(15, N=203)=13.515$, $p=0.563$].

3.3.2 Baby outcome (live or stillbirths)

There was no statistical significance in baby outcome (live or stillborn) between the HIV positive and negative groups [$\chi^2(1, N=210)=0.034$, $p=0.853$] and between primigravid and multigravid women [$\chi^2(1, N=210)=2.852$, $p=0.091$]. However, there was a significant difference between the normotensive and pre-eclamptic groups [$\chi^2(1, N=210)=7.183$, $p=0.007$] and between normotensive pregnant *vs* the EOPE *vs* the LOPE groups [$\chi^2(2, N=210)=25.909$, $p<0.001$]. Of the total population 32.4% were normotensive women and 67.6% were pre-eclamptic (36.6% were EOPE and 31.0% were LOPE). There were 100% live births (100%) in the normotensive group whilst in the pre-eclamptic group there were only 90.1% live births. A total of 100.0% live births were reporteded in the LOPE group, however only 81.8% live births in the EOPE group. There also was a relationship between live and stillborn babies across all six study groups [$\chi^2(5, N=210)=26.020$, $p<0.001$].

3.3.3 Baby weight

There was no statistical significance in baby weight between the HIV positive and negative groups [$t(210)=-0.659$, $p=0.511$; Independent samples t-test]. However, baby weight was significantly higher in the normotensive group (3.32±0.41 kg) compared to the pre-eclamptic group [2.35±0.92 kg, $t(210)=8.28$; $p<0.001$; Independent samples t-test]. There was a statistically significant difference between groups as determined by one-way ANOVA [$F(2,207=113.36$, $p<0.001$] with a higher birth weight in the normotensive group (3.32±0.41kg) compared to the LOPE group (2.99±0.62kg) and the EOPE group (1.81±0.79kg, $p<0.001$). There was a significant difference in baby weight between all six study groups [$F(5,204=45.81$, $p<0.001$; ANOVA].

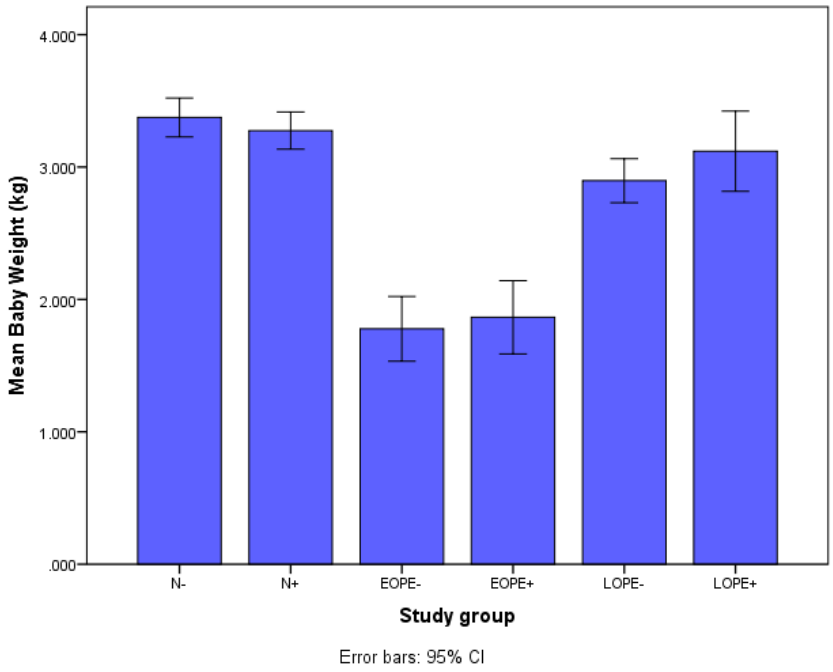


Figure 3.10: Baby weight (kg) in all six study groups

3.3.4 Admission to Neonatal Intensive Care Unit

There was no significant difference in admission to Neonatal Intensive Care Unit (NICU) between the HIV positive and negative groups [$\chi^2(1, N=192)=1.073$, $p=0.300$]. However, there was a significant difference in admission of neonates to the NICU between the normotensive and pre-eclamptic groups [$\chi^2(1, N=192)=25.254$, $p<0.001$]. Only 8.6% of neonates were admitted to NICU in the normotensive group compared to 91.4% in the pre-eclamptic group.

There was a significant difference in admission of neonates to the NICU between the normotensive pregnant *vs* the EOPE group *vs* the LOPE groups [$\chi^2(2, N=192)=66.94$, $p<0.001$]. A total of 74.6% of neonates where admitted to NICU from the EOPE group compared to 19.0% in the LOPE group and 8.6% in the normotensive group. There was a significant difference in admission of neonates to the NICU between the normotensive HIV negative (5.2%) *vs* normotensive HIV positive (3.4%) *vs* EOPE HIV negative (41.4%) *vs* EOPE HIV positive (31.0%) *vs* LOPE HIV negative (13.8%) and LOPE HIV positive groups[5.2%; $\chi^2(1, N=192)=25.254$, $p<0.001$].

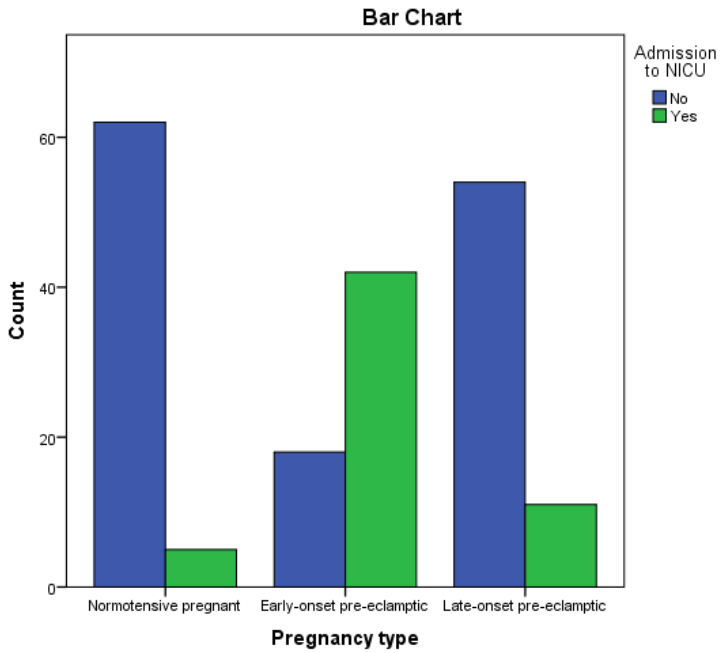


Figure 3.11: No. of babies admitted to NICU across pregnancy types

3.3.5 Gender of the baby

A chi-square test was performed and no relationship was found between the gender of the baby (male or female) and HIV status of the mother [$\chi^2(1, N=206)=0.051$ $p=0.821$] and also between the normotensive *vs* pre-eclamptic group [$\chi^2(1, N=206)=3.49$ $p=0.062$]. This study, however found a relationship between the gender of the baby and whether the women were primigravid or multigravida [$\chi^2(1, N=206)=4.79$ $p=0.029$] and normotensive or EOPE or LOPE groups [$\chi^2(2, N=206)=7.12$ $p=0.028$]. Of the total population 35.9% were primigravid whilst 64.1% were multigravid. A total of 43.2% of primigravid women had female babies whilst 56.8% had male babies. A total of 59.1% of multigravid women had female babies and 40.9% had male babies. Of the total population 32.0% were normotensive women, 36.4% were EOPE and 31.6% were LOPE. A total of 43.9% normotensive women had female babies whilst 56.1% had male babies, 65.3% of the EOPE group had female babies whilst 34.7% had male babies and 49.2% of the

LOPE group had females babies and 50.8% males babies. There was no difference in the gender of the baby across the six study groups [$\chi^2(5, N=206)=8.69, p=0.122$].

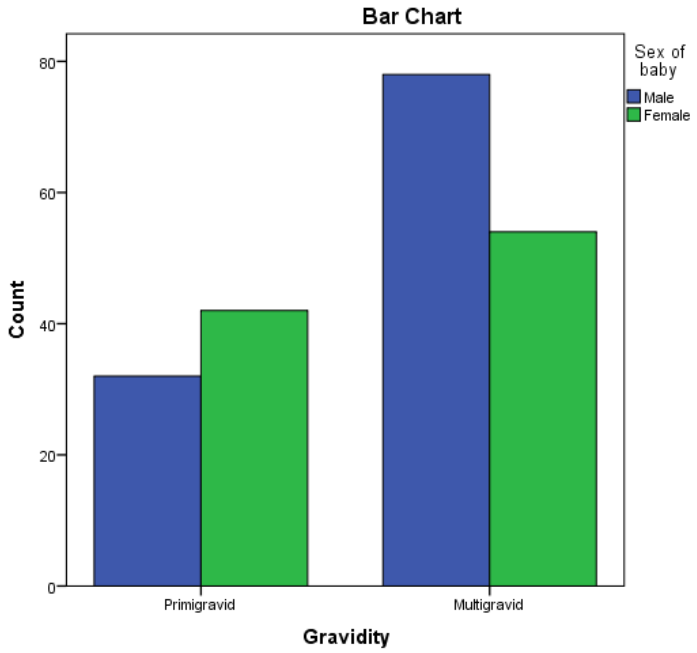


Figure 3.12: Gender of babies (male or female) between the primigravid and multigravid groups

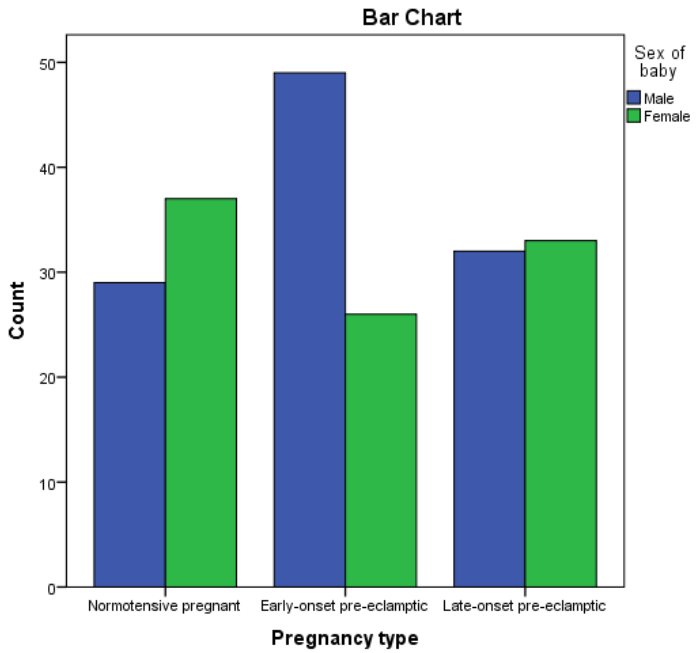


Figure 3.13: Gender of babies (male or female) across pregnancy groups

3.4 LABORATORY DATA

The laboratory data for all six study groups are shown in Table 3.3.

3.4.1 Haemoglobin

The mean haemoglobin±SD of the total study population was 10.71±1.40 g/dL. There was no statistical significance in haemoglobin level between the HIV positive and negative groups [t(183)=0.579, p=0.563; Independent samples t-test] and between the normotensive and pre-eclamptic groups [t(183)=-0.280, p=0.780; Independent samples t-test]. Also, there was no significant difference in haemoglobin between the normotensive pregnant *vs* the EOPE *vs* the LOPE groups [F(2,182=0.992, p=0.373; ANOVA] and across all six study groups [F(5,179=0.681, p=0.638; ANOVA].

3.4.2 Platelets

The mean platelet±SD of the total study population was 210.27±62.50 10^9/L. There was no significant difference in platelets between HIV positive and negative groups [t(161)=-1.010, *p*=0.314; Independent samples t-test] and between the normotensive and pre-eclamptic groups [t(161)=1.866, *p*=0.064; Independent samples t-test]. Also, there was no significant difference in platelets between the normotensive pregnant *vs* the EOPE *vs* the LOPE groups [*F*(2,160=1.731, *p*=0.180; ANOVA) and across all six study groups [*F*(5,157=0.850, *p*=0.516; ANOVA].

3.4.3 Urea

The mean urea±SD of the total study population was 2.61±1.37 mmol/L. There was no statistical significance in urea concentration between the HIV positive and negative groups [U=1486.00, *p*=0.319; Mann-Whitney U test] and between the normotensive and pre-eclamptic groups [U=106.00, *p*=0.072; Mann-Whitney U test].

The distribution of urea was significantly different between the normotensive pregnant *vs* the EOPE *vs* the LOPE groups [$\chi^2(2)=8.567$, *p*=0.014; Kruskal-Wallis test]. Urea was higher in the EOPE group (mean rank=66.27) followed by the LOPE group (mean rank=51.35) and the normotensive group (mean rank=29.00). There was also a significant difference in urea across all six study groups [$\chi^2(5)=12.552$, *p*=0.028; Kruskal-Wallis test].

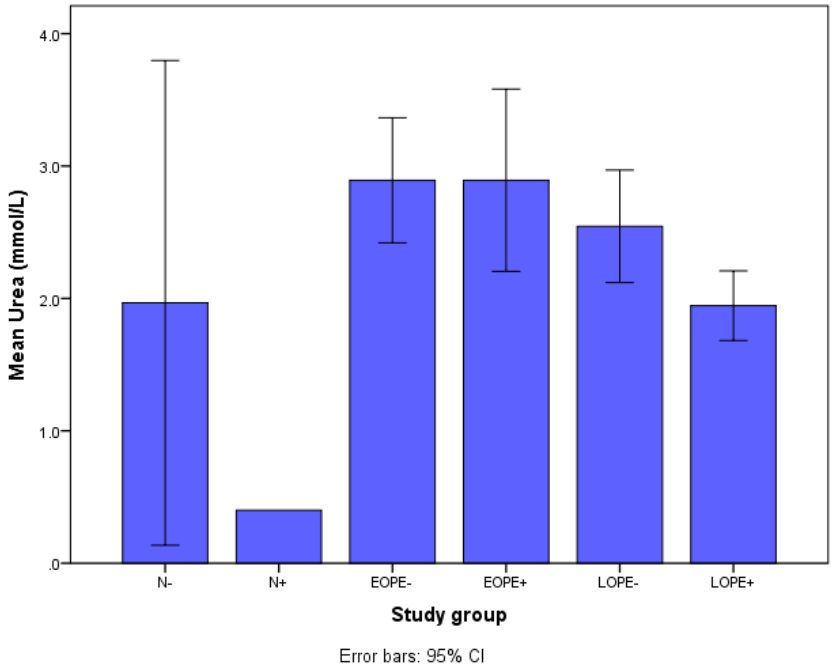


Figure 3.14: Urea (mmol/L) across all six study groups

3.4.4 Creatinine

The mean creatinine±SD of the total study population was 50.27±15.23 µmol/L. There was no statistical significance in creatinine between the HIV positive and negative groups [U=1560.50, p=0.831; Mann-Whitney U test] and between the normotensive and pre-eclamptic groups [U=161.00, p=0.915; Mann-Whitney U test].

The distribution of creatinine was not significantly different between the normotensive pregnant *vs* the EOPE *vs* the LOPE groups [$\chi^2(2)$=0.619, p=0.929; Kruskal-Wallis test] and across all six study groups [$\chi^2(4)$=1.333, p=0.856; Kruskal-Wallis test].

3.4.5 Alanine aminotransferase

The mean alanine aminotransferase (ALT)±SD of the total study population was 19.69±9.74 U/L. There was a statistical significance in ALT between the HIV positive and negative groups [U=606.50, p=0.008; Mann-Whitney U test] with higher levels in the HIV positive group (mean rank=51.54) compared to the HIV negative group (mean rank=37.14). There was no significant difference in ALT between the normotensive and pre-eclamptic groups [U=30.50, p=0.140; Mann-Whitney U test].

The distribution of ALT was the same between the normotensive pregnant *vs* the EOPE *vs* the LOPE groups [$\chi^2(2)=2.614$, p=0.271; Kruskal-Wallis test] and across all six study groups [$\chi^2(5)=10.577$, p=0.060; Kruskal-Wallis test].

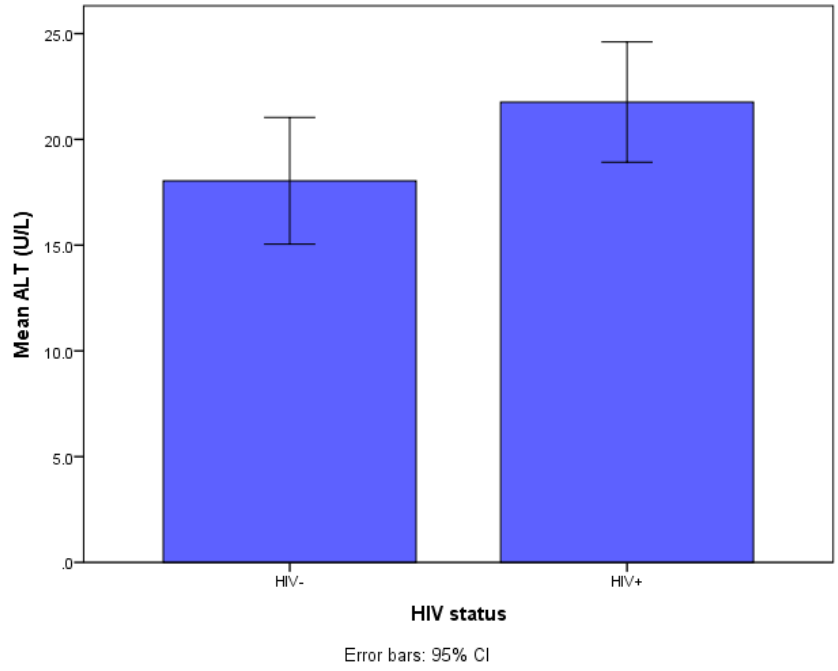


Figure 3.15: ALT levels (U/L) between the HIV positive and negative groups

3.4.6 Aspartate aminotransferase

The mean aspartate aminotransferase (AST)±SD of the total study population was 25.79±9.60 U/L. There was no statistical significance in AST between the HIV positive and negative groups [U=317.50, p=0.123; Mann-Whitney U test] and between the normotensive and pre-eclamptic groups [U=23.00, p=0.189; Mann-Whitney U test]. The distribution of AST was the same between the normotensive pregnant *vs* the EOPE *vs* the LOPE groups [$\chi^2(2)$= 2.396, p=0.302; Kruskal-Wallis test] and across all six study groups [$\chi^2(5)$=5.818, p=0.324; Kruskal-Wallis test].

3.4.7 Lactate dehydrogenase

The mean lactate dehydrogenase(LDH)±SD of the total study population was 542.05±210.05 U/L. There was no statistical significance in LDH between the HIV positive and negative groups [t(18)=-0.025, p=0.981; Independent samples t-test] and across all six study groups [F(3,16=2.192, p=0.129; ANOVA].

3.4.8 Total bilirubin

The mean bilirubin±SD of the total study population was 5.74±4.27 μmol/L. There was no statistical significance in total bilirubin between the HIV positive and negative groups [U=732.50, p=0.102; Mann-Whitney U test] and between the normotensive and pre-eclamptic groups [U=50.50, p=0.370; Mann-Whitney U test]. The distribution of total bilirubin was the same between the normotensive pregnant *vs* the EOPE *vs* the LOPE groups [$\chi^2(2)$=1.071, p=0.585; Kruskal-Wallis test] and across all six study groups [$\chi^2(5)$=8.985, p=0.110; Kruskal-Wallis test].

3.4.9 Total protein

The mean protein±SD of the total study population was 62.88±9.66 g/L. There was a significant difference in total protein between HIV positive and negative groups [t(89)=-4.529, p<0.001; Independent samples t-test]. Total protein in the HIV positive group was higher (67.58±9.02) than the HIV negative group (59.20±8.55). However, there was no significant difference in total protein between the normotensive and pre-eclamptic groups [t(89)=0.386, p=0.700; Independent samples t-test] and between the normotensive pregnant *vs* the EOPE *vs* the LOPE groups [F(2,88=0.564, p=0.571; ANOVA]. There was a significant difference in total protein across all six study groups [F(5,85=4.211, p=0.002; ANOVA].

Table 3.3 Blood test results across the six study groups

	Normotensive HIV negative	Normotensive HIV positive	Early onset pre-eclamptic HIV negative	Early onset pre-eclamptic HIV positive	Late onset pre-eclamptic HIV negative	Late onset pre-eclamptic HIV positive	*p* value
n	26	32	41	30	34	22	185
Haemoglobin (g/dL)	10.74±1.34	10.60±1.24	10.72±1.58	10.38±1.53	10.83±1.44	11.05±1.12	0.638
Platelets (10^9/L)	224.94±71.00	228.95±52.82	202.49±70.36	209.60±56.86	200.15±64.03	213.33±51.58	0.516
Urea (mmol/L)	1.7(1.4-2.8)	0.4(0.4)	2.25(1.2-7.5)	2.4(0.6-10.7)	2.4(1.2-5.2)	2.05(1.2-3.3)	0.028*
Creatinine (µmol/L)	45(41-65)	-	51.0(27-110)	48(30-77)	47(25-91)	48.5(24-68)	0.856
ALT (U/L)	33(33)	21(21)	15(8-61)	20(9-45)	17(8-32)	21(11-41)	0.060
AST (U/L)	32(32)	34(34)	21.5(15-55)	24(15-51)	23(15-44)	29(19-40)	0.324
LDH (U/L)	-	-	646.57±251.12	652.67±222.00	393.40±145.86	478.00±89.61	0.129
Total bilirubin (µmol/L)	4(4)	2(2)	5.5(1-14)	3(1-26)	4(1-10)	5(2-12)	0.110
Total protein (g/L)	-	-	58.94±9.80	66.27±9.14	59.47±6.51	69.18±9.14	0.002*

Haemoglobin, platelets, LDH and total protein are expressed as mean±SD and data analysis performed by ANOVA followed by Tukey post-test.

Urea, creatinine, ALT, AST and total bilirubin are expressed as median (range) and data analysis performed by Kruskal-Wallis.

Statistical significances at $p<0.05$ are highlighted by *.

3.5 QUANTIFICATION OF PERIPHERAL NATURAL KILLER CELLS AND THEIR RECEPTORS USING MULTICOLOUR FLOW CYTOMETRY

3.5.1 Estimation of peripheral Natural Killer cells

3.5.1.1 Lymphocytes

The mean lymphocyte±SD for the total study population was 101377.38±93597.30. There was no statistical significance in lymphocyte count between the HIV positive and negative groups [U=5194.00, p=0.248; Mann-Whitney U test]. However, a statistically significant difference was observed between the normotensive pregnant and pre-eclamptic groups [U=4038.00, p<0.001; Mann-Whitney U test] and among all pregnancy types [$\chi^2(2)$=14.74, p=0.001; Kruskal-Wallis test]. There was an increase in lymphocyte count in the pre-eclamptic (mean rank=121.07) compared to the normotensive group (mean rank=90.51) and an increase in the LOPE (mean rank=128.77) compared to the EOPE group (mean rank=113.24). A statistically significant difference was also observed across all six study groups [$\chi^2(5)$=22.81, p<0.001; Kruskal-Wallis test].

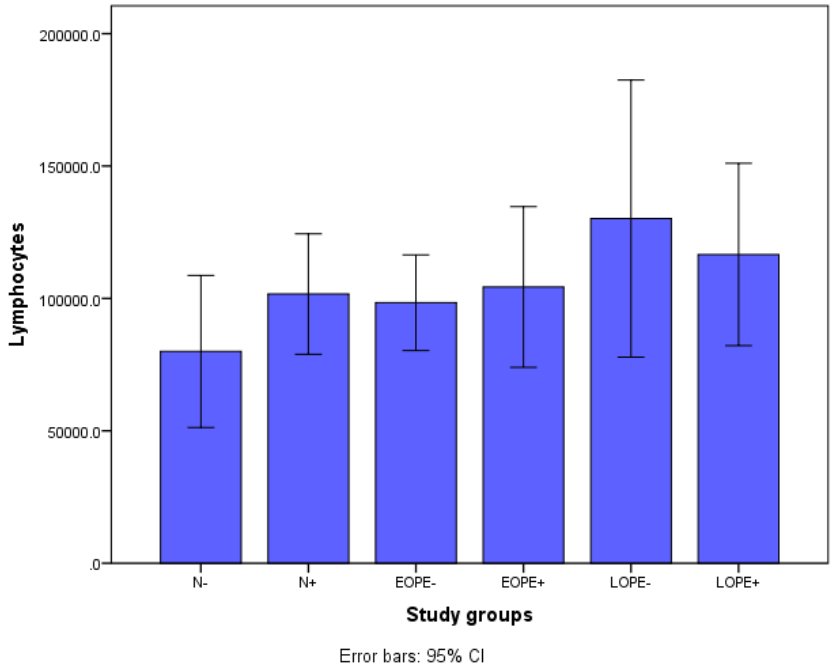


Figure 3.16: Lymphocytes in all six study groups

3.5.1.2 Natural Killer cells

The mean Natural Killer cells±SD of the entire study population was 50378.39±64637.25. There was no statistical significance in NK ($CD3^-$) cells between the HIV positive and negative groups [U=4704.50, p=0.068; Mann-Whitney U test]. A statistically significant difference was observed in NK cells in the normotensive pregnant and pre-eclamptic groups [U=4284.50, p=0.008; Mann-Whitney U test] and among all pregnancy types [$\chi^2(2)=7.34$, p=0.026; Kruskal-Wallis test]. There was an increase in NK cells in the pre-eclamptic group (mean rank=115.56) compared to the normotensive group (mean rank=93.08) and an increase in the EOPE (mean rank=118.30) compared to the LOPE group (mean rank=112.92). A statistically significant difference was also observed across all six study groups [$\chi^2(5)=18.56$, p=0.002; Kruskal-Wallis test].

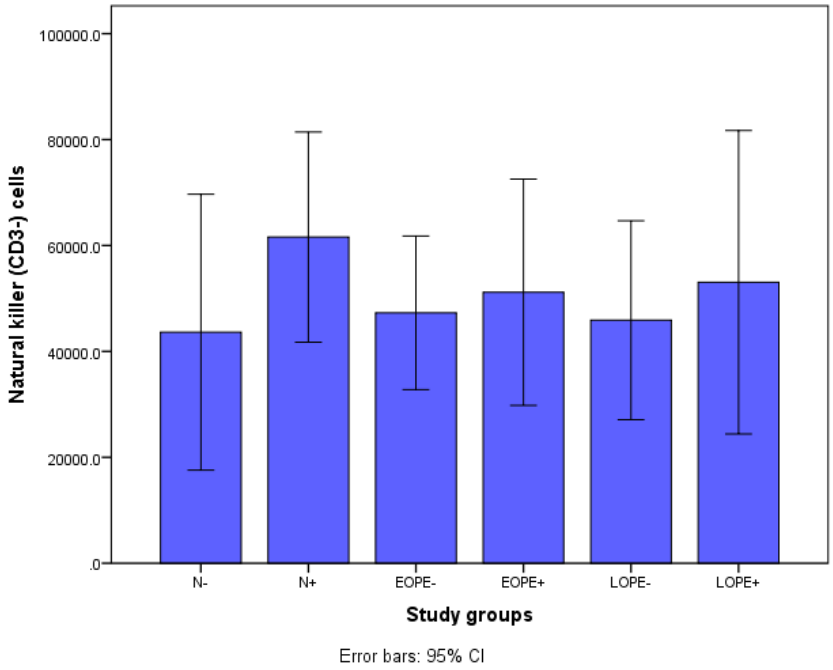


Figure 3.17: Natural Killer ($CD3^-$) cells in all six study groups

3.5.1.3 Peripheral Natural Killer cells

The mean peripheral Natural Killer cells±SD of the total study population was 1945.62±3140.17. There was a statistically significant difference in peripheral NK ($CD56^{dim}CD16^+$) cells between the HIV positive and negative groups [U=4450.00, p=0.021; Mann-Whitney U test]. Peripheral NK cells were higher in the HIV negative (mean rank=114.41) compared to HIV positive group (mean rank=95.13). There also was a significant difference between the normotensive pregnant and pre-eclamptic groups [U=4311.50, p=0.012; Mann-Whitney U test] and among all pregnancy types [$\chi^2(2)$=7.40, p=0.025 Kruskal-Wallis test]. There was an increase in peripheral NK cells in the pre-eclamptic group (mean rank=114.51) compared to the normotensive group (mean rank=93.37) and an increase in the LOPE (mean rank=120.33) compared to the EOPE group (mean rank=108.59). A statistically significant difference was also observed across all six study groups [$\chi^2(5)$=13.92, p=0.016; Kruskal-Wallis test].

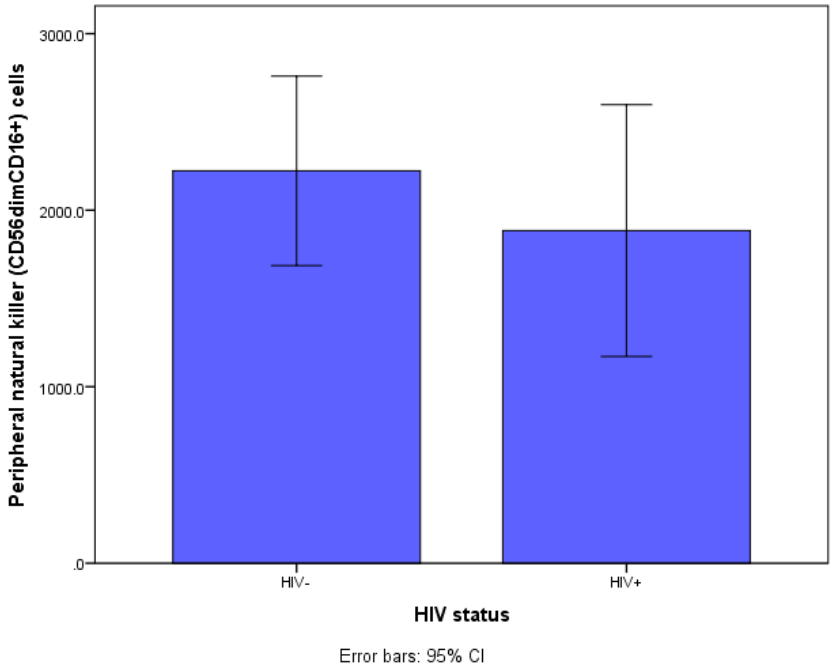


Figure 3.18: Peripheral Natural Killer ($CD56^{dim}CD16^{+}$) cells between the HIV positive and negative groups

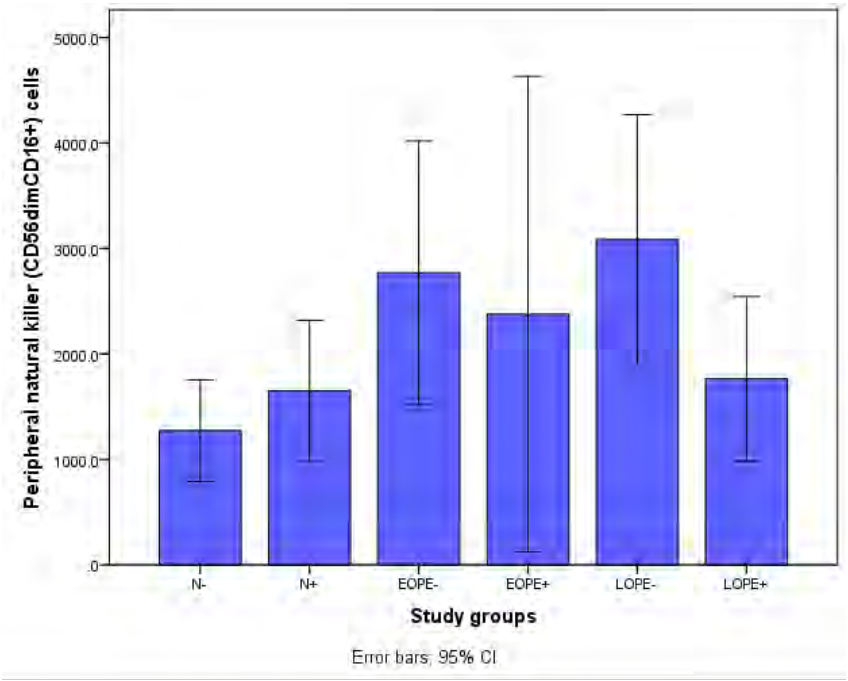


Figure 3.19: Peripheral Natural Killer ($CD56^{dim}CD16^{+}$) cells in all six study groups

3.5.2 Estimation of peripheral Natural Killer cell receptors (CD69, CD94, NKG2A, NKG2C and NKG2D)

3.5.2.1 CD69

The mean CD69±SD of the total study population was 500.70±1167.12. There was a statistical significant difference in CD69 receptor between the HIV positive and negative groups [U=4496.50, p=0.028; Mann-Whitney U test]. There was a decrease in CD69 in the HIV positive (mean rank=95.58) compared to the negative (mean rank=113.98) group. Also, a statistically significant difference was observed in CD69 in the normotensive pregnant and pre-eclamptic groups [U=4454.00, p=0.029; Mann-Whitney U test] and among all pregnancy types [$\chi^2(2)$=6.34, p=0.042 Kruskal-Wallis test]. There was an increase in CD69 in the pre-eclamptic group (mean rank=113.27) compared to the normotensive group (mean rank=94.88) and an increase in the LOPE (mean rank=120.24) compared to the EOPE group (mean rank=106.18). No difference among all six study groups [$\chi^2(5)$=10.83, p=0.055; Kruskal-Wallis test].

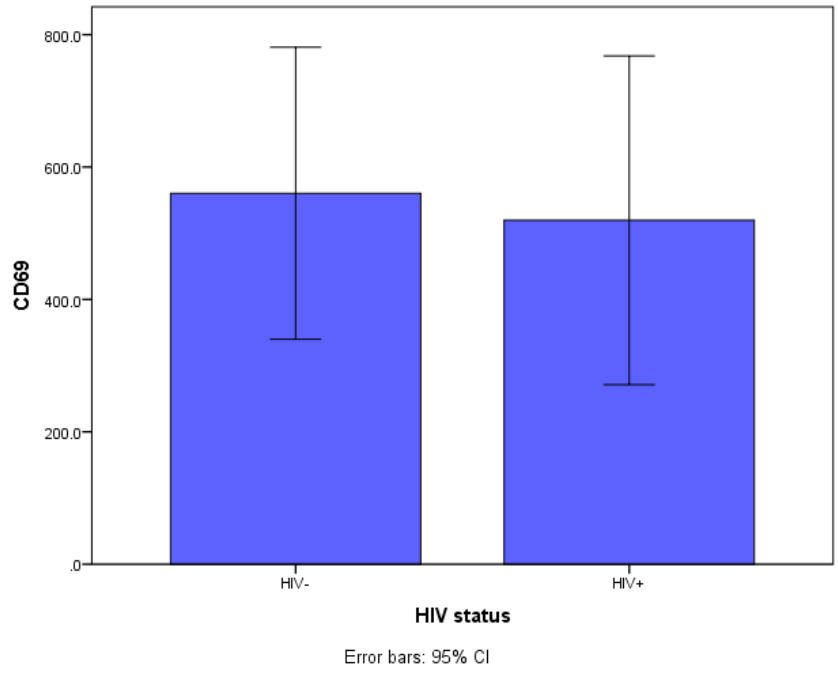


Figure 3.20: CD69 receptor between the HIV positive and negative groups

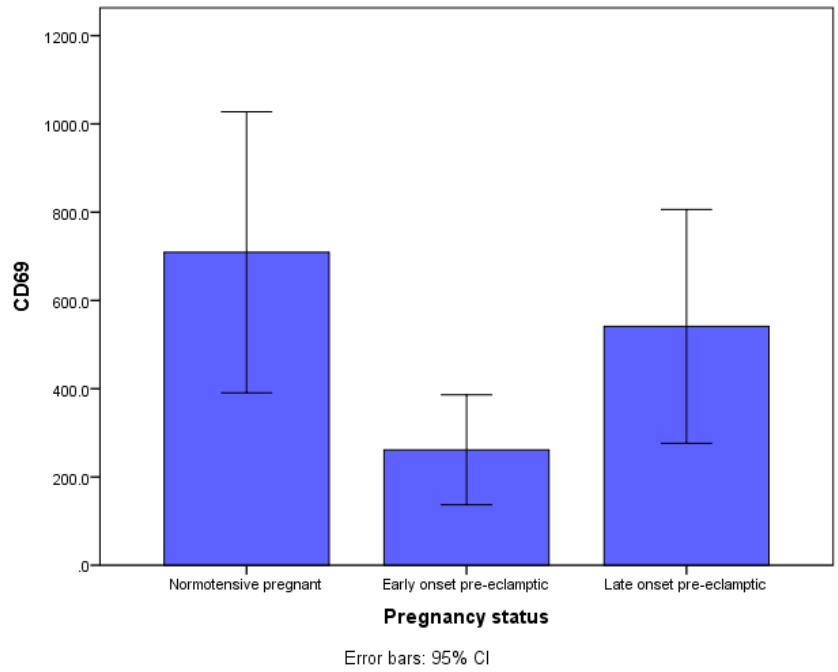


Figure 3.21: CD69 receptor across pregnancy groups

3.5.2.2 CD94

The mean CD94±SD of the total study population was 983.26±2584.25. There was a statistical significant difference in CD94 receptor between the HIV positive and negative groups [U=4463.50, p=0.030; Mann-Whitney U test], between the normotensive pregnant and pre-eclamptic groups (U=4255.00, p=0.011; Mann-Whitney U test), among all pregnancy types [$\chi^2(2)$=6.41, p=0.041 Kruskal-Wallis test] and all six study groups [$\chi^2(5)$=11.31, p=0.046; Kruskal-Wallis test]. There was an increase in CD94 in the pre-eclamptic group (mean rank=114.00) compared to the normotensive group (mean rank=92.75) and an increase in the EOPE (mean rank=114.32) compared to the LOPE group (mean rank=113.69).

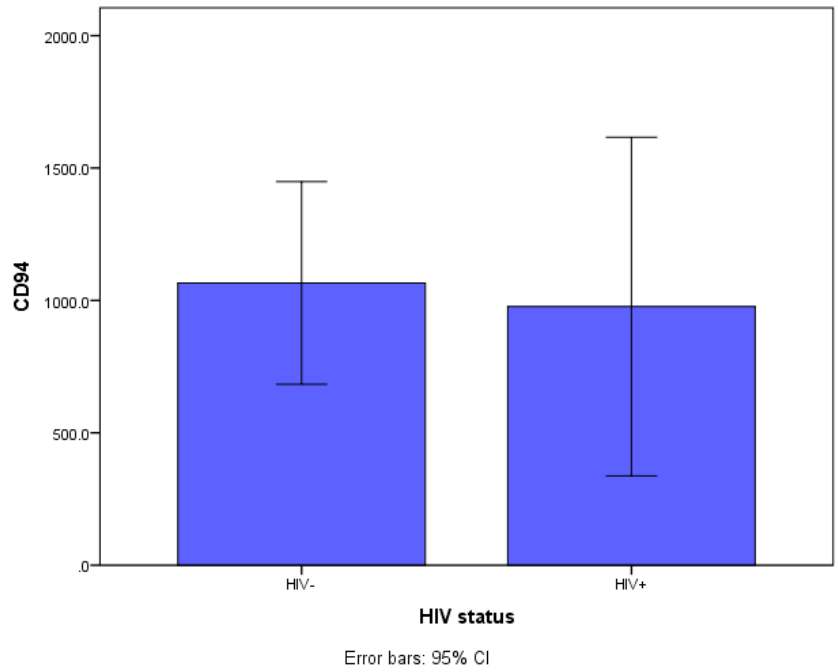


Figure 3.22: CD94 receptor between the HIV positive and negative groups

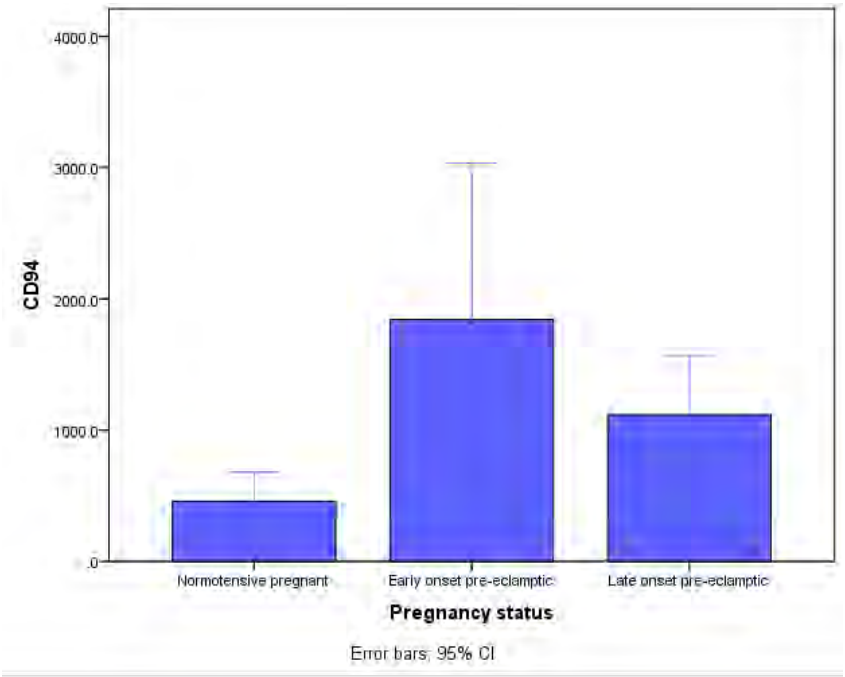


Figure 3.23: CD94 receptor across pregnancy groups

3.5.2.3 NKG2A

The mean NKG2A±SD of the total study population was 450.59±911.10. There was a statistical significant difference in NKG2A receptor between the HIV positive and negative groups [U=4308.00, p=0.012; Mann-Whitney U test]. There was no significant difference between the normotensive pregnant and pre-eclamptic groups [U=4809, p=0.212; Mann-Whitney U test] however, there was a significant difference among all pregnancy types [$\chi^2(2)=4.62$, p=0.041 Kruskal-Wallis test] with an increase in the LOPE group (mean rank=112.03) compared to the normotensive (mean rank=110.29) and EOPE (mean rank=87.39) groups and across all six study groups [$\chi^2(5)=12.60$, p=0.027; Kruskal-Wallis test].

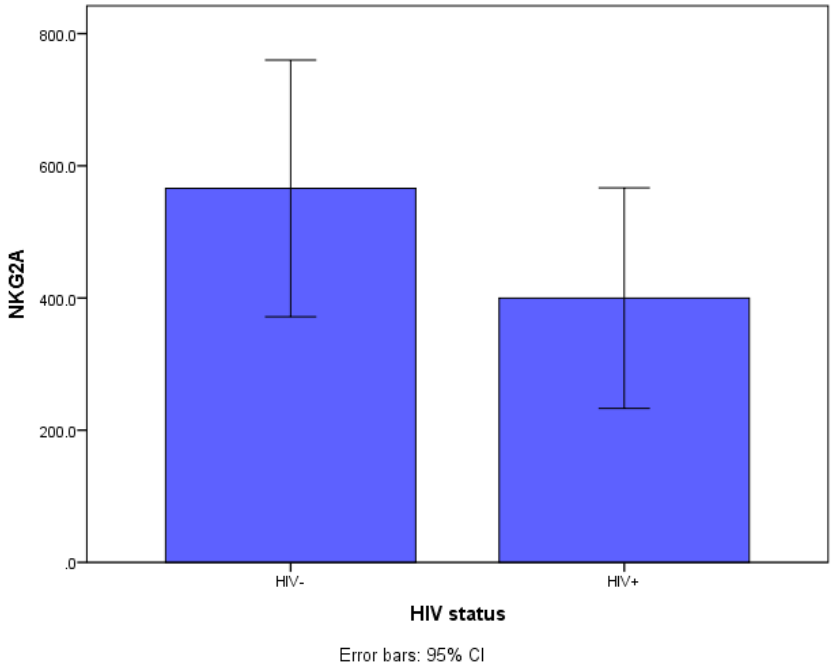


Figure 3.24: NKG2A receptor between the HIV positive and negative groups

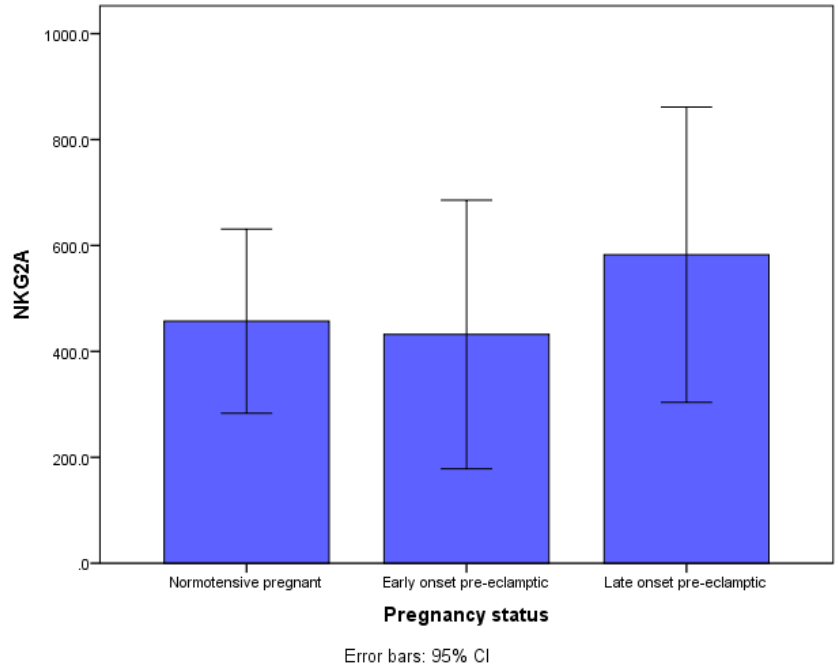


Figure 3.25: NKG2A receptor across pregnancy groups

3.5.2.4 NKG2C

The mean NKG2C±SD of the total study population was 709.23±1953.26. There was no statistical significance in NKG2C receptor between the HIV positive and negative groups [U=4740.50, p=0.126; Mann-Whitney U test], between the normotensive pregnant and pre-eclamptic groups [U=5273.00, p=0.863; Mann-Whitney U test], among all pregnancy types [$\chi^2(2)=0.036$, p=0.982 Kruskal-Wallis test] and across all six study groups [$\chi^2(5)=5.20$, p=0.392; Kruskal-Wallis test].

3.5.2.5 NKG2D

The mean NKG2D±SD of the total study population was 1312.30±1876.93. There was no statistical significance in NKG2D receptor between the HIV positive and negative groups [U=4540.50, p=0.047; Mann-Whitney U test], between the normotensive pregnant and pre-

eclamptic groups (U=4575.50, p=0.074; Mann-Whitney U test), among all pregnancy types [$\chi^2(2)$=4.06, p=0.131; Kruskal-Wallis test] and across all six study groups [$\chi^2(5)$=8.08, p=0.152; Kruskal-Wallis test].

Table 3.4 Natural Killer cells and their receptors across the six study groups

	Normotensive HIV negative	Normotensive HIV positive	Early onset pre-eclamptic HIV negative	Early onset pre-eclamptic HIV positive	Late onset pre-eclamptic HIV negative	Late onset pre-eclamptic HIV positive	*p* value
n	46	49	30	29	35	25	214
Lymphocytes	86538 (24850-403027)	49684.50 (7497-441135)	80529 (23194-199130)	82375 (29483-347314)	91497 (32458-934230)	100353 (23508-424997)	<0.001*
$CD3^-$	36214.5 (4912-276851)	13635 (1977-390276)	33096 (3203-189791)	32625.50 (8698-217234)	33521 (6938-328753)	31338 (4470-330569)	0.002*
$CD56^{dim}CD16^+$	484.50(8-9576)	747.50(5-8628)	767(11.81-11878)	431(1-31059)	1135(36-13913)	995(1-6661)	0.016*
CD94	146(0-8741)	188(0-3862)	502(0-11706)	176(1-29137)	310(14-9657)	215(0-4296)	0.046*
CD69	19.5(0-9323)	49(0-6959)	71(0-2420)	43.5(0-1454)	97(0-4657)	66(0-2687)	0.055
NKG2A	48(0-3982)	225.5(0-4075)	16(0-3909)	22(0-5357)	227(0-6703)	21(0-2255)	0.027*
NKG2C	93(6902)	179(0-1978)	106(0-8677)	26(0-22578)	222(0-5210)	57(0-2684)	0.392
NKG2D	277(0-9229)	669(3-7037)	530.5(0-11722)	329(0-29661)	762(21-13693)	357(0-4714)	0.152

Natural Killer cells and their receptors are expressed as median (range) and data analysis performed by Kruskal-Wallis.

Statistical significances at $p<0.05$ are highlighted by *.

3.6 QUANTIFICATION OF TH1 AND TH2 MATERNAL SERUM CYTOKINE LEVELS

The mean± SD maternal serum Th1 (IL-2, IL-12(70), IFN-γ, TNF-α and GM-CSF) and Th2 (IL-4, IL-5, IL-10 and IL-13) cytokine levels of all 6 study groups are outlined in Table 2. There were no significant differences in maternal serum Th1 (IL-2, p=0.175; IL-12(70), p=0.923; IFN-γ, p=0.860; TNF-α, p=0.924 and GM-CSF, p=0.775) and Th2 (IL-4, p=502; IL-5, p=0.420; IL-10, p=0.795 and IL-13, p=0.177) cytokine levels between the HIV positive and negative groups.

The Kruskal-Wallis test showed that the distribution of Th1 and Th2 cytokines were the same for all pregnancy groups (normotensive, early and late onset pre-eclampsia) and across all six study groups (Table 3). Although there was an increase in Th1 (IL-2, IFN-γ and GM-CSF, TNF-α) and Th2 (IL-13) cytokine levels and a decrease in Th1 (IL12p70) and Th2 (IL-4, IL-5 and IL-10) cytokine levels in normotensive patients compared to the pre-eclamptic group, these differences were not significant. Also, there was a decrease in Th1 (IL-2, IFN-γ, and GM-CSF) and Th2 (IL-4 and IL-5) cytokine levels with concurrent increase in Th1 (IL-12p70 and TNF-α) and Th2 (IL-10 and IL-13) cytokine levels in the early onset compared to the late onset pre-eclamptic group, these differences were also not significant.

However, GM-CSF levels were significantly different [$\chi^2(5)=12.03$, p=0.034; Kruskal-Wallis test] when comparing each of the six study groups (Figure 1).

Table 3.5 Th1/Th2 maternal serum levels (pg/ml) across all six study groups

	Normotensive pregnant		EOPE		LOPE			
(pg/ml)	HIV negative	HIV positive	HIV negative	HIV positive	HIV negative	HIV positive	Study population	*p*-Value
n	30	30	24	26	35	25	170	
IL-2	0.035 (0.035-212.87)	0.035 (0.035-191.77)	0.04 (0.035-38.08)	0.035 (0.035-385.46)	0.035 (0.035-80.98)	0.035 (0.035-120.93)	0.035 (0.035-385.46)	0.357
IL-12p70	3.18 (0.055-8084.00)	0.055 (0.055-163.22)	0.82 (0.55-36.14)	9.31 (0.55-241.26)	4.33 (0.055-213.53)	0.11 (0.055-41.91)	1.81 (0.055-8084.00)	0.105
IFN-γ	0.005 (0.005-221.68)	0.005 (0.005-674.21)	0.005 (0.00-134.17)	0.005 (0.00-2101.60)	0.01 (0.005-683.75)	0.005 (0.005-493.84)	0.005 (0.00-2101.60)	0.086
TNF-α	0.43 (0.025-8.08)	0.25 (0.025-14.46)	0.025 (0.025-8.13)	2.36 (0.025-271.22)	0.23 (0.025-11.34)	0.52 (0.025-10.06)	0.43 (0.025-271.22)	0.091
GM-CSF	0.24 (0.240-36.90)	0.24 (0.240-50.21)	0.24 (0.240-15.47)	0.24 (0.240-121.90)	0.24 (0.240-37.20)	0.24 (0.24-27.54)	0.240 (0.240-121.90)	0.034*
IL-4	0.22 (0.01-1.39)	0.038 (0.01-3.15)	0.12 (0.01-1.06)	0.22 (0.01-13.35)	0.37 (0.01-3.82)	0.12 (0.01-2.87)	0.18 (0.010-13.35)	0.144
IL-5	0.03 (0.025-40.62)	0.025 (0.025-61.51)	0.025 (0.025-35.08)	8.61 (0.025-132.48)	0.20 (0.025-60.71)	0.20 (0.038-61.51)	0.025 (0.025-132.48)	0.188
IL-10	0.105 (0.06-41.90)	0.515 (0.105-12.85)	0.25 (0.105-8.54)	0.515 (0.105-241.11)	0.11 (0.105-20.67)	0.105 (0.105-8.62)	0.105 (0.060-241.11)	0.446
IL-13	7.00 (0.10-33.07)	6.17 (0.79-20.04)	4.74 (0.10-11.81)	6.17 (0.10-44.92)	5.81 (0.10-24.89)	6.21 (0.10-16.73)	6.17 (0.100-44.92)	0.334

Th1 and Th2 cytokines are expressed as median (range) and data analysis performed by Kruskal-Wallis.

Statistical significances at $p<0.05$ are highlighted by *

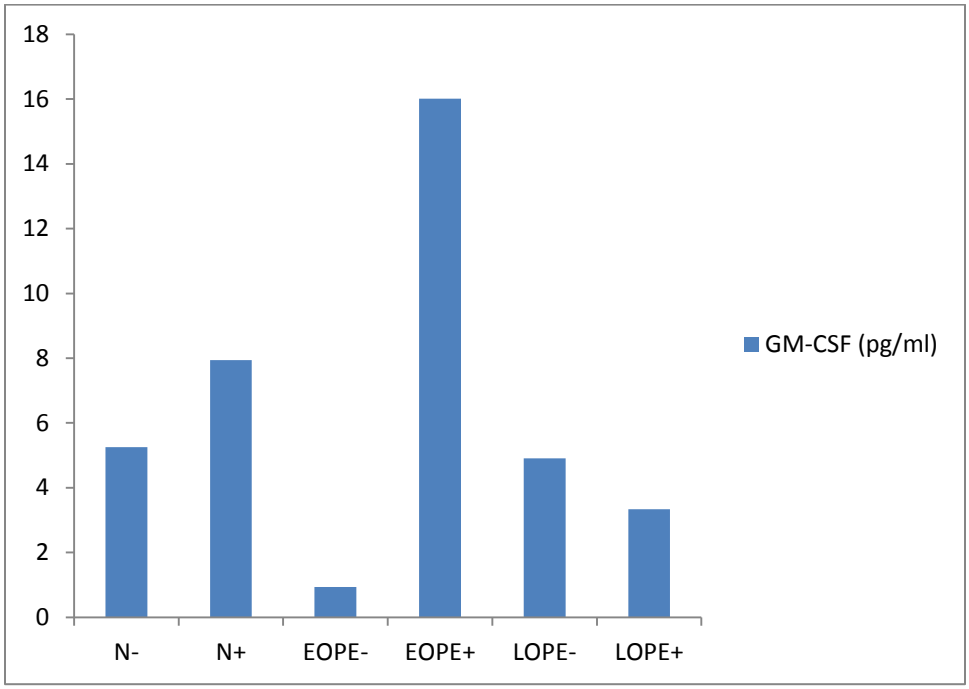


Figure 3.26: GM-CSF (pg/ml) maternal serum cytokine levels in all six study groups

3.6.1 Correlation between Th1 and Th2 maternal serum cytokines in pregnancy groups

Th1 and Th2 cytokines increased simultaneously in the normotensive group (p<0.001) except IL-13 which increased only with an increase in Th2 cytokines, IL-4 (p=0.004, r=0.454) and IL-10 (p<0.001, r=0.365). Th1 and Th2 cytokines increased concurrently in the EOPE group. There was an increase in Th1 and Th2 cytokines (p<0.001) except IL-13 which did not increase or decrease with the Th1 cytokines, GM-CSF (p=0.088, r=0.246) and IL-2 (p=0.334, r=0.141) in the LOPE group.

3.6.2 Correlation between NK cell receptor expression and Th1/Th2 maternal serum cytokines within pregnancy groups

In the normotensive group, NK cells decreased simultaneously with TNF-α (p=0.013, r=-0.332) and IL-10 (p=0.046, r=-0.270). CD69 expression decreased concurrently with all Th1 cytokines. Significant correlation between CD69 and the Th2 cytokines, IL-5 (p=0.008, r=-0.355) and IL-10 (p=0.048, r=-0.268) was found. Only the significant correlations in the EOPE and LOPE groups are shown in Tables 3.6 and 3.7 respectively.

Table 3.6 Correlation between NK cell receptor expression and Th1/Th2 maternal serum cytokines within the EOPE group

NK cell receptor expression	Th1/Th2 cytokine	p-Value	Coefficient of correlation (r)
pNK cells	IL-5	0.036*	0.297
CD94	IL-12(p70)	0.017*	0.337
CD94	IFN-γ	0.004*	0.403
CD94	TNF-α	0.003*	0.413
CD94	GM-CSF	0.002*	0.421
CD94	IL-4	0.027*	0.313
CD94	IL-5	0.001*	0.453
CD94	IL-13	0.013*	0.350
NKG2A	IFN-γ	0.020*	0.329
NKG2C	IL-5	0.022*	0.324
NKG2D	IL-12(p70)	0.040*	0.291
NKG2D	IFN-γ	0.007*	0.378
NKG2D	TNF- α	0.025*	0.316
NKG2D	GM-CSF	0.008*	0.369
NKG2D	IL-5	0.003*	0.408

Statistical significances at p<0.05are highlighted by *

Table 3.7 Correlation between NK cell receptor expression and Th1/Th2 maternal serum cytokines within the LOPE group

NK cell receptor expression	Th1/Th2 cytokine	*p*-Value	Coefficient of correlation (*r*)
CD69	IFN-γ	0.011*	-0.393
CD69	TNF-α	0.016*	-0.376
CD69	GM-CSF	0.006*	-0.418
CD69	IL-5	0.029*	-0.341
CD94	IL-12(p70)	0.003*	0.454
CD94	IFN-γ	0.013*	0.383
CD94	TNF- α	0.018*	0.368
CD94	GM-CSF	0.012*	0.388
CD94	IL-5	0.011*	0.394
CD94	IL-10	0.048*	0.311
NKG2A	IL-12(p70)	0.030*	0.340
NKG2D	IL-5	0.048*	0.311

Statistical significances at $p<0.05$are highlighted by *

CHAPTER 4

DISCUSSION

4.1 Introduction – Immune response in HIV associated pre-eclampsia

Sub-Saharan Africa remains the epicenter of the global HIV pandemic. With the increased accessibility to antiretroviral treatment in South Africa there has been a decrease from 40.5% (2008-2010) to 34.7% (2011-2013) of maternal deaths attributable to non-pregnancy related infections (mostly HIV-related) (Department of Health, Saving Mothers Reported, 2015). The other two leading causes of maternal deaths in South Africa are obstretric haemorrhage (15.8%) and hypertensive disorders of pregnancy (14.8%) of which 54.2% is attributable to pre-eclampsia eclampsia and eclampsia (Department of Health, Saving Mothers Reported, 2015). This deadly trio accounts for two thirds of maternal mortality in South Africa, 65% of which could be preventable and mostly due to inadequate access to healthcare and lack of trained health care professionals. (Department of Health, Saving Mothers Reported, 2015). Furthermore, KwaZulu-Natal accounts for the highest maternal deaths (22.0%) compared to the other provinces in South Africa with 42.5% due to non-pregnancy related infections (mostly HIV-related) and 8.3% to pre-eclampsia. This deadly duo therefore warrants investigation and is the focus of our study.

The pathology and etiology of pre-eclampsia is not fully understood. In the first trimester, trophoblast cells invade the myometrium and create the maternal-foetal interface called the decidua. Trophoblasts modify the wall of the spiral artery, converting them to large, low resistance sinusoids thus allowing an adequate blood supply to the placenta and the growing fetus (Bachmayer et al., 2006). Pre-eclampsia occurs when trophoblast migration is deficient and

there is failure of the physiological conversion of the myometrial spiral arteries (Walker, 2011). Blood flow to the placenta is reduced, hence nutrient and oxygen transfer across the placenta limits foetal growth. Trophoblast cell debris that is released into the maternal circulation induces an inflammatory response in the second trimester of pregnancy (Bachmayer et al., 2006). This explains the hypertension, oedema and proteinuria which are characteristically associated with this disease. The invading trophoblast, maternal immunological cells and Natural Killer cells play an important role in normal pregnancy development (Walker, 2011).

Natural Killer (NK) cells target viral and tumour cells (Vacca et al., 2013) and play an important role in ensuring reproductive success (Fukui et al., 2011). Abnormalities in NK cell response amongst other immune responses may cause placental dysfunction and resultant hypertension and high levels of proteinuria (Cerdeira and Karumanchi, 2012). There are two types of NK cells namely, uterine and peripheral NK cells. Uterine NK cells constitute 70-90% of all blood lymphocytes (Fukui et al., 2011). In early pregnancy NK cells within the decidua are known to play a role in placentation, angiogenesis and regulation of trophoblast invasion (Bachmayer et al., 2009). Peripheral NK cells constitute 5-10% of all blood lymphocytes (Fukui et al., 2011). NK cells occur either as $CD56^{bright}$ $CD16^{-}$ or $CD56^{dim}$ $CD16^{+.}$ The former are the major pro-inflammatory cytokine producers, are less cytotoxic and found mainly in the decidua whilst the latter are cytotoxic and found mainly in peripheral blood (Bachmayer et al., 2009; Fukui et al., 2011). Studies have shown the usefulness of uterine NK cells to ensure successful pregnancies however, the function of peripheral NK cells in pregnancy and moreover in pathological pregnancies such as pre-eclampsia are not well known (Fukui et al., 2011).

The activity of NK cells are regulated by inhibitory and activating receptors. $CD56^{dim}CD16^{bright}$ NK cells express the inhibitory killer cell immunoglobulin-like receptors (KIR) which are found predominately in the peripheral blood. $CD56^{bright}$ $CD16^{dim}$ lack KIR expression but express high levels of the activating CD94/NKG2 receptors which are found in the peripheral tissues. CD94/NKG2 receptors include the inhibitory receptor CD94/NKG2A and the stimulatory receptors CD94/NKG2C and NKG2D (Bachmayer et al., 2009). These receptors play an important role in the formation of the decidua and placenta (Bachmayer et al., 2006).

NK cells are triggered by cytokines, IL-12, IL-15 and IL-18. Once activated NK cells express CD69 which activate IFN-ɣ production that contribute to a type I response in the circulation in pre-eclampsia (Bachmayer et al., 2009). In healthy non-pregnant women there is a balance between the Th1 and Th2 response (Laresgoiti-Servitje et al., 2010). However, in successful normal pregnancy there is a shift from the Th1 to a Th2 immune response (Laresgoiti-Servitje et al., 2010). In pathological pregnancies such as pre-eclampsia, spontaneous miscarriages and intrauterine growth restriction (IUGR), the regulation of the maternal immune system is further altered and the shift to a Th2 immune response does not occur (Laresgoiti-Servitje et al., 2010). Wegmann et al. (1993) proposed that the placenta is a Th2 organ that stimulates the production of Th2 cytokines in the maternal immune system. When the development of the placenta is abnormal, the maternal immune system responds by producing Th1 cytokines. Transforming growth factor-β cytokines surge thereby retarding cytotrohophoblast migration (Laresgoiti-Servitje et al., 2010; Fukai et al., 2011). Moreover, syncytiotrophoblast debris is increased in pre-eclamptic maternal circulation due to an ischemic placenta thereby triggering the Th1 response (Redman et al., 2012).

Although the role of NK cells is to destroy viral cells, HIV has the ability to suppress the function of NK cells. The role of NK cells in HIV is well documented but their role in HIV associated pre-eclampsia has produced conflicting results(Conde-Agudelo et al., 2008). Some studies reported that untreated HIV infection lowers the rate of preeclampsia whilst treated HIV infection restores the rate of pre-eclampsia. Other studies reported that there is no difference in treated and untreated HIV infection in preeclampsia (Mattar et al., 2004). In an attempt to elucidate the influence of HIV on NK cells in pre-eclampsia this study used multicolour flow cytometry to compare peripheral NK cells in HIV positive and negative normotensive, early and late onset pre-eclamptic women.

In HIV infected patients, there is a decrease in Th1 cytokines with a concurrent increase in Th2 cytokine production (Clerichi and Shearer, 1993). Th2 cytokines respond to extracellular infection and are known to suppress the immune system (Kalkunte et al., 2011). However, there are conflicting results on the secretion of cytokines in pre-eclampsia and HIV (Chatterjee et al., 2011). In an attempt to understand the maternal immune response in HIV associated pre-eclampsia, our study used multiplex enzyme-linked immunosorbent assay (ELISA) to compare the maternal serum levels of Th1 and Th2 cytokines in HIV positive and negative pre-eclamptic (early and late onset) with normotensive pregnant Black South Africans.

Other studies have looked at the maternal immune response (both NK cells and Th1 and Th2 cytokines) in HIV and pre-eclampsia in isolation and further have not sub-stratified the pre-eclamptic group into early and late onset. In view of the high prevalence of pre-eclampsia

superimposed with HIV in South Africa, this novel study will attempt to determine the role of the maternal immune response in HIV associated pre-eclampsia.

4.2 Clinical data and demographics

4.2.1 Maternal age

Our study did not find a significant difference in maternal age between the normotensive and pre-eclamptic groups, however other studies have found that older women are at a higher risk of developing pre-eclampsia than younger women (Tessema et al., 2015; Gupta and Chari, 2015). Older women are more prone to cardiovascular disease as the blood vessels may lose compliance and also the haemodynamic adaptation during pregnancy becomes more difficult (Tessema et al., 2015). However, young maternal age has also been identified as a risk factor for preterm birth, low foetal birth weight and foetal growth restriction (Gold et al., 2014). The Tenth interim reported on Confidential Enquiries into Maternal Deaths in South Africa, 2011 and 2012 reporteded that teenagers are at increased risk of dying due to complications of hypertension in pregnancy (Fawcus et al., 2013). This may be due to limited sperm exposure which could lead to an inflammatory response to the paternal antigens thereby eliciting a Th1 immune response which is associated with poor placental development and foetal growth (Dekker and Robillard, 2007).

Maternal age in the HIV positive group was significantly higher (p=0.006) than the HIV negative group. In 2012, South Africa reporteded that non-pregnancy related infections (mostly HIV related) were highest between 25-40 years of age (Fawcus et al., 2013). Younger women are more educated about the disease and possibly take precautions to prevent HIV infection.

4.2.2 Maternal weight and BMI

Gupta and Chari, (2015) support our findings of no significant differences in maternal weight and BMI in pre-eclampsia compared to normotensive pregnancy. Other studies have found BMI to be a risk factor for pre-eclampsia development in HIV uninfected pregnant women (Machado et al., 2014). However, obesity is a huge problem globally (Roberts et al., 2011) and in South Africa 42.0% of women are overweight which is the highest in Sub-Saharan Africa and third highest in the world (Ng et al., 2014). Overweight and obesity are known risk factors of pre-eclampsia. Futhermore, overweight has been associated with oxidative stress consisting of an imbalance of lipid metabolism and an inflammatory state that is also associated with pre-eclampsia. However, during pregnancy especially in the third trimester overweight may be due to fluid retention (Machado et al., 2014).

4.2.3 Parity and gravidity

Parity (no. of live births, p=0.023) and gravidity (no of pregnancies, p=0.028) were higher in the HIV positive group than the HIV negative group. Multiple pregnancies may increase the risk of HIV transmission. Multigravid women with pre-eclampsia in a prior pregnancy have an increased risk of developing pre-eclampsia in a subsequent pregnancy (Roberts et al., 2011; Machado et al., 2014).

4.2.4 CD4+ cell count and ARV treatment

The role of HIV-infection in the development of pre-eclampsia is controversial. Hall et al., 2014 and Kalumba et al. (2012) found that pre-eclampsia was less common in HIV positive women.

Wimalasundera et al., (2002) found a lower rate of pre-eclampsia among untreated HIV positive women when compared to negative women or HIV positive on treatment. Several studies found no significant difference in pre-eclampsia between HIV negative and infected women on treatment. Being on HAART at conception was associated with a 2.3 times increased risk of developing pre-eclampsia during pregnancy (Machado et al., 2014).

In our study all pregnant women were on ARV treatment, either HAART or Fixed Dose Combination (FDC). Our study was limited as we did not include an HIV positive ARV treatment naïve group so as to determine the effect of ARV treatment in HIV associated pre-eclampsia. Of the total population, 32.8% of women had a CD4+ cell count <350 cells/mm^3 and 67.2% had a CD4+ cell count >350 cells/mm^3. Our study was further limited by missing CD4+ cell count data (41% of the total HIV population), hence the effect of CD4+ count on pre-eclampsia could not be accurately determined. A total of 45.5% of women with a CD4+ cell count <350 cells/mm^3 live in a rural area whilst 54.5% live in an urban area. A total of 15.6% of women with a CD4+ cell count >350 cells/mm^3 live in a rural area and 84.4% live in an urban area (p=0.008). This shows that access to ARV treatment may be limited in the rural area compared to the urban area. Access may also be limited by cost of transport from a rural area to hospitals and clinics in an urban area.

4.3 Neonatal data

4.3.1 Method of delivery

Our study found no significant differences in method of delivery (normal vaginal, normal induced, elective caesarean section and emergency caesarean section) between HIV positive and

negative groups (p=0.618) as well as amongst pregnancy types (p=0.308). Increasing rates of early elective delivery either labour induction or caesarean section before 40 weeks gestation has been reporteded internationally. Changes in elective delivery are changing the distribution of gestational age at or near term (Roberts et al., 2011). Also, the prevention of mother to child transmission (PMTCT) of HIV has progressed from recommending caesarean section (Machado et al., 2014).

4.3.2 Gestational age, baby weight and baby outcomes (live/stillbirths)

In pre-eclampsia a failure of the physiological transformation of uterine arteries results in a reduced placental supply of oxygen and nutrients (Nakimuli et al., 2014). The only known treatment for pre-eclampsia is delivery of the placenta which leads to premature delivery and adverse effects on neonatal outcomes such as small for gestational age for delivery, lower birth weight and an increased risk of stillbirths (Wang et al., 2009; Backes et al., 2011). The results of our study confirms the previous study as gestational age (p=0.001) and baby weight (p=0.001) were significantly lower in the pre-eclamptic compared to the normotensive group, also being lower in the early compared to the late onset pre-eclamptic group. Also, 18.2% of stillbirths occurred in the early compared to none in the late onset pre-eclamptic and normotensive groups. Although there were no significant differences of gestational age (p=0.976), baby weight (p=0.511), baby outcome (live or stillborn; p=0.853) and admission to NICU (p=0.300) between the HIV positive and negative groups irrespective of pregnancy status. HIV infection has been associated with adverse birth outcomes (Chen et al., 2012) such as preterm delivery, small gestational age infants and stillbirths which were more common in HIV positive than negative women. Arechavaleta-Velascoet et al. (2006) showed that adeno-associated virus-2 infection can

induce placental dysfunction by inhibiting invasion of extravillous trophoblast cells and by causing trophoblast cell death (Conde-Agudelo et al., 2008). Further studies should determine the risk of ARV treatment on placental development and neonatal outcome.

4.3.3 Admission to NICU

A total of 74.6% of neonates where admitted to NICU from the EOPE group compared to 19.0% in the LOPE group and 8.6% in the normotensive group. Duhig and Shennan, (2015) and Ilekis, et al., (2007) also reporteded an increased risk of stillbirths in EOPE. Early diagnosis of pre-eclampsia may be possible if pregnant women seek antenatal care early in pregnancy (Tessema et al., 2015). It has also been found that women diagnosed with EOPE also are at a greater risk of cardiovascular disease in later life (Iiekis et al., 2007).

4.4 Laboratory data

4.4.1 Haemoglobin

Our study found no significant differences in haemoglobin concentration between HIV positive and negative groups (p=0.563) and the pre-eclamptic and normotensive groups (p=0.780). Aghamohammadi, et al. (2011) showed that high haemoglobin levels in the first trimester increases the risk of pregnancy induced hypertension. Increased concentrations of haemoglobin may cause vasoconstriction and abnormal endothelial cell function. Due to vasoconstriction the number of red blood cells increases but blood volume does not increase in pre-eclampsia which results in a limited supply of oxygen and nutrients to the growing fetus (Aghamohammadi et al., 2011). This in turn results in a small gestational age baby and lower birth weight. (Machado et al., 2014).

4.4.2 Platelets

Our study found no significant difference in platelets between the normotensive and pre-eclamptic groups (p=0.064). In normal pregnancy there is an increase in platelet aggregation but decrease in circulation with gestation. In pre-eclampsia due to the failure of cytotrophoblast invasion into maternal spiral arteries and subsequent dysfunction of the endothelial cells, results in increased platelets in uteroplacental circulation and decreased platelets in maternal circulation early in pregnancy (Juan et al., 2011).

4.4.3 Urea

Serum urea concentration was higher in the early onset pre-eclamptic group followed by the late onset pre-eclamptic group and the normotensive group (p=0.014). There also was a significant

difference in urea across all six study groups (p=0.028). Jumaah (2012) reporteded similar findings of higher urea in the pre-eclamptic compared to the normotensive group. Pre-eclampsia is associated with renal function abnormalities (Jumaah, 2012). An increased resistance of the afferent arterioles reduces blood flow to the kidneys and causes renin to activate the renin angiotensin aldosterone system which increases blood pressure and reabsorption of sodium ions into the bloodstream. This coupled with a decreased glomerular filtration rate and plasma volume contraction contributes to increased amount of urea in the blood (Zar et al., 2011).

4.4.4 Creatinine

There was no significant difference in creatinine between the HIV positive and negative groups (p=0.831), between the normotensive and pre-eclamptic groups (p=0.915), between the normotensive pregnant *vs* the EOPE *vs* the LOPE groups (p=0.929) and across all six study groups (p=0.856). Weerasekera et al., (2003) also showed no difference in creatinine levels in the pre-eclamptic compared to the normotensive group.

4.4.5 Alanine transaminase

There was a significant difference in alanine transaminase (ALT) between the HIV positive and negative groups (p=0.008) with higher levels in the HIV positive group compared to the HIV negative group. The liver is a site for HIV replication and when the liver is damaged, transaminases such as ALT which are made in the liver leak into the bloodstream (Netto et al., 2009).

4.4.6 Aspartate aminotransferase

There was no significant difference in aspartate aminotransferase (AST) between the HIV positive and negative groups (p=0.123), between the normotensive and pre-eclamptic groups (p=0.189), between the normotensive vs early onset pre-eclamptic vs the late onset pre-eclamptic groups (p=0.302) and across all six study groups (p=0.324). Higher levels of the liver enzymes AST and ALT were found in early onset pre-eclamptic patients which was associated with a higher risk of developing severe pre-eclampsia (Mei-Dan et al., 2013).

4.4.7 Lactate dehydrogenase

There was no significant difference in LDH between the HIV positive and negative groups (p=0.981) and across all six study groups (p=0.129). Higher serum LDH levels were shown in HIV positive patients compared to HIV negative patients. LDH plays an important role in glucose metabolism and during infection high levels are released. Furthermore HAART has been shown to have toxic effects on various organs which further results in an increase in LDH levels (Ramana et al., 2013).

4.4.8 Total bilirubin

There was no significance difference in total bilirubin between the HIV positive and negative groups (p=0.102), between the normotensive and pre-eclamptic groups (p=0.370), between the normotensive pregnant *vs* the EOPE *vs* the LOPE groups (p=0.585) and across all six study groups (p=0.110). McDonald et al., (2012) supports our finding of no difference in HIV positive and negative groups on HIV treatment.

4.4.9 Total protein

Total protein in the HIV positive group was higher (67.58±9.02) than the HIV negative group (p<0.001; 59.20±8.55). There was a significant difference in total protein across all six study groups (p=0.002). Similar results were reporteded by Sarro et al. (2010). HIV infection induces changes in serum proteins such as globulins which occur above the normal range of 21 - 37 g/L and decreased albumin levels. Normally albumins are the most abundant protein found in blood (Erick et al., 2012).

4.5 Innate immune system – Lymphocytes

Pre-eclampsia is triggered by placental antigens which results in an exagerated maternal inflammatory response. Abnormal levels of immune factors suggest a possible role of the innate immune system in particular NK cells which are responsible for the production of cytokines and the expression of activating or inhibitory receptors (Bueno-Sánchez et al., 2013).

In our study, there was no significance difference in lymphocytes between the HIV positive and negative groups (p=0.133). ARV treatment may have contributed to increasing the lymphocyte population to similar levels to that of HIV negative patients. A significant difference was observed between the normotensive pregnant and pre-eclamptic groups (p<0.001) and among all pregnancy types (p<0.001) and in all six study groups (p<0.001). There was an increase in lymphocytes in the pre-eclamptic group compared to the normotensive group and an increase in the late onset compared to the early onset pre-eclamptic group. Mosimann et al. (2013) showed a significant decrease in the total lymphocyte population from the first to the second trimester (p=0.015) and no significant change from the second to the third trimester (p=0.286) in normotensive pregnancies.

4.5.1 Natural Killer cells

NK cells ($CD3^-$) comprise 15% of peripheral blood lymphocytes and provide an immediate defense against infection (Acar et al., 2011). It is known that NK cells are reduced in the latter stage of HIV infection and in pregnancy (Kane et al., 1996). In our study there was no significant difference in NK cells between the HIV positive and negative groups (p=0.198). Our study participants may be at various stages of HIV infection and since all HIV positive patients were

on ARV treatment this may have played a role in improving the NK cell population. There was a significant increase in NK cells in the pre-eclamptic group compared to the normotensive group ($p<0.001$) and an increase in the EOPE to the LOPE groups ($p<0.001$). This increase may be explained by the release of syncytiotrophoblast-derived micro-particles (STBM) from the placenta of pre-eclamptic women (Germain et al., 2007). A study that looked at the *in vitro* activated mononuclear cells from mildly pre-eclamptic patients showed a decrease in NK cells (van Nieuwenhoven et al., 2008). Bachmayer et al. (2006) showed a lower percentage of NK cells at delivery in both the pre-eclamptic and normotensive pregnant groups compared to a non-pregnant group ($p=0.06$) whilst Mosimann et al. (2013) reporteded a significant decrease in the total NK cell count ($p=0.009$) from the first to the third trimester of pregnancy. A limitation of our study was that we did not evaluate peripheral NK cells across the trimesters of pregnancy.

4.5.2 Peripheral Natural Killer cells

There are two types of NK cells namely, uterine ($CD56^{bright}CD16^{+}$) and peripheral ($CD56^{dim}CD16^{+}$) NK cells (Fukui et al., 2011). Uterine NK cells are the major pro-inflammatory cytokine producers, are less cytotoxic and found mainly in the decidua whilst the latter are cytotoxic, produce less cytokines and found mainly in peripheral blood (Bachmayer et al., 2009 and Fukui et al., 2011). There was a significant difference in peripheral NK cells between the HIV positive and negative groups ($p=0.024$). Peripheral NK cells were higher in the HIV negative group compared to HIV positive group. This is surprising as peripheral NK cells possess more cytotoxic properties and would be expected to be present in larger amounts in the HIV positive compared to the negative group. However, HIV infection is known to suppress the cytotoxic properties of NK cells which may explain these results.

There was a significant increase in peripheral NK cells in the pre-eclamptic group (mean rank=114.51) compared to the normotensive group (mean rank=93.37; p=0.012) and an increase in the LOPE (mean rank=120.33; p=0.025) compared to the EOPE group (mean rank=108.59) and across all six study groups (p=0.016). Mosimann et al. (2013) also showed a decrease in peripheral NK cells in normotensive pregnancy. Pre-eclampsia is associated with exaggerated inflammation and increased production of cytokines (Schumacher, 2014). This study confirms this as increased accumulation of peripheral NK cells were observed in pre-eclamptic compared to the normotensive pregnancies. However, Bueno-Sánchez et al. (2013) reporteded no difference in $CD56^{dim}$ population in the severe pre-eclamptic group compared to the normotensive group (p=0.11).

4.5.3 CD69

CD69, a C-type lectin receptor is an early, nonspecific NK cell activation marker. When NK cells are activated upon infection or inflammation there is an increase in the levels of CD69 (Fogel et al., 2013). However, our study showed a decrease in CD69 in the HIV positive (mean rank=95.58) compared to the negative (mean rank=113.98; p=0.343). All patients in our study were on ARV treatment which may have played a role in decreasing CD69 in HIV positive patients. A significant difference was observed between the normotensive pregnant and pre-eclamptic groups (p=0.003) and among all pregnancy types (p=0.007). In normal pregnancy cytotoxic NK cells are decreased suggesting that activated CD69 expressing NK cells play an important role in the control of trophoblast growth and placental development (Mosimann et al., 2013). However, a conflicting reported by Bachmayer et al. (2006) showed no difference in CD69 in the pre-eclamptic compared to the normotensive group. Also a limitation of this study is that other factors may stimulate CD69 expression such as T cell receptors (Borrego et al., 1999).

4.5.4 CD94, NKG2A and NKG2C receptors

CD94/NKG2 receptor is a member of the C-type lectin superfamily which consists of five receptors, NKG2A, NKG2C, NKG2D, NKG2E and NKG2F. NKG2A and NKG2B are inhibitory receptors which inhibits NK cell function whilst NKG2C and NKG2D are NK cell activating receptors (Bachmayer *et al.*, 2009). NKG2A negatively regulates cytotoxic activity and Th2 cytokine production and NKG2C triggers NK cell effector functions (Lanier et al., 1998). IL-12/IL-15 or IFN and IL-10 stimulates an increase in NKG2A which may negatively affect NKG2C activity (Bueno-Sánchez et al., 2013).

In our study there was a significant difference in NKG2A between the HIV positive and negative groups (p=0.012), among all pregnancy types (p=0.041) and across all six study groups (p=0.027). There was no difference in NKG2A between the normotensive pregnant and pre-eclamptic groups (p=0.212). There was no significant difference in NKG2C (p=0.126) between the HIV positive and negative groups, between the normotensive pregnant and pre-eclamptic groups (p=0.863), among all pregnancy types (p=0.982) and across all six study groups (p=0.392). An increase in NKG2C cells compared to NKG2A cells have been reporteded in HIV infected patients (Bachmayer et al., 2006). Data are conflicting with others studies which have shown a significant increase in percentage of NKG2A and NKG2C receptors in severely pre-eclamptic women compared to normotensive women (Kusumi et al., 2006; Bueno-Sánchez et al., 2013). Bachmayer et al. (2006) supports our findings of similar percentages of peripheral NK cell NKG2A and NKG2C receptors in pre-eclamptic and normotensive pregnant women however, higher levels of both receptors were reporteded in pre-eclampsia compared with

healthy pregnant women.

Kusumi et al. (2006) found that decidual NK cells express inhibitory NKG2A and activating NKG2C cells concurrently to control trophoblast invasion in the developing placenta whereas peripheral NK cells express these receptors equally. Inhibitory NK receptors may prevent maternal rejection of a fetus and activating NK receptors may also be important because trophoblasts never excessively invade into decidua. Thus, the balance of inhibitory and activating NK receptors is crucial for the proper growth of trophoblasts in placenta (Yokoyama and Riley, 2008; Kusumi et al., 2006). The invading trophoblasts have direct contact with maternal peripheral lymphocytes hence the expression of activating NKG2C may attack the trophoblasts and prevent them from invading maternal vessels in pre-eclampsia (Kusumi et al., 2006).

4.5.5 NKG2D receptor

There was a significant difference in the stimulatory receptor NKG2D receptor between the HIV positive and negative groups (p=0.047). There was no significant difference between the normotensive pregnant and pre-eclamptic groups (p=0.074), among all pregnancy types (p=0.131) and across all six study groups (p=0.152). Bachmayer et al. (2006) supports our finding of similar percentages of peripheral NK cell NKG2D receptor in pre-eclamptic and normotensive pregnant women. However, a lower expression of NKG2D was shown in pre-eclamptic compared to the normotensive group (Sohlberg et al., 2014).

4.6 Th1 and Th2 maternal immune response

4.6.1 Th1 and Th2 response in pre-eclampsia

In pre-eclampsia, abnormal trophoblast invasion and inadequate spiral artery remodeling produces a hypoxic microenvironment with resultant increased placental oxidative stress. The resulting flux of anti-oxidant and anti-angiogenic balance contributes to systemic endothelial dysfunction augmenting the inflammatory response (Kalkunte et al., 2011; Chatterjee et al., 2011). This leads to the subsequent clinical manifestation of pre-eclampsia (Perez-Sepulveda et al., 2014). The abnormal trophoblast invasion may be attributed to the altered maternal immune response to the semi allogenic fetus which leads to the production of neutrophils and monocytes in the decidua and within peripheral blood. Consequently this stimulates the production of pro-inflammatory (Th1) cytokines whilst anti-inflammatory (Th2) cytokines are reduced (Bachmayer et al., 2006). Our study however, revealed no shift from a Th1 (IL-2, p=0.357; IL-12(70), p=0.105; IFN-γ, p=0.086; TNF-α, p=0.091) response to a Th2 (IL-4, p=0.144; IL-5, p=0.188; IL-10, p=0.446 and IL-13, p=0.334) response across the HIV positive and negative normotensive, EOPE and LOPE groups.

This study showed a significant difference in the level of GM-CSF across each of the 6 study groups. Granulocyte-macrophage colony-stimulating factor (GM-CSF) levels are elevated in the presence of HIV infection as they are responsible for stimulating monocyte derived macrophages that fight infection (Le Bouteiller et al., 2011). Chatterjee *et al.* (2011) showed that the incidence of pre-eclampsia is decreased in women with HIV infection. In addition, in normal pregnancy there is a shift from the Th1 to the Th2 maternal immune response irrespective of HIV status with the Th2 cytokines being dominant in circulation whereas in pre-eclampsia the shift does not

occur with the Th1 cytokines being dominant in circulation whilst in HIV infection there is a counter shift from the Th1 to the Th2 response with the Th2 cytokines being more prominent. Hence in HIV associated pre-eclampsia there could be a counteraction in the Th1 and Th2 cytokine levels hence a neutralization of Th1 and Th2 response (Fig. 2).

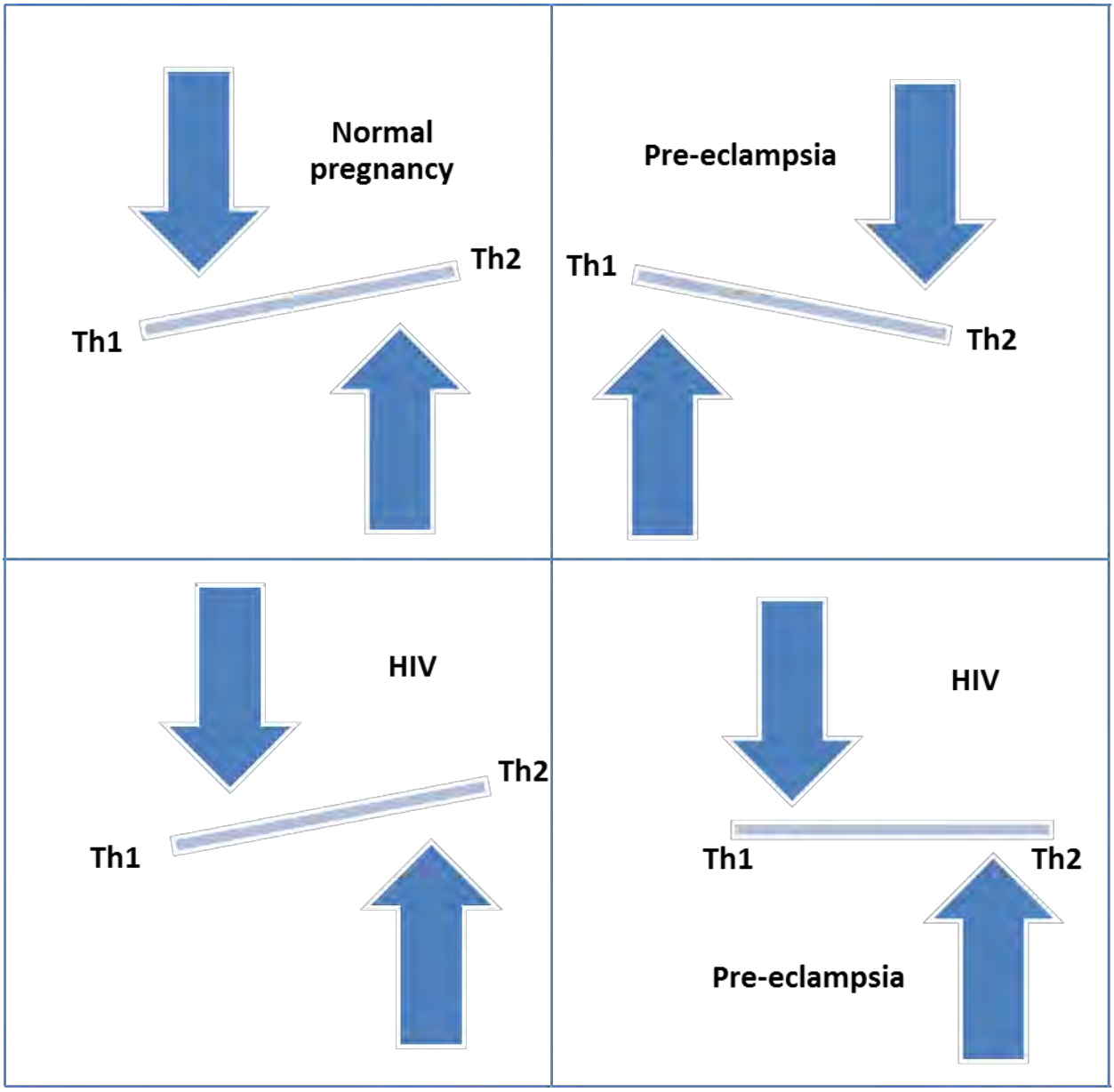


Figure 4.1: Proposed Th1 and Th2 maternal immune response in HIV+ associated pre-eclampsia. In normal pregnancy there is a shift from the Th1 to the Th2 maternal immune response with the Th2 cytokines being dominant in circulation whereas in pre-eclampsia this shift does not occur with the Th1 cytokines dominant in circulation whilst in HIV infection there is a counter shift from the Th1 to the Th2 response with the Th2 cytokines more prominent. Hence in HIV+

associated pre-eclampsia there could be a counteraction in the Th1 and Th2 cytokine levels hence a neutralization of Th1 and Th2 response.

4.6.2 Th1 immune response in pre-eclampsia

In our study, IL-12(p70) was slightly decreased in the normotensive compared to the pre-eclamptic cohort, irrespective of HIV status. Additionally, there was an increase in IL-12 (p70), in the EOPE compared to the LOPE group, these differences were also not significant. Dysregulated immune activation has been advocated as a contributor to pre-eclampsia and HELLP development (Becker, 2004). However, data are conflicting in that Mansouri *et al.* (2007) found an increased IL-12 level in pre-eclamptic women whilst Pinheiro *et al.* (2013) reporteded no difference in IL-12 between the normotensive and pre-eclamptic groups. The conflicting reporteds of IL-12 levels may be attributed to variability in gestational age at time point of collection of serum in the evolving pre-eclamptic disease etiology. Notably, the kinetics of IL-12 production and metabolism are also not fully elucidated. Our study also correlates IFN-γ increase with IL-12 (p70) elevation in all pregnancy groups (p<0.001). IL-12 promotes IFN-γ production for successful implantation and placentation (Bachmayer et al., 2009).

Albeit not significant, our study reporteds an increase in TNF-α levels at term in normotensive compared to pre-eclamptic women. Also, we show a trend of increase in TNF-α in the EOPE compared to the LOPE group, also non-significant. Many studies support our findings of no significant difference in TNF-α levels in pre-eclampsia compared to normotensive pregnancy (Becker, 2004; Pinheiro et al., 2013; Szarka et al., 2010). On the other hand, other studies have shown elevated levels in pre-eclamptic women (Alanbay et al., 2012). TNF-α is known to

promote apoptosis and systemic endothelial dysfunction which leads to oxidative stress (Vitoratos et al., 2012). Daher et al. (1999) showed low levels of TNF-α in the first trimester of normal pregnancies with an increase in gestational age. The erraticism of TNF-α levels may be confounded by gestational ages at < or > 34 weeks in the PE groups.

In a recent study, Kumar *et al.* (2013) reporteded that the levels of IL-10, TNF-α and IFN-γ between 14 and 18 weeks of gestation may act as potential biomarkers in the diagnosis of pre-eclampsia. However, other studies have reporteded elevated IFN-γ in the pre-eclamptic group compared to the normotensive group (Mansouri et al., 2007; Pinheiro et al., 2013). Opposing results have been demonstrated by others (Jonsson, 2005; Arriaga-Pizano L et al., 2005). IFN-γ is known to play an important role in decidual vascular remodeling and angiogenesis. The expression of IFN-γ by trophoblast cells is increased early in normotensive pregnancy and gradually decreases as pregnancy progresses (Kumar et al., 2013). Yet again gestational age may confound IFN-γ levels.

4.6.3 Th2 immune response in pre-eclampsia

In our study there was a decrease in IL-4 and IL-10 in normotensive patients compared to the pre-eclamptic group, albiet not significant. This Th2 cytokine decline (IL-4 and IL-10) between the normotensive and pre-eclamptic groups is similar to the reported by Kumar *et al.* (2013). Also, we reported a decrease in IL-4, and an increase in IL-10 in the EOPE compared to the LOPE group irrespective of HIV status, these differences were also not significant. Our results were obtained at term gestational ages however; increased IL-4 levels in the second trimester and puerperium have been associated with pre-eclampsia development. Pinheiro *et al.* (2013) showed

decreased IL-4 levels in pre-eclampsia compared to the normotensive group. IL-10 plays an important role in infection by reducing inflammation and tissue damage (Kalkunte et al., 2011). In pregnancy, IL-10 suppresses pro-inflammatory cytokine production at the maternal foetal interface, vascular dysfunction, endothelial dysfunction and prevents placental apoptosis (Chatterjee et al., 2011). Reduced levels of IL-10 may result in poor placentation and production of anti-angiogenic molecules, soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng) (Kalkunte et al., 2011). IL-10 levels decrease in the third trimester compared to the first and second trimesters (Kalkunte et al., 2011). Pinheiro *et al.* (2013) showed increased IL-10 levels in normotensive compared to the pre-eclamptic group. Limitations to the evaluation of IL-10 levels include its short half-life, hence it may be impossible to correctly determine the production of IL-10 in pre-eclampsia (Pinheiro et al., 2013).

4.6.4 Th1 and Th2 immune response in HIV

During HIV-1 infection changes in the Th1/Th2 cytokine balance is due to an allergic response to viral proteins which up-regulate the production of Th2 cytokines (Becker, 2004). Allergic responses and HIV-1 infection are similar in that Th2 cytokines, IL-4 induces the production of IgE antibodies which results in the depletion of CD4+ cells thereby preventing the production of IFN-γ. Also, IL-5 induces eosinophilia and enhances the replication of HIV in CD4+ lymphocytes and IL-13 which also up-regulates IgE production has been shown to reduce viral load but also block the production of IL-12 (Becker, 2004; Mikovits et al., 1994). IL-4 further prevents the production of Th1 cytokines, IL-2, IL-12 and IFN-γ which are responsible for stimulating the production of cytotoxic T cells (CTLs). These responses are different in HIV-1 infection where the interaction with viral proteins is suppressed by the immune system which

prevents the production of CTLs (Becker, 2004). However, Graziosi *et al.* (1996) found low expression of IL-2 and IL-4 in HIV positive non-pregnant patients (Graziosi et al., 1996). This study however, demonstrates no difference in Th1 and Th2 cytokines when comparing the HIV positive to the negative groups irrespective of pregnancy status. Romagnini *et al.* 1994 also does not support the Th1 to Th2 shift hypothesis proposed by Clerici and Shearer (1993), instead suggesting that HIV may prefer Th2 cytokines for replication (Kalkunte et al., 2011; Le Bouteiller et al., 2011).

4.6.5 Correlation between NK cell receptor expression and Th1/Th2 maternal serum cytokines within pregnancy groups

NK cells play an important role against pathogens and infection through their cytolytic activity and production of pro-inflammatory (Th1) cytokines (Vacca et al., 2013). NK cells also require the stimulus of pro-inflammatory cytokines such as IL-2, IL12 and IFN-γ to proliferate. In our study we aimed to determine the correlation between peripheral NK cells and their receptors with circulating Th1 and Th2 cytokines. In the normotensive pregnant group there was a simultaneous decrease in NK cells and IL-10 ($p=0.046$). These results are supported by Kalkunte et al. (2011) where IL-10 levels decreased in the third trimester compared to the first and second trimesters (Kalkunte et al., 2011). It is known that NK cells are reduced in the latter stage of pregnancy (Kane et al., 1996). In the EOPE group there was a concurrent increase in peripheral NK cells and IL-5 ($p=0.036$). Pre-eclampsia is associated with exaggerated inflammation which explains the increase in the cytotoxic peripheral NK cells in this study. IL-5 induces eosinophilia which occurs due to an exaggerated inflammatory response (Becker, 2004).

4.7 Strengths and limitations of the study

This novel study with a larger total sample population and pre-eclamptic groups compared to other studies (Mansouri et al., 2007; Pinheiro et al., 2013; Szarka et al., 2010; Kumar et al., 2013; Jonsson, 2005), showed that levels of peripheral NK cells are higher in the pre-eclamptic group compared to the normotensive group. Also, our study revealed no significant difference in Th1 and Th2 maternal serum levels in HIV positive vs HIV negative, normotensive vs the pre-eclamptic groups and in HIV associated pre-eclampsia. Unlike other studies the pre-eclamptic group in our study was stratified by gestational age (early and late onset) and HIV status (positive and uninfected). All HIV positive patients were on ARV treatment which may have played a role in restoring the maternal immune response. The duration of HIV patients on ARV treatment was unknown which may also be a limiting factor of this study. Also, the variability in maternal serum cytokine levels in pre-eclampsia may be attributed to sample type (serum or plasma) (Mansouri et al., 2007). Serum is recommended for bioassays however, cytokine levels may be affected during coagulation (Zhou et al., 2010). Patients were not sampled at different gestational ages up to delivery.

4.8 Conclusion and future research

To our knowledge, the quantification of peripheral NK cells as well as, Th1 and Th2 maternal serum cytokines in HIV associated pre-eclampsia is novel. Our study supports the hypothesis that the levels of peripheral NK cells are higher in the HIV positive pre-eclamptic group compared to the HIV positive normotensive group. Our study also suggests a neutralisation effect of Th1 and Th2 cytokines in HIV associated pre-eclampsia. Additionally, our study found that peripheral CD69 NK cells were higher in pre-eclamptic compared to the normotensive group

and GM-CSF was significantly different across each of the six study groups. Further studies may implicate CD69 and GM-CSF as possible biomarkers for early diagnosis of pre-eclampsia development with the ultimate goal of decreasing maternal and foetal morbidity and mortality rates globally.

CHAPTER FIVE

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CHAPTER SIX

APPENDICES

APPENDIX I: POSTGRADUATE PERMISSION

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

Dr M.V. Mabandla
Academic Leader Research
School of Laboratory Medicine and Medical Sciences
College of Health Sciences
University of KwaZulu Natal
Private Bag X54001
Durban
4000
email: mabandlam@ukzn.ac.za
Tel: (031) 260 8602
Fax: (031) 260 7132
16 April 2013

Dear Ms A Ajith

The role of peripheral natural killer cells in immunocompromised preeclamptic and normotensive pregnant black South Africans

This letter serves to confirm that I as the Academic Leader for Research in the School of Laboratory Medicine and Medical Sciences has approved your protocol application for the above-mentioned study. Your application has been forwarded to the UKZN Biomedical Research Ethics Committee (BREC) for final approval.

Yours sincerely

Musa Vuyisile Mabandla, PhD

APPENDIX II: INSTITUTIONAL ETHICS CLEARANCE (BE176/13)

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

13 November 2013

Miss A Ajith
1569 Sarnia Road
Queensburgh
Durban
4094
ajithk@ukzn.ac.za

Dear Ms Ajith

PROTOCOL: The role of peripheral natural killer cells in immunocompromised preeclamptic and normotensive pregnant Black South Africans. REF: BE176/13.

EXPEDITED APPLICATION

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 18 April 2013.

The study was provisionally approved pending appropriate responses to queries raised. Your responses received on 07 November 2013 to queries raised on 31October 2013 have been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given full ethics approval and may begin as from 13 November 2013.

This approval is valid for one year from 13 November 2013. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

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

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2004), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

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

The sub-committee's decision will be **RATIFIED** by a full Committee at its next meeting taking place on: **10 December 2013.**

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

Yours sincerely

Professor D.R Wassenaar
Chair: Biomedical Research Ethics Committee

APPENDIX III: DEPARTMENT OF HEALTH PERMISSION

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

Health Research & Knowledge Management sub-component
10 – 103 Natalia Building, 330 Langalibalele Street
Private Bag x9051
Pietermaritzburg
3200
Tel.: 033 – 3953189
Fax.: 033 – 394 3782
Email.: hrkm@kznhealth.gov.za
www.kznhealth.gov.za

Reference : HRKM238/14
Enquiries: Mrs G Khumalo
Telephone : 033 – 395 3189

Dear A Ajith

Subject: Approval of a Research Proposal

1. The research proposal titled **'THE ROLE OF PERIPHERAL NATURAL KILLER CELLS IN IMMUNOCOMPROMISED PREECLAMPTIC AND NORMOTENSIVE PREGNANT BLACK SOUTH AFRICANS'** was reviewed by the KwaZulu-Natal Department of Health (KZN-DoH).

The proposal is hereby **approved** for research to be undertaken at King Edward VIII & Prince Mshiyeni Memorial Hospitals.

2. You are requested to take note of the following:
   a. Make the necessary arrangement with the identified facility before commencing with your research project.
   b. Provide an interim progress report and final report (electronic and hard copies) when your research is complete.

3. Your final report must be posted to **HEALTH RESEARCH AND KNOWLEDGE MANAGEMENT, 10-102, PRIVATE BAG X9051, PIETERMARITZBURG, 3200** and e-mail an electronic copy to hrkm@kznhealth.gov.za

For any additional information please contact Mrs G Khumalo on 033-395 3189.

Yours Sincerely

Dr. E Lutge
Chairperson, KwaZulu-Natal Health Research Committee
Date: 10/09/14

APPENDIX IV: PERMISSION FROM PRINCE MSHIYENI MEMORIAL HOSPITAL MANAGER

PERMISSION TO CONDUCT A RESEARCH STUDY/TRIAL

This must be completed and submitted to the Medical Superintendent/s / Hospital Manager/s for signature.

For **King Edward VIII Hospital (KEH) and Inkosi Albert Luthuli Central Hospital (IALCH)** studies please submit the document together with the following:

1. Research proposal and protocol.
2. Letter giving provisional ethical approval.
3. Details of other research presently being performed by yourself if in the employ of KEH, (individually or as a collaborator).
4. Declaration of all funding applications / grants, please supply substantiating documentation.
5. Complete the attached KEH Form – *"Research Details"*

Once the document has been signed it should be returned to: Biomedical Research Ethics Administrator, Room N03, Govan Mbeki Building, Westville Campus, University of KwaZulu-Natal.

To: Chief Medical Superintendent / Hospital Manager

Permission is requested to conduct the above research study at the hospital/s indicated below:

Site 1 address:
PRINCE MSHIYENI MEMORIAL HOSPITAL

Investigator/s:
Principal: MISS A ATITH
Co-investigator: PROF T MAHOMED
Co-investigator: PROF J MOODLEY

Signature of Chief Medical Superintendent/Hospital Manager:
_____ Date: _____

Site 2 address:
_____

Investigator/s
Principal: _____
Co-investigator: _____
Co-Investigator: _____

Signature of Chief Medical Superintendent / Hospital Manager:
_____ Date: _____

NB: Medical Superintendent/s / Hospital Manager/s to send a copy of this document to Natalia

APPENDIX V: LETTER OF STUDY INFORMATION AND CONSENT

STUDY NO.

Ikhasi nemvume enolwazi yokuhlanganyela ocwaningweni

Usuku:

Nkosazane/Nkosikazi

Sawubona, nginguNkosazane Anushka Ajith, umcwaningi kanye nesitshudeni se-PhD eNyuvesi YaKwaZulu-Natali.

Senza ucwaningo lokuthola ukuthi yini ebangela umfutho wamandla egazi ophezulu kubantu besifazane abakhulelwe. Awunawo umfutho wamandla egazi ophezulu (i-hypertension). Ucwaningo yindlela nje yokuthola noma yokufunda impendulo embuzweni. Kulolu cwaningo, sizodinga ukuthi sithole ithisipuni legazi elivela kuwe. Ishubhu legazi elengeziwe lizothathwa kuwe ngesikhathi esifanayo nalesi okuthathwa ngaso elinye igazi elijwayelekile ukuze kuncishiswe nanoma yikuphi ukungaphatheki kahle ongahle ube nakho. Ukuthathwa kwegazi angeke kuphazamise ukukhulelwa kwakho noma umntwana wakho. Angeke siphazamise ukwelashwa kwakho. Odokotela esibhedlela bazokunika ukunakekelwa okujwayelekile.

Sicela wazi ukuthi ungakwazi ukwala ukungena ocwaningweni, angeke uphathwe ngokuhlukile kwabanye. Wena azikho izindleko ozozithwala ngokubamba iqhaza ocwaningweni. Ucwaningo olwenziwa manje angeke lukusize kulokhu kukhulelwa kodwa luyokusiza kokunye esikhathini esizayo.

Uma uvuma ukubamba iqhaza kulolu cwaningo, uzoba ngomunye weziguli eziningana ezibambe iqhaza kulolu cwaningo e-Prince Mshiyeni Memorial Hospital. Ulwazi maqondana nokubeletha kwakho luzotholakala eshadini lakho lokwelashwa bese lufakwa ekhasini lolwazi oluqoqiwe. Angeke ukhonjwe ngananoma iyiphi indlela njengoba umhlanganyeli ngamunye ezonikwa inombolo yocwaningo futhi nolwazi lwakho luyohlala lungaziwa ukuthi ngolukabani. Lonke ulwazi oluqoqiwe luzogcinwa kukhompiyutha (laptop) yomphenyi omkhulu ezovikelwa nge-password. Amakhophi abhalwe ephepheni azogcinwa ehhovisi lomphenyi elikhiywayo. Lonke ulwazi oluqoqiwe luzogcinwa isikhathi socwaningo kanye neminyaka emihlanu ngemuva kocwaningo.

Igazi lakho angeke ligcinelwe ukuhlaziywa okuqhubekayo noma ukukhishwa luyiswe kwamanye amazwe. Ngeke kwenziwe izivivinyo zofuzo egazini lakho.

Lolu cwaningo luhlolwe ngokwezimilo futhi lwavunywa yi- UKZN Biomedical research Ethics Committee (inamba yemvume BE176/13).

Uma kwenzeka uba nezinkinga noma okukukhazathayo/ imibuzo ungathintana nami kunombolo yeselula: 072 855 8414, Emsebenzini: 031 260 4750 nakwi-imeyili: ajith@ukzn.ac.za noma i-UKZN Biomedical Research Ethics Committee, imininingwane yokuthintana yile elandelayo:

BIOMEDICAL RESEARCH ETHICS ADMINISTRATION

Research Office, Westville Campus
Govan Mbeki Building
Private Bag X 54001
Durban
4000

KwaZulu-Natal, SOUTH AFRICA

Ucingo: 27 31 2604769 - Ifeksi: 27 31 2604609

I-imeyili: BREC@ukzn.ac.za

IMVUME

Mina _____ ngazisiwe ngocwaningo olunesihloko esithi “Iqhaza lamaseli abulala ngokwemvelo angabalulekile kuBantu Abamnyama abakhulelwe baseNingizimu Afrika abanomfutho wamandla egazi ophezulu abanezivikeli mzimba ezingaphenduli kahle kanye nabanomfutho wamandla egazi ojwayelekile” *(“The role of peripheral Natural Killer cells in immunocompromised preeclamptic and normotensive pregnant Black South Africans”)* nguNkosazane Anushka Ajith.

Ngiyayiqonda inhloso kanye nezinqubo zocwaningo ezenzelwa ukuthola ukuthi yini ebangela umfutho wamandla egazi ophezulu kubantu besifazane abakhulelwe. Isipuni segazi sizothathwa kuwe ngesikhathi esifanayo nalesi okuthathwa ngaso elinye igazi elijwayelekile.

Ngiye nganikezwa ithuba lokubuza imibuzo ngocwaningo futhi imibuzo yami iphendulwe ngendlela enganelisayo.

Ngiyavuma ukuthi ukuhlanganyela kwami kulolu cwaningo kungukuzithandela kwami ngokuphelele futhi ngingayeka noma nini futhi lokhu ngeke kube nomthelela ekwelashweni kwami noma kokunye ukunakekelwa kwami engivame ukuba nelungelo lokukuthola.

Ngazisiwe ngananoma yisiphi isinxephezelo esitholakalayo noma imithi yokwelashwa uma kwenzeka ukulimala kimi ngenxa yezinqubo eziphathelene nocwaningo.

Uma nginanoma yimiphi eminye imibuzo/ukukhathazeka noma imibuzo ephathelene nocwaningo ngiyaqonda ukuthi ngingathinta umcwaningi kuselula: 072 855 8414, Emsebenzini: 031 260 4750 nakwi-imeyili: ajith@ukzn.ac.za

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I-imeyili: BREC@ukzn.ac.za

_____ _____

Isignesha yomhlanganyeli Usuku

(ukuthathwa kwegazi)

_____ _____

Isignesha yomhlanganyeli Usuku

(ukuvulwa kwamafayela ami esibhedlela)

_____ _____

Isignesha kafakazi Usuku

(Lapho kufanele khona)

_____ _____

Isignesha yomhumushi Usuku

(Lapho kufanele khona)

STUDY NO.

Ikhasi nemvume enolwazi yokuhlanganyela ocwaningweni

Usuku:

Nkosazane/Nkosikazi

Sawubona, nginguNkosazane Anushka Ajith, umcwaningi kanye nesitshudeni se-PhD eNyuvesi YaKwaZulu-Natali.

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IMVUME

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_____ _____

Isignesha yomhlanganyeli **Usuku**

(ukuthathwa kwegazi)

_____ _____

Isignesha yomhlanganyeli **Usuku**

(ukuvulwa kwamafayela ami esibhedlela)

_____ _____

Isignesha kafakazi **Usuku**

(Lapho kufanele khona)

_____ _____

Isignesha yomhumushi **Usuku**

(Lapho kufanele khona)

APPENDIX VI: DATA COLLECTION TOOL

THE ROLE OF PERIPHERAL NATURAL KILLER CELLS IN IMMUNOCOMPROMISED PREECLAMPTIC AND NORMOTENSIVE PREGNANT BLACK SOUTH AFRICANS

IP/OP no: ☐ **Study no:** ☐

Category (tick): (more than 1 category may require a tick)

1. Pregnant Normotensive HIV +ve, CD4 < 350: ☐
   - HAART ☐
   - Triple regime (single tablet) ☐
2. Pregnant Normotensive HIV +ve, CD4 > 350: ☐
   - PMTCT (dual) ☐
   - Triple regime (single tablet) ☐
3. Pregnant Normotensive HIV -ve: ☐

PLEASE CHECK EXCLUSION CRITERIA LIST ☐

THE FOLLOWING BLOODS MUST BE TAKEN FOR THE PURPOSES OF THIS STUDY. PLEASE TICK IF THE BLOODS WERE TAKEN

1. **Sterile VAC 4ml K_3EDTA lavender tube** ☐
2. **Sterile VAC 5ml serum gel SST red yellow tube** ☐

General hospital information

Admission date		PMMH no	

Demographics

Age (years)			
Area of Residence (tick)	Rural	Urban	
Cigarette smoking (y/n)		No. of cigarettes/day	
Alcohol		Quantity/day	
Recreational drugs (cocaine)		Quantity/day	

Maternal treatment

Type of Treatment	Yes	No	If Yes, no. of days
Magnesium sulphate			
Aldomet			
Monoohydralazine			
Nifedipine			
Dihydralazine (nepresol)			
Labetalol			
Others			

Clinical data

<table>
<tr><td colspan="2">Parity</td><td colspan="2">P: G:</td><td colspan="2">Weeks gestation on admission</td><td></td></tr>
<tr><td colspan="7">Reason for previous pregnancy loss (If any)</td></tr>
<tr><td colspan="2">Highest BP</td><td colspan="5">Systolic: Diastolic:</td></tr>
<tr><td colspan="2">Maternal weight</td><td></td><td colspan="2">Maternal height</td><td>BMI</td><td></td></tr>
<tr><td colspan="2">Oedema (tick)</td><td>ankle</td><td>Up to knee</td><td>Up to groin</td><td colspan="2">Generalised (facial)</td></tr>
<tr><td rowspan="9">Lab results

(or attach copy of results)</td><td rowspan="3">Proteinuria</td><td colspan="5">Dipstick</td></tr>
<tr><td colspan="5">Lab 24hr protein (if done)</td></tr>
<tr><td colspan="5">Creatinine clearance (if done)</td></tr>
<tr><td rowspan="6">Full blood count</td><td>Red cell count</td><td></td><td>White cell count</td><td></td></tr>
<tr><td>Haemoglobin</td><td></td><td>Neutrophils</td><td></td></tr>
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<tr><td>Mean cell volume</td><td></td><td>Monocytes</td><td></td></tr>
<tr><td>Mean cell Hb</td><td></td><td>Eosinophils</td><td></td></tr>
<tr><td>Platelets</td><td></td><td>Basophils</td><td></td></tr>
</table>

	Urea and electrolyte	Sodium		Urea	
		Potassium		Creatinine	
		Chloride		Anion gap	
		CO2		Serum creatinine	
	Liver function tests	Total protein		Alkaline phos	
		Albumin		AST	
		Globulin		ALT	
		Alb : Glob		LDH	
		Total bilirubin			

Antenatal foetal investigations

Type (tick)	Gestational age done		Note any abnormalities
Sonar			
Doppler		RI =	
Electronic foetal HR			

Birth details

Weeks of gestation at time of birth	
Date of birth	**Time of birth**
Gender of baby	

Indication for delivery (tick one)	Maternal interest	Foetal Distress	Combination of Maternal and foetal interest.
		CTG abnormal	
		MSL	
		IUGR	
	Explain above if relevant	Explain above if Relevant	Explain above if relevant
	Diagnosis: Eclampsia, severe abruptio infection		

Method of Delivery (tick one)	Normal vaginal		Caesarean	
	Spontaneous		Elective	
	Induced		Emergency	

Complications in labour.	Eclampsia –related (tick)	Severe pre-eclampsia	Imminent eclampsia
	Abruptio-placentae		
	Other (explain)		
Mother outcome	Admitted to ICU	Death	

Baby details at birth

APGAR	1 min		5 min	
Baby (tick)	Live		Stillborn (early neonatal	

			death)	
	Neonatal death (up to 28 days)			
Baby weight (kgs)				
Admission to NICU				

FOLLOW UP DATA PRIOR TO DISCHARGE FROM HOSPITAL

Date:_____ Inpatient / Outpatient visit: _____

Oedema (tick)	ankle	Up to knee	Up to groin	Generalised (facial)

Any other observations/clinical data/information of relevance for mother:

(Maternal complications / morbidity)

_____
_____
_____
_____

Baby weight: _____ Maternal BP: _____

Feeding choice	formula	Breast	flash heating	not fed	TPN
Cranial scan					

Morbidities in early NN period

Resp Distress	HMD, TTN, Pneum ?Mas, other	
CNS	Asphyxia, meningitis	
Metabolic	hypoglycaemia, electrolyte imbalance	Other
hypothermia,		
Infections	Minor	Skin,
		eye,
		umbilicus,
		Suspected sepsis
		normal WCC +CRP
	Major	Pneumonia,
		Septicaemia (positive BC),
		meningitis (positive culture
		NEC,
		susp sepsis + low wcc and raised CRP (negative culture)

All positive cultures = severe infections. CPAP and ventilalion = severe illness

Any other observations/clinical data/information of relevance for child:

(Neonatal complications / morbidity)

_____
_____
_____
_____

FOLLOW UP DATA AFTER DISCHARGE FROM HOSPITAL

Date:_____ Inpatient / Outpatient visit: _____

Oedema (tick)	Ankle	Up to knee	Up to groin	Generalised (facial)

Baby weight: _____ Maternal BP: _____

Any other observations/clinical data/information of relevance for mother or child:

_____
_____
_____
_____

_____

HIV status of baby 6 weeks post delivery	HIV +ve (PCR)	HIV –ve
CD4+ cell count		
Baby NVP and AZT		(7 days or 28 days)
Bactrim yes/no		

Late morbidities

Neurological impairment	
BPD	
ROP	
Nutritional	

Outcomes

Alive well	
Alive ill - record morbidities as above	Minor infections, HIV related infections, ROP and Audiology if small babies (<34 weeks), feeding choices
ENND	

THE ROLE OF PERIPHERAL NATURAL KILLER CELLS IN IMMUNOCOMPROMISED PREECLAMPTIC AND NORMOTENSIVE PREGNANT BLACK SOUTH AFRICANS

IP/OP no: ☐ **Study no:** ☐

Category (tick): (more than 1 category may require a tick)

4. Preeclamptic HIV +ve, CD4 < 350: ☐

   HAART ☐

   Triple regime (single tablet) ☐

5. Preeclamptic HIV +ve, CD4 > 350: ☐

   PMTCT (dual) ☐

   Triple regime (single tablet) ☐

6. Preeclamptic HIV -ve: ☐

7. Onset preeclampsia before 33 wks ☐

8. Onset of preeclampsia at or after 34wks ☐

PLEASE CHECK EXCLUSION CRITERIA LIST ☐

<u>THE FOLLOWING BLOODS MUST BE TAKEN FOR THE PURPOSES OF THIS STUDY. PLEASE TICK IF THE BLOODS TAKEN</u>

3. **Sterile VAC 4ml K_3EDTA lavender tube** ☐
4. **Sterile VAC 5ml serum gel SST red yellow tube** ☐

General hospital information

Admission date		PMMH no.	

Demographics

Age (years)			
Area of Residence (tick)	Rural	Urban	
Cigarette smoking (y/n)		No. of cigarettes/day	
Alcohol		Quantity/day	
Recreational drugs (cocaine)		Quantity/day	

Maternal treatment

Type of Treatment	Yes	No	If Yes, no. of days
Magnesium sulphate			
Aldomet			
Monoohydralazine			
Nifedipine			
Dihydralazine (nepresol)			
Labetalol			
Others			

Clinical data

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		Platelets		Basophils	
	Urea and electrolyte	Sodium		Urea	
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		CO2		Serum creatinine	
	Liver function tests	Total protein		Alkaline phos	
		Albumin		AST	
		Globulin		ALT	
		Alb : Glob		LDH	
		Total bilirubin			

Antenatal foetal investigations

Type (tick)	Gestational age done		Note any abnormalities
Sonar			
Doppler		RI =	
Electronic foetal HR			

Birth details

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<tr><td colspan="2">Weeks of gestation at time of birth</td><td colspan="3"></td></tr>
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<tr><td colspan="2">Gender of baby</td><td colspan="3"></td></tr>
<tr><td rowspan="4">Indication for delivery

(tick one)</td><td rowspan="4">Maternal interest</td><td>Foetal

Distress</td><td colspan="2" rowspan="4">Combination of Maternal and foetal interest.</td></tr>
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Baby details at birth

APGAR	1 min		5 min	
Baby (tick)	Live		Stillborn (early neonatal death)	
	Neonatal death (up to 28 days)			
Baby weight (kgs)				
Admission to NICU				

FOLLOW UP DATA PRIOR TO DISCHARGE FROM HOSPITAL

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Any other observations/clinical data/information of relevance for mother:

(Maternal complications / morbidity)

_____
_____
_____
_____

Baby weight: _____ Maternal BP: _____

Feeding choice	formula	Breast	flash heating	not fed	TPN
Cranial scan					

Morbidities in early NN period

Resp Distress	HMD, TTN, Pneum ?Mas, other	
CNS	Asphyxia, meningitis	
Metabolic	hypoglycaemia, electrolyte imbalance	Other
hypothermia,		
Infections	Minor	Skin,
		eye,
		umbilicus,
		Suspected sepsis
		normal WCC +CRP
	Major	Pneumonia,
		Septicaemia (positive BC),
		meningitis (positive culture
		NEC,
		susp sepsis + low wcc and raised CRP (negative culture)

All positive cultures = severe infections. CPAP and ventilalion = severe illness

Any other observations/clinical data/information of relevance for child:

(Neonatal complications / morbidity)

_____
_____
_____
_____

FOLLOW UP DATA AFTER DISCHARGE FROM HOSPITAL

Date:_____ Inpatient / Outpatient visit: _____

Oedema (tick)	Ankle	Up to knee	Up to groin	Generalised (facial)

Baby weight: _____ Maternal BP: _____

Any other observations/clinical data/information of relevance for mother or child:

_____
_____
_____
_____

HIV status of baby 6 weeks post delivery	HIV +ve (PCR)	HIV –ve
CD4+ cell count		
Baby NVP and AZT		(7 days or 28 days)
Bactrim yes/no		

Late morbidities

Neurological impairment	
BPD	
ROP	
Nutritional	

Outcomes

Alive well	
Alive ill - record morbidities as above	Minor infections, HIV related infections, ROP and Audiology if small babies (<34 weeks), feeding choices
ENND	