THE ROLE OF CD69 IN HIV-ASSOCIATED PRE-ECLAMPTIC AND NORMOTENSIVE PREGNANT BLACK SOUTH AFRICANS

by

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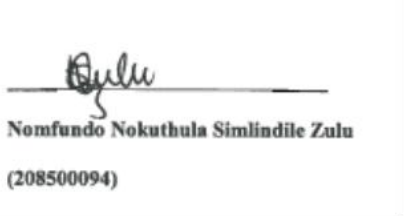
PREFACE

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 & Imaging Centre, College of Health Sciences, University of KwaZulu-Natal, South Africa under the supervision of Ms. A. Ajith, Professor T. Naicker and Professor J. Moodley



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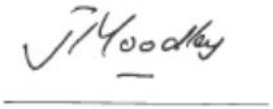

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DECLARATION

I, Nomfundo Nokuthula Simlindile Zulu declare that:

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

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DEDICATION

To my mother, Sindisiwe Gertrude Zulu

&

My daughter, Nompilo Luyanda Sindiswa Buthelezi

PEER REVIEWED PUBLICATIONS AND CONFERENCE PRESENTATIONS

- Zulu, N.N., Ajith, A., Moodley, J. and Naicker, T., 2014. The Role of CD69 in Normotensive Black South Africans. Microscopy Society of Southern Africa Proceedings, Stellenbosch, South Africa, 44, p43. (Appendix V, page 99)
- Zulu, N.N., A Ajith, A., Moodley, J. and Naicker, T., 2015. The Role of Natural Killer Cells in HIV-associated Normotensive and Pre-eclamptic Pregnancies. College of Health Science Research Symposium Proceedings, University of KwaZulu-Natal, South Africa, p. 127. (Appendix VI, page 100)

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LIST OF ABBREVIATIONS

µl	Microliters
ANOVA	Analysis of variance
ARV	Antiretroviral drugs
BMI	Body Mass Index
CI	Confidence Interval
DDMRI	Doris Duke Medical Research Institute
EOPE	Early-onset Pre-eclampsia
F	Figure of
FMO	Fluorescence Minus One
GA	Gestational age
GWG	Gestational weight gain
HAART	Highly active anti-retroviral therapy
HELLP	Elevated liver enzymes and low platelets
HIV	Human Immunodeficiency Virus
ICAM	Intracellular Adhesion Molecules
IFN-γ	Interferon-γ
IL	Interleukin
Kg	Kilograms
Kg/m^2	Kilograms per square meter
KIR	Killer Immunoglobulin-like Receptors
K-RITH	KwaZulu-Natal Research Institute for Tuberculosis and HIV
LOPE	Late-onset Pre-eclampsia
Mg	Milligrams
Ml	Milliliters
mmHg	Millimeters of mercury
NK cells	Natural Killer Cells
PE	Pre-eclampsia
PlGF	Placental Growth Factor

pNK cells	Peripheral Natural Killer cells
PPWR	Post-partum weight retention
RBC	Red blood cells
rpm	Revolutions Per Minute
SD	Standard Deviation
sFlt-1	Soluble fms-like tyrosine kinase-1
TB	Tuberculosis
TGF- β	Transforming Growth Factor-β
Th1	T-helper 1
Th2	T-helper 2
UKZN	University of KwaZulu-Natal
uNK cells	Uterine Natural Killer cells
VCAM	Vascular Cellular Adhesion Molecules
VEGF	Vascular Endothelial Growth Factor
WBC	White blood cells
WHO	World Health Organization

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ABSTRACT

The role of Natural Killer (NK) cells in pregnancy progression post implantation is inconclusive. NK cells render the maternal immune system more tolerant towards the growing fetus, yet their extensive activation may also result in failed pregnancies. CD_{69} surface glycoprotein is an early specific marker for activated NK cell. The aim of the study was to determine the role of CD_{69}-expressing NK cells in HIV-associated PE in Black South African women. This was done in an attempt to investigate potential techniques that may be used for early diagnosis of PE prior to the manifestation of clinical symptoms. The correlation between PE and presence of HIV in women will also be determined.

Following institutional ethical approval and informed consent, clinical characteristics and 4 Milliliters (ml) venous blood were collected each patient. Study consisted of 102 pre-eclamptic and 58 normotensive pregnant women recruited at Prince Mshiyeni Memorial Hospital, a regional hospital in the eThekwini region of KwaZulu-Natal, South Africa. The study groups were sub-stratified according to HIV-status, while the pre-eclamptic group was sub-stratified according to gestational age (GA) into early-onset (EOPE) and late-onset (LOPE) PE. White blood cells (WBC) were extracted from the samples and then stained with fluorochrome-conjugated CD_3, CD_{56}, CD_{16} and CD_{69} and analysed on the multicolour flow cytometer. NK cells are CD_3 negative, CD_{16} positive and CD_{56} positive. Results were assessed on the FlowJo software and exported to SPSS for statistical analysis.

There was no significant difference for maternal age, weight, body mass index, parity and gravidity based on HIV status, pregnancy type and across study groups. GA and birth weight differed significantly between pre-eclamptic and normotensive pregnancies, but not according to HIV status. Birth weight was higher in the normotensive compared to LOPE and EOPE group ($p< 0.001$). GA was lowest in the EOPE compared to normotensive and LOPE groups ($p< 0.001$).

Lymphocyte count was significantly different based on HIV-status as it was higher in HIV-positive compared to HIV-negative participants ($p= 0.002$). There were lower levels of lymphocytes in normotensive participants compared to EOPE and LOPE ($p< 0.001$). Additionally, the highest levels of lymphocytes were seen in the HIV-positive LOPE study group ($p< 0.001$). A significant difference was observed in the amount of CD_3 negative lymphocytes (NK cells) between the HIV-positive and HIV-negative group ($p= 0.011$). There were higher levels of CD_3 negative cells in the HIV-positive compared to the HIV-negative group. CD_3 negative NK cells were higher in the pre-eclamptic compared to the normotensive group ($p< 0.001$). Additionally, this study established high levels of the CD_3 negative NK cells in the HIV-positive EOPE study group ($p< 0.001$).

Uterine NK (uNK) cell levels were not different according to HIV status. In contrast, peripheral NK (pNK) cells differed between the HIV groups ($p=0.005$), being higher in the HIV-negative compared to the HIV-positive group. Moreover, there were no significant differences observed between the pregnancy types and study groups with regard to both NK

cell subsets. There was no significant difference in the level of CD_{69}-expressing NK cells in both the NK cell subgroups with respect to HIV status. With regard to pregnancy type, only the pNK cell subgroup showed significant difference in levels of CD_{69} positive cells irrespective of HIV status (p= 0.009). CD_{69} positive pNK cells were the highest in the EOPE group compared to the LOPE and normotensive pregnant group.

The results of this study show an elevation of CD_{69} positive pNK cells in HIV-associated pre-eclamptic pregnancies compared to normotensive pregnancies. This could be due to the dual paradigm of its immune-regulatory function or its contribution to the cytotoxic effect of NK cells. Moreover, with further research CD_{69} may be used as an early biomarker for PE development before the onset of clinical symptoms.

CHAPTER 1

INTRODUCTION

1.1. Pre-eclampsia (PE)

PE is a human specific disorder defined as sustained new onset of systolic blood pressure ≥140 Millimeters of mercury (mmHg) and diastolic blood pressure ≥90 mmHg at least 4 hours apart, and proteinuria after 20 weeks' gestation in a previously normotensive patient (Moodley 2013). Proteinuria is defined as urine dipstick protein of 1+ or more (on at least two occasions) or a 24 hour urine protein of at least 300 Milligrams (mg) (Powe, Levine et al. 2011, Moodley 2013). The exact aetiology of PE remains elusive. PE remains a vital area of research due too the devastatingly high maternal and neonatal morbidity and mortality statistics.

PE may be described as a disorder emerging first from poor placentation and then presenting itself as a multi-system maternal syndrome (Powe, Levine et al. 2011, Walker 2011). Delivery of the placenta results in resolution of the disorder hence most of the aetiology of this disease lies within the placenta (Steegers, von Dadelszen et al. 2010). PE presents with various clinical features and does not manifest itself uniformly, making it difficult to diagnose and treat (Powe, Levine et al. 2011, Walker 2011).

PE has a wide clinical spectrum and in most circumstances presents as a mild disease with few clinical signs (Young, Levine et al. 2010, Trogstad, Magnus et al. 2011). In severe cases of PE women may develop headaches or visual changes, pain in the upper abdomen arising from acute liver injury, pulmonary oedema, acute renal failure, haemolysis and/or thrombocytopenia (Young, Levine et al. 2010). PE mostly affects systemic blood vessels, and in this way may be seen affecting the functioning of critical organs including the liver and brain. The effects of this disease may be seen long after pregnancy.

Immune maladaptation has been largely associated with the pathogenesis of this syndrome. This study examines activated pNK cells, a vital component of the innate immune system. It aims to investigate the involvement of pNK cells in pathological pregnancies, an area of research that has not been fully explored. The information produced will contribute towards understanding immune maladaptation in PE.

1.1.1. PE epidemiology

Globally, approximately 2-8% of pregnant women develop PE (Ghulmiyyah and Sibai 2012, Rebelo, Schlüssel et al. 2013). Most of PE diagnoses occur in developing countries like South African (see figure 1.1). PE is considered the most commonly encountered medical complication during pregnancy, and is also associated with increased morbidity in both mother and child post-partum. (Steegers, von Dadelszen et al. 2010, Walker 2011).

In 2010, PE was reported to be the third leading cause of maternal deaths in USA accounting for about 16-20% maternal deaths (Young, Levine et al. 2010, Ghulmiyyah and Sibai 2012). In South Africa, the latest Confidential Enquiries into Maternal Deaths between 2011-2013 reports that non-pregnancy related infections, obstetric haemorrhage and hypertension accounts for above 65% of maternal deaths (Pattinson 2014). Moreover, most of the maternal deaths arising from hypertension are directly linked to PE.

The Millennium Development Goal recommended an attainment of 38 deaths per 100 000 live births by the year 2015. Currently, South Africa has 310 deaths per 100 000 live births. This is unacceptably high as similar low to middle income countries like Brazil have 70 deaths per 100 000 deliveries. Problems in accessing professional medical care and late prenatal care are the main reasons for the high number of maternal and neonatal mortality and morbidity in South Africa (Young, Levine et al. 2010, Hall, Gebhardt et al. 2013). Many lower income countries have poor resource settings and there is a lack of patient education on this disorder compared to high income countries (Steegers, von Dadelszen et al. 2010, Powe, Levine et al. 2011). Additionally, the incident of new cases of PE are increasing globally due to an increased prevalence of predisposing disorders such as chronic hypertension, diabetes, and obesity especially in developing countries (Steegers, von Dadelszen et al. 2010).

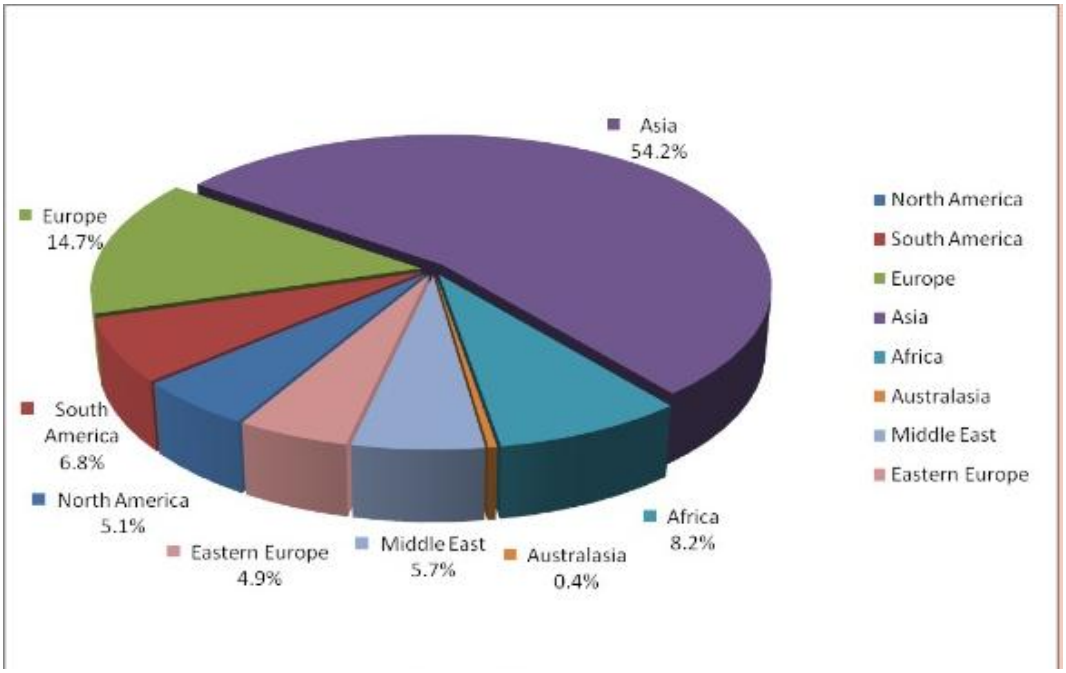

Figure 1.1: Distribution of incidences of pre-eclampsia in the world. (http://cureresearch.com/pre-eclampsia/ Accessed on the 15 March 2016)

1.1.2. PE classification

- PE can either be classified as early-onset PE (EOPE) or late-onset PE (LOPE) depending on when PE developed during pregnancy. EOPE usually develops before 34 weeks of gestation, while LOPE develops at or after 34 weeks of gestation (Li, Chen et al. 2014). EOPE affects about 5-20% while LOPE affects about 80% of pregnant women (Huppertz 2008). EOPE is regarded as a foetal disorder defined by placental dysfunction and more adverse maternal and neonatal outcomes, while LOPE is considered to be a maternal disorder resulting from underlying maternal disorders (Raymond and Peterson 2011).

- PE can also be classified as a mild, moderate or severe depending on the severity of the clinical characteristics. Mild and moderate PE occurs in most of the women who develops the disorder.

  Severe PE may also occur in variant forms in different women. One form of severe PE is the haemolysis, elevated liver enzymes and low platelets (HELLP) syndrome, which occurs in about 20% of women with severe PE (Young, Levine et al. 2010, Powe, Levine et al. 2011). The HELLP syndrome affects the liver function and the coagulation system, where some of the women may develop disseminated intravascular coagulation which is dangerous for both mother and foetus (Young, Levine et al. 2010, Powe, Levine et al. 2011). Another form of severe PE is eclampsia, this is a type of severe PE characterised by the occurrence of convulsions (Ghulmiyyah and Sibai 2012). Eclampsia has a high mortality rate for both the mother and the foetus, and is seen to have a higher incident rate of about 10-30 times in developing countries than in high income countries (Steegers, von Dadelszen et al. 2010, Ghulmiyyah and Sibai 2012). Most of the deaths seen in eclampsia are due to cerebrovascular complications and other complications of the blood vessels (Powe, Levine et al. 2011). Furthermore, severe PE is a major cause of profound maternal morbidity including stroke, renal failure and liver rupture (Powe, Levine et al. 2011, Walker 2011). Severe PE is also detrimental to the growing foetus and may cause adverse perinatal outcomes such as prematurity and intrauterine growth restriction (Powe, Levine et al. 2011, Walker 2011).

1.1.3. Risk factors

The actual aetiology of PE is unknown, yet various factors have been implicated in increasing the risk of its development:

- Geographical altitude- PE rates are higher in women living in higher altitudes because of the lower atmospheric oxygen content, introducing hypoxic conditions (Powe, Levine et al. 2011). Placental development and the functionality of the placenta are affected by these hypoxic conditions, predisposing the women to a higher risk of developing PE (Powe, Levine et al. 2011).

- History of PE- A personal or family history of PE significantly increases the risk of PE development (Steegers, von Dadelszen et al. 2010).

- First pregnancy- The risk of developing PE is as high as 7.5% during the first pregnancy. This has led to classifying PE as a disorder of nulliparous women (Young, Levine et al. 2010).

- Parity- Primiparity is the cardinal risk factor for PE development. With multiparous women, the risk of developing the disease in following pregnancies is greater if the disease was seen early in their previous pregnancy (Trogstad, Magnus et al. 2011, Nawaz, Sultan et al. 2014).

- New paternity- PE is also associated with multiparous women especially those with a new partner, due to a limited exposure to the new paternal antigens (Basso, Christensen et al. 2001).

- Maternal age- Most studies have shown that PE is most highly seen in very young (<18 years) and very old women (>40 years) of child barring age (Cleary-Goldman, Malone et al. 2005).

- Obesity- The risk of PE is higher in obese women compared to women with normal weight (Trogstad, Magnus et al. 2011).

- Multiple pregnancy- PE is more common in women who are carrying twins, triplets or other multiples. Increased placental mass, mole development within the uterus and a multifetal pregnancy are seen as a threat resulting in increased risk of developing this condition (Duckitt and Harrington 2005).

- Interval between pregnancies- Having babies less than two years or more than 10 years apart leads to a higher risk of PE development (Skjærven, Wilcox et al. 2002).

- History of metabolic conditions- Conditions such as chronic high blood pressure, cardiovascular diseases, migraine headaches, type 1 or type 2 diabetes, kidney disease, a tendency to develop blood clots, or systemic lupus erythematosus increases the risk of PE development (Duckitt and Harrington 2005, Steegers, von Dadelszen et al. 2010)

- Use of contraceptives during sexual intercourse is also seen as a pre-disposing factor in the development of PE (Powe, Levine et al. 2011).

- Hormonal factors- Circulation of a higher or lower amount of hormones needed for the progression of pregnancy have been associated with increased risk of PE development. Various hormones involved in the dilation of blood vessels are lowered in pre-

eclamptic women, causing vasoconstriction and restricting placental perfusion (Lam, Lim et al. 2005).

- Smoking- In a study done by England *et al* (2002) smoking was labelled as a protective mechanism against PE development. This fact can be intensively debated as smoking may indirectly increase the risk of the woman acquiring predisposing disorders of PE like cardiovascular diseases.

- Ethnicity- Some ethnic groups and communities of low socioeconomic status are at a higher risk of developing PE (Steegers, von Dadelszen et al. 2010, Ghulmiyyah and Sibai 2012).

- Nutritional factors- Some dietary preferences may make the mother more susceptible to developing the disorder (Trogstad, Magnus et al. 2011).

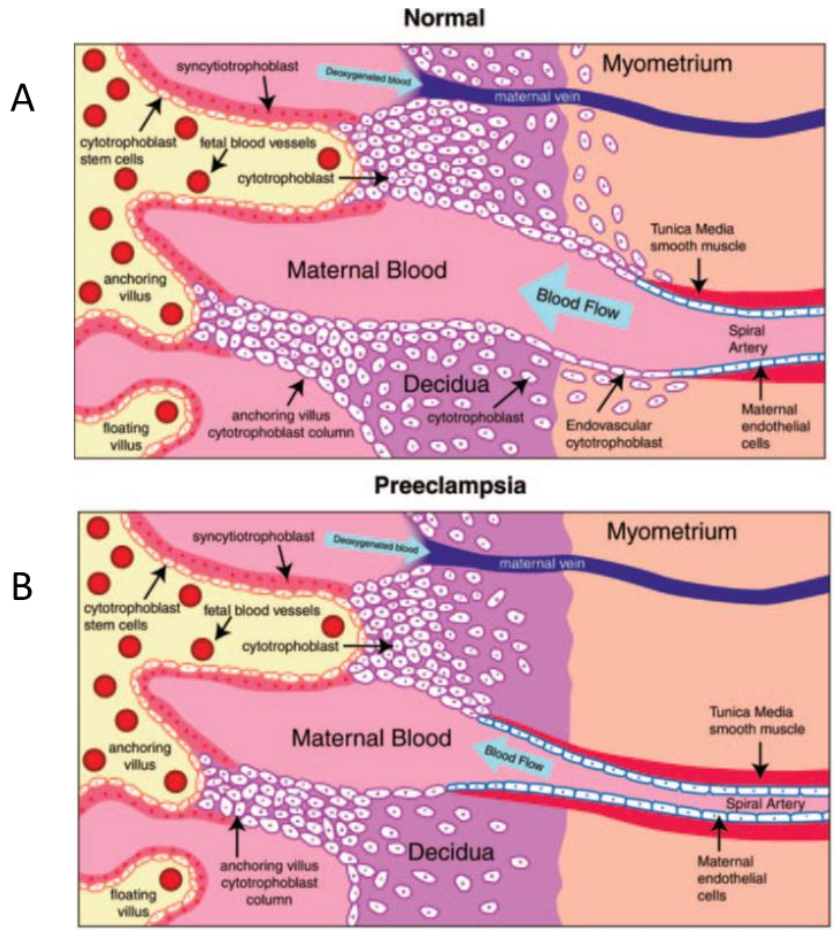

Figure 1.2: A. represents events leading to normal placentation and B. illustrates events leading to abnormal placentation, its consequences on the maternal blood vessels and blood flow. In normal placental development, cytotrophoblasts originating from the anchoring villi invade the maternal spiral arteries ensuring its dilation for maximum blood supply to the foetus (Naicker, Khedun et al. 2003, Lam, Lim et al. 2005).

1.1.4. Pathogenesis of PE

1.1.4.1. Abnormal placentation

The key purpose of the placenta is the transfer of nutrients and vital gases to the developing foetus, and also to eliminate waste from the foetus through the mother's blood supply. Based

on this, it is seen that the placenta plays an important role in the success of the pregnancy (Mosimann, Wagner et al. 2013). Evaluation of the placenta from women with mild and severe PE have revealed several abnormalities within the placenta, including infarcts, prolonged inflammation, atherosclerosis and blood clots (Powe, Levine et al. 2011).

In normal pregnancy, trophoblast cells migrate from the tips of the anchoring villi, passing through the decidua and the inner myometrium in a set timed sequence (Lam, Lim et al. 2005). Congruently, extra villous trophoblast cells invade the maternal spiral arterioles and remodel them into large-capacitance, low-resistance vessels (Naicker, Khedun et al. 2003, Trogstad, Magnus et al. 2011). The muscular wall of the artery is replaced with a fibroid type material in which are embedded trophoblast cells. Additionally the endothelial phenotype changes (Naicker, Khedun et al. 2003, Ghulmiyyah and Sibai 2012). This conversion is referred to as a physiological change, ensuring a four-fold blood supply to the growing foetus (Naicker, Khedun et al. 2003).

In PE, trophoblast migration of spiral arteries is limited to the decidua. There is no physiological conversion of spiral arteries within the myometrium, and the cytotrophoblasts fails to adopt an invasive endothelial phenotype (Naicker, Khedun et al. 2003). This results in failure for the lumen of the arteries to modify into large calibre arteries (Moodley 2013). This lack of physiological transformation does not ensure maximal blood supply to and from the baby. The lack of oxygen supplied to the placenta creates a hypoxic environment with resultant oxidative stress and a release of various anti-angiogenic factors (Moodley 2013).

1.1.4.2. Angiogenic imbalance

Blood vessels are vital in the succession of pregnancy, as these are the main network between the maternal system and the growing foetus (Shibuya 2008). For the foetus to survive; nutrients, oxygen and other compounds have to be transported between the maternal system and the growing foetus through the blood vessels (Shibuya 2008, Wang, Rana et al. 2009).

In PE, the hypoxic microenvironment causes the placenta to release high levels of anti-angiogenic factors and low levels of pro-angiogenic factors, thus disrupting the maternal endothelium (Young, Levine et al. 2010). The reason for the modification from the usual angiogenic stability into an anti-angiogenic state may be linked to the oxidative stress arising from the ischaemic conditions (Young, Levine et al. 2010). This shift is said to be the main cause of the maternal hypertensive syndrome, proteinuria as well as other clinical signs of PE and eclampsia (Lam, Lim et al. 2005, Young, Levine et al. 2010).

Various angiogenic factors and cytokines play a vital role during blood vessel formation (angiogenesis) and maintenance. Angiogenic factors and cytokines that are important for the formation and maintenance of blood vessels in pregnancy include: Vascular Endothelial Growth Factor (VEGF), Endocrine Gland derived Vascular Endothelial Growth Factor (EG-VEGF), Transforming Growth Factor-β (TGF- β) and Placental Growth Factor (PlGF). These factors and cytokines are pro-angiogenic and will support the formation of blood vessels during pregnancy. Deficiency and improper balance between these angiogenic factors has detrimental effects on the progression of pregnancy (Naicker, Khedun et al. 2003, Wang, Rana

et al. 2009, Powe, Levine et al. 2011). Prior studies have also shown that in diseased states the placenta releases excessive amount of anti-angiogenic factors into the maternal blood resulting in the distinctive characteristics of PE both locally and systemically (Young, Levine et al. 2010). Anti-angiogenic factors that are released during PE include: endothelin-1, fibronectin, soluble endoglin (sEng), and soluble fms-like tyrosine kinase-1 (sFlt-1). During PE these anti-angiogenic factors inhibit the action of pro-angiogenic factors by binding to them, preventing the binding of pro-angiogenic binding to endothelium receptors like endoglin receptors (ENG) TGF- β receptors (Alk5) (see figure 1.3).

a) Vascular endothelial growth factors (VEGF)

VEGF is a pro-angiogenic factor involved in the formation of new blood vessels in the embryo and the placenta for the advancement of pregnancy (Shibuya 2008, Powe, Levine et al. 2011). VEGF also plays an important role in established blood vessels and tissues of other organs to maintain efficient blood flow (Lam, Lim et al. 2005, Powe, Levine et al. 2011). VEGF is involved in the succession of endothelial cells and in making endothelial cells more permeable (Wang, Rana et al. 2009). In some organs, for example the kidney and heart, VEGF may have vasodilator effects involving it in the control of the systemic blood pressure thus the control of hypertension (Powe, Levine et al. 2011). The incorrect expression or/and deletion of the gene responsible for VEGF production may cause death of the foetus at the embryonic phase (Shibuya 2008, Wang, Rana et al. 2009). This may be due to irregular formation of blood vessels in both embryo and placenta in the early stages of pregnancy. In the maternal body, VEGF irregularities affect multiple organs including the brain, liver and kidney (Powe, Levine et al. 2011).

VEGFs bind to receptors present on vascular endothelial cells to modulate and regulate the endothelial cells (Figure 1.2) (Shibuya 2008). The binding of VEGFs to endothelial cells maintains their integrity and wellbeing and facilitates the relaxation of blood vessels, preventing both the proteinuria and hypertension (Wang, Rana et al. 2009). In some organs like the kidney, VEGFs are involved in maintaining the fenestrated nature of endothelial cells (Lam, Lim et al. 2005). Presence of abnormal VEGF molecules in the kidney affect the fenestrated nature of the endothelial cells, making them incapable to perform their function of preventing large molecules like proteins to filter through into the urine. Therefore, imbalance of the expression of pro-angiogenic and antiangiogenic factors also plays an important role in the development of proteinuria, which is an important characteristic in diagnosing PE (Wang, Rana et al. 2009, Powe, Levine et al. 2011).

b) Transforming Growth Factor- β (TGF-β)

TGF-β plays different roles in different tissues and is not fully understood in its functions. In angiogenesis, absence of TGF-β and its isoforms have harmful effects on the development of new blood vessels in both the embryo and placenta, and in most to all cases results in the death of the foetus (Wang, Rana et al. 2009, Powe, Levine et al. 2011). Most studies have also implicated TGF-β in the trans-signalling regulation of VEGF, moreover indicating its importance in angiogenesis (Wang, Rana et al. 2009, Powe, Levine et al. 2011). In mature and newly formed blood vessels, the facilitated binding of TGF-β is involved in the homeostasis and vasodilation of the blood vessels (Figure 1.2) (Powe, Levine et al. 2011).

c) Placental Growth Factor (PlGF)

During pregnancy the placenta also releases PlGF (Lam, Lim et al. 2005). Absence of PlGF is seen to have no adverse effects on the maternal body (Powe, Levine et al. 2011). In instances like myocardial infarction and wound healing absence of PlGF may have detrimental effects, suggesting that this growth factor is important for angiogenesis in the pathological conditions (Wang, Rana et al. 2009). In pregnancies without any complications the function of PlGF is not fully understood.

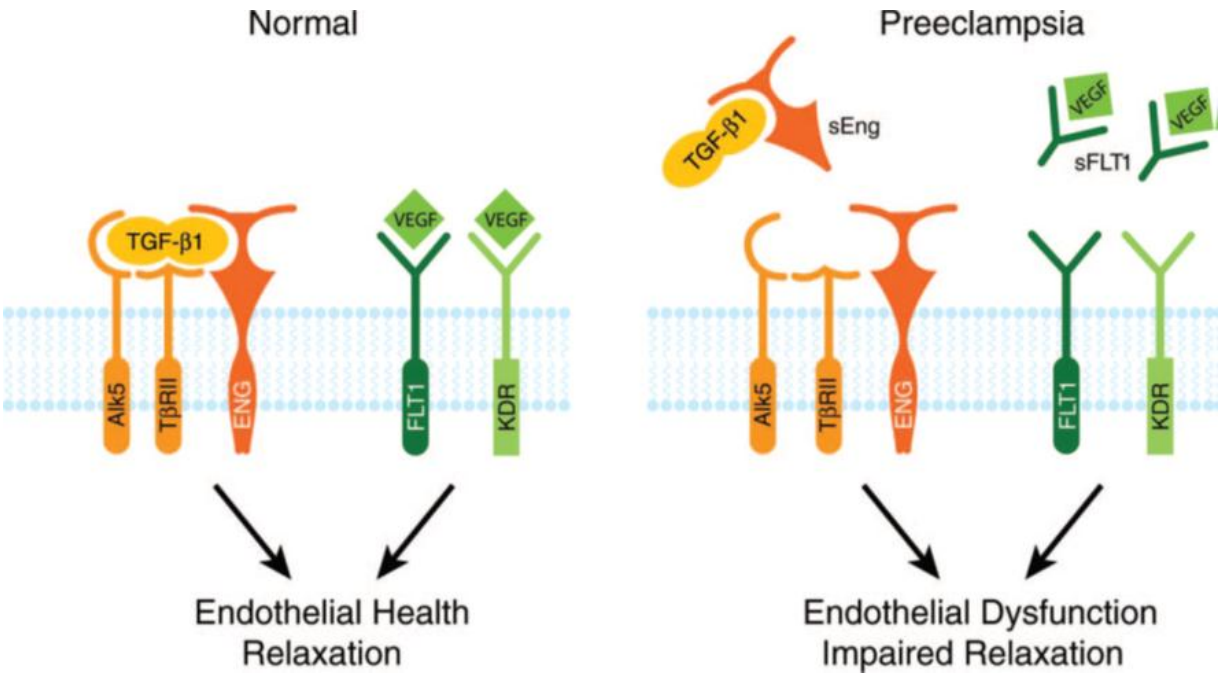

Figure 1.3: The role of angiogenic factors in normotensive pregnancies and in pre-eclamptic pregnancies (Powe, Levine et al. 2011).

1.1.5. Clinical features of PE

PE is very complicated in its manifestation and diagnosis. Hypertension and proteinuria are at a diagnostic level in pregnancy when systolic blood pressure is ≥140 mmHg, diastolic blood pressure is ≥90 mmHg and there is ≥300 mg of proteins in a 24 hour urine specimen (Young, Levine et al. 2010, Moodley 2013). In the absence of one or both of the clinical features, laboratory testing of organ functionality may be used to diagnose existence of PE in pregnant women (Powe, Levine et al. 2011). Traditionally oedema was also used as a diagnostic triad of PE. This was found to be problematic as most normotensive women also present with oedema during their pregnancy, this is as oedema is not only specific to pre-eclamptic patients (Young, Levine et al. 2010). Presentation of glomerular endotheliosis and HELLP in pregnant women may also be seen as a sign of the existence of PE (Young, Levine et al. 2010, Powe, Levine et al. 2011). Another clinical feature that has been associated with PE is podocyturia, this is the shedding of viable amounts of podocytes in urea (Powe, Levine et al. 2011).

1.1.6. Management of PE

A pharmaceutical cure for the disease has not been developed to date, and administration of therapeutics/drugs to pregnant women may be associated with teratogenic effects on the neonate. Early detection of the clinical characteristic of PE (hypertension and proteinuria), delivery of the placenta, and management of the hypertension through maternal diet and exercise still remain the fundamental approach to resolve the disease and prevent serious consequences of PE (Young, Levine et al. 2010, Trogstad, Magnus et al. 2011, Walker 2011).

A decision of induced premature delivery is mostly used in developed countries to protect the health of the mother (Young, Levine et al. 2010, Powe, Levine et al. 2011). Yet, the method of premature delivery has shown to have adverse results in the survival of the neonate and may result in low birth weight, morbidity and mortality (Young, Levine et al. 2010, Powe, Levine et al. 2011). This may be due to inadequate development of the foetus as it has not reached its full developmental ability.

The lack of medications to treat PE requires that the foetus is closely monitored using ultrasound examination and foetal heart rate testing to ensure the survival of the foetus (Young, Levine et al. 2010). The maternal blood pressure, heart rate and urine protein are closely supervised to ensure that the disease does not manifest into any other conditions that may be fatal to the mother or the foetus (Steegers, von Dadelszen et al. 2010, Powe, Levine et al. 2011). It is advisable to monitor both mother and child post-delivery to prevent complications, morbidity and mortality (Moodley 2013). In the case of eclampsia, doctors are forced to administer treatments that may be detrimental to the foetus due to the severe implications of this condition. Pregnant women who are eclamptic are given magnesium sulfate prophylaxis to seize the occurrence of convulsions (Powe, Levine et al. 2011).

The clinical symptoms of PE are seen only after 20 weeks of gestation. This fact brings about a need to use other methods of diagnosis, thus assisting in the management of the disorder. Biological molecules have been proposed as early biomarkers for PE, these are seen to be

elevated in pre-eclamptic pregnancies before the onset of clinical indicators. Biological molecules that have been proposed include:

1) CD_{69} receptor: an early specific marker for activated NK cells (Lund, Boysen et al. 2012).

2) Lipid concentrations and C-reactive protein: inflammatory cytokines that are increased in over weight and obese women (Rebelo, Schlüssel et al. 2013).

3) Long pentraxin receptor: a soluble pattern recognition receptor that is up-regulated in inflammatory responses (Than, Romero et al. 2008).

4) Soluble endoglin and sFlt-1: anti-angiogenic factors present in high amounts in pre-eclamptic women (Lam, Lim et al. 2005).

1.2. HIV and PE

South Africa represents a unique and ideal site to study co-morbidities of both PE and HIV. Non pregnancy related female infections (predominately HIV) and hypertension (predominately PE) contribute to about 50% of maternal deaths in South Africa (Pattinson 2014). Moreover; the province of KZN, in which this study was conducted, is considered the epicentre of the global HIV pandemic.

Data that is available on the effect of HIV infection on the progression of PE is inconsistent. Some studies have found that PE is less common in HIV infected women compared to the general population, this is linked to the lowered immune-reactivity associated with HIV

infection (Hall, Gebhardt et al. 2013, Kalumba, Moodley et al. 2013). In contrast, a study by Boyajian *et al* (2012) indicated that HIV-positive woman developed a more severe form of PE due to their dysfunctional immune system. To date, there has been no agreement between HIV infection and PE development and progression.

1.3. Immune system in pregnancy

In normal pregnancy a mild inflammatory response is seen due to the foreign paternal pathogens (Mor, Cardenas et al. 2011, Mosimann, Wagner et al. 2013). In PE the inflammatory response is exaggerated leading to maternal intolerance towards the pregnancy. This maternal intolerance is seen as one of the aetiologies of PE, associating the immune system to the development of PE (Steegers, von Dadelszen et al. 2010, Walker 2011).

The human immune system is divided into two intercalated systems; the adaptive immune system and the innate immune system. The adaptive immune system is said to be the more specific reaction, using a larger spectrum of receptors (Castle 2000, Vivier, Raulet et al. 2011). It consists of the cellular and a humoral response, and is able to create memory cells that assist in a more rapid response upon encountering the same antigen (Castle 2000, Vivier, Raulet et al. 2011). The innate immune system is considered to be the less studied component, it is regarded to be more rapidly reactive and consists of a cellular and a non-cellular compartment (Castle 2000, Vivier, Raulet et al. 2011).

The focus of this study is to investigate a component of the innate immune system, the NK cells. NK cells are an important component of the innate immune system, these cells are key players as the first defence against viral infections and tumour development (Vacca, Moretta et al. 2011, Vivier, Raulet et al. 2011). NK cells also play a crucial role in development of normal pregnancy (Vacca, Moretta et al. 2011). The function of NK cells in pregnancy have been extensively debated among researchers. On one hand, NK cells are supportive in the progression of normal pregnancy. Alternatively, increased activated NK cell counts may contribute to increased foetal and maternal deaths as well as failed in vitro fertilization attempts (Thum, Bhaskaran et al. 2004, Mosimann, Wagner et al. 2013). This is as the activated NK cells may recognize the paternal antigens as foreign harmful materials and activate an inflammatory response.

1.3.1. Cytokines involved in pregnancy

For a proficient reaction of the immune system, immune cells of both the adaptive and innate system have to work collectively to attempt to eliminate infections. Interaction between the different immune cells is accomplished by hormone-like proteins known as cytokines (Castle 2000, Vivier, Raulet et al. 2011). Cytokine are important in promoting and/or halting the inflammation response, and are available in varying biochemical structures (Lund, Boysen et al. 2012). These proteins play an important role in the immune system, and are specific in their role profile (Vivier, Raulet et al. 2011).

During pregnancy there is production of a variety of cytokines which supports the progression of the pregnancy. In a normal pregnancy, cytokines produced are said to up-regulate a T-helper 2 (Th2) immune response, where as in pre-eclamptic pregnancies cytokines produced promote a T-helper 1 (Th1) immune response (Figure 1.3) (Castle 2000). The Th1 response is more of an acquired immunity and utilises cell-mediated reactions to eliminate most pathogenic antigens (Hosono, de Boer et al. 2003). Th1 involved cytokines and interleukins (IL) are described as cytokines that promote the inflammatory response, these include IL-2, IL-12, Tumour Necrosis Factor-α and Interferon-γ (IFN-γ) (Lund, Boysen et al. 2012, Jewett, Man et al. 2013). The Th2 immune response is less efficient in eliminating infections, and is associated with a higher amount of anti-inflammatory cytokines including IL-4, IL-5, Il-10 and IL-13 (Castle 2000, Hosono, de Boer et al. 2003).

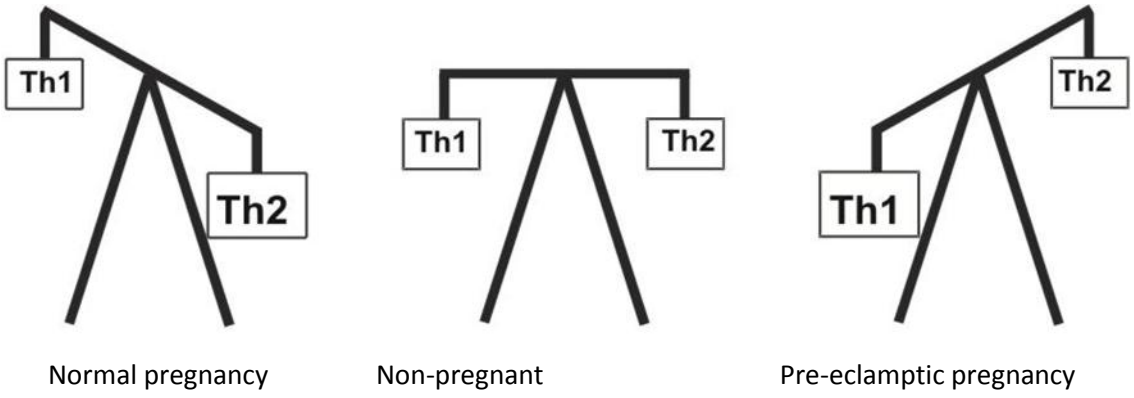

Figure 1.4: Different immune responses between the different pregnancy states.

IL-15 and IL-2 are cytokines with overlapping properties responsible for the activation of NK cells (Vivier, Raulet et al. 2011, Lund, Boysen et al. 2012). IL-15 and IL-2 are responsible for NK cell development, survival and activation in order for the NK cells to perform their

function (Vivier, Raulet et al. 2011). IL-15 is produced in multiple tissues by monocytes, macrophages and dendritic cells, while IL-2 is produced by activated lymphatic T-cells (Vacca, Moretta et al. 2011, Vivier, Raulet et al. 2011). In humans; IL-2 is seen to be more abundant then IL-15, performing most of the functions responsible for the activation of NK cells (Lund, Boysen et al. 2012). Activation of NK cells release various cytokines including IFN-γ and IL-8 (Vivier, Raulet et al. 2011). The release of IFN-γ and IL-8 by activated NK cells contribute in tissue and spiral artery remodelling as well as the formation of new blood vessels (Vacca, Moretta et al. 2011, Vivier, Raulet et al. 2011).

During pregnancy the placenta releases various cytokines including IL-4 and IL-18 to modulate NK cells to lose their killing capabilities, thus assisting in the progression of pregnancy by protecting the paternal antigens (Roussev, Dons'koi et al. 2013). The modulated NK cells express properties of the uNK cells, alternatively making the population of uNK cells within the uterus greater.

Other cytokines important for the functioning of NK cells include Intracellular Adhesion Molecules (ICAM) and Vascular Cellular Adhesion Molecules (VCAM). ICAMs and VCAMs are chemotactic factors, responsible for the migration of NK cells into tissues and secondary lymphoid organs (Vivier, Raulet et al. 2011). These chemotactic factors are important for recruiting NK cells to sites where they are needed the most. In pregnancy these chemotactic factors assist in the recruitment of pNK cells into the uterus to form uNK cells (Tang, Alfirevic et al. 2011).

1.3.2. NK cell sub-groups

NK cells may be divided into two sub-groups, uterine Natural Killer (uNK) and peripheral Natural Killer (pNK) cells. Both these subsets of NK cells play a vital role in the success of pregnancy. The uNK play an important role in implantation, other placental functions and foetal survival necessities (Mosimann, Wagner et al. 2013). The pNK cells play an important role in the maternal systemic blood stream.

Difference between the two sub-groups of NK cells is mostly seen in their cytotoxicity levels. uNK cells are said to be less cytotoxic and produce more cytokines compared to pNK cells (Figure 1.4) (Mosimann, Wagner et al. 2013). During pregnancy, changes in the maternal body are required for supporting the pregnancy, demanding a change in the systemic immune system (Tang, Alfirevic et al. 2011). To facilitate pregnancy uNK cells are seen to be more predominate in the uterus, this is to modulate the body to be more tolerant of the paternal antigens (Mosimann, Wagner et al. 2013).

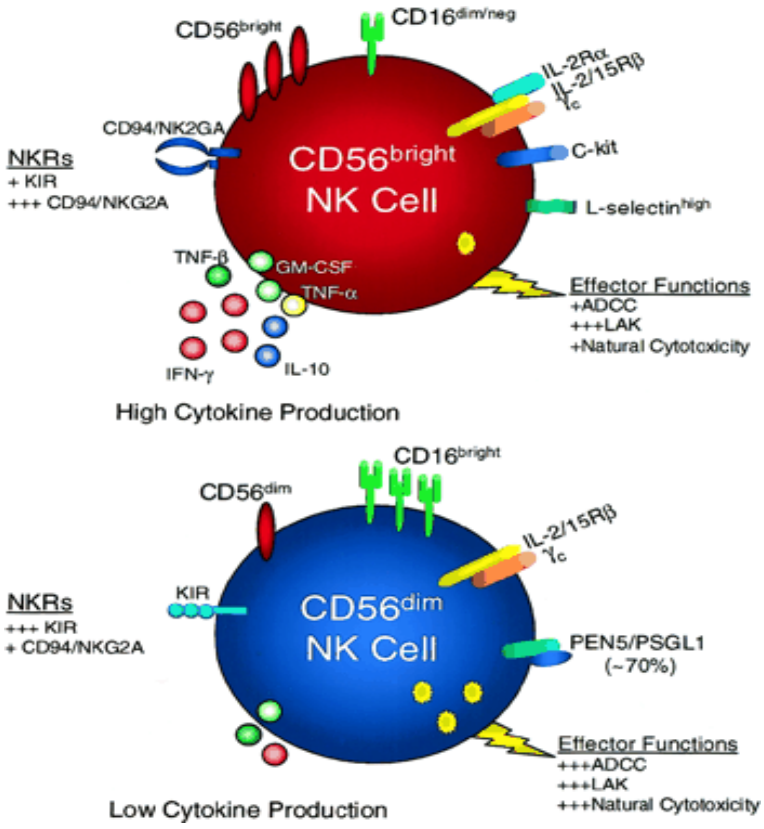

Figure 1.5: Differences in uNK ($CD_{56}^{Bright)}$ and pNK (CD_{56}^{Dim}) cells. uNk cells show to be less cytotoxic compared to pNk cells considering the amount of cytokine they release and the surface glycoproteins they display (Cooper, Fehniger et al. 2001).

1.3.3. NK cell receptors

The receptors responsible for the activation of NK cells (activator receptors) are mainly Natural Cytotoxicity Receptors, Killer Immunoglobulin-like Receptors (KIR), NKG_2D, CD_{94}/ NKG_2C, CD_{84}, CD_{16} and CD_{69} (Figure 1.5) (Vivier, Raulet et al. 2011). Binding of specific antigens/cytokines to the activating receptors activates the NK cells, while binding of specific antigens/cytokines to inhibition receptors like CD_{94}/NKG_2A and Natural Killer Cell Receptors inhibits the functioning of NK cells (Figure 1.5) (Vacca, Moretta et al. 2011, Vivier, Raulet et al. 2011).

NK cells are recognized by their expression of both activator and inhibitor cell surface receptors (Vivier, Raulet et al. 2011, Mosimann, Wagner et al. 2013). NK cells usually do not express the CD_3 receptor on their cell surface, and possess both CD_{16} and CD_{56} (Mosimann, Wagner et al. 2013). The two subsets of NK cells, uNK and pNK cells differ in the expression of activation and inhibition receptors (Figure 1.4) (Vivier, Raulet et al. 2011, Mosimann, Wagner et al. 2013). The uNK cells lack most of the activator receptors (CD_{16} and CD_{69}) and possess a high number of inhibitor receptors like CD_{56}, thus they are said to be CD_{56}^{Bright} (Figure 1.4) (Vivier, Raulet et al. 2011, Mosimann, Wagner et al. 2013). While pNK cell subgroup express a lot of activator receptors and low number of inhibitor receptors like CD_{56}, thus they are referred to as CD_{56}^{Dim} (Figure 1.4) (Vivier, Raulet et al. 2011, Mosimann, Wagner et al. 2013). Due to the difference in surface receptors, NK cell sub-groups differ in function. The uNK cell sub-group is more capable of perform a more protective function in the uterus, whilst pNK cells are more suited to perform their function in the peripheral body.

The focus of this study is on the CD_{69} surface glycoprotein of NK cells. CD_{69} is one of the early specific markers for activated NK cells (Mosimann, Wagner et al. 2013). It is involved in the activation and differentiation process of NK cells and other lymphocytes (Sancho, Gómez et al. 2005). Expression of CD_{69} is triggered by various mechanisms including the presence of cytokines IL-2 and IL-15 (Hosono, de Boer et al. 2003, Lund, Boysen et al. 2012). CD_{69} is said to be an immune-regulatory molecule expressed by immune cells during an immune response and at chronic inflammation (Sancho, Gómez et al. 2003, Sancho, Gómez et al. 2005). CD_{69} is therefore highly expressed on cytotoxic NK cells, indicating presence of inflammation. In normal pregnancy NK cells are less cytotoxic and express less activator

receptors on their surface, this is why uNK cells are predominate in the uterus. For this reason, CD_{69} may be used as an early biomarker for PE and other pathological inflammatory conditions due to its early expression on immune cells.

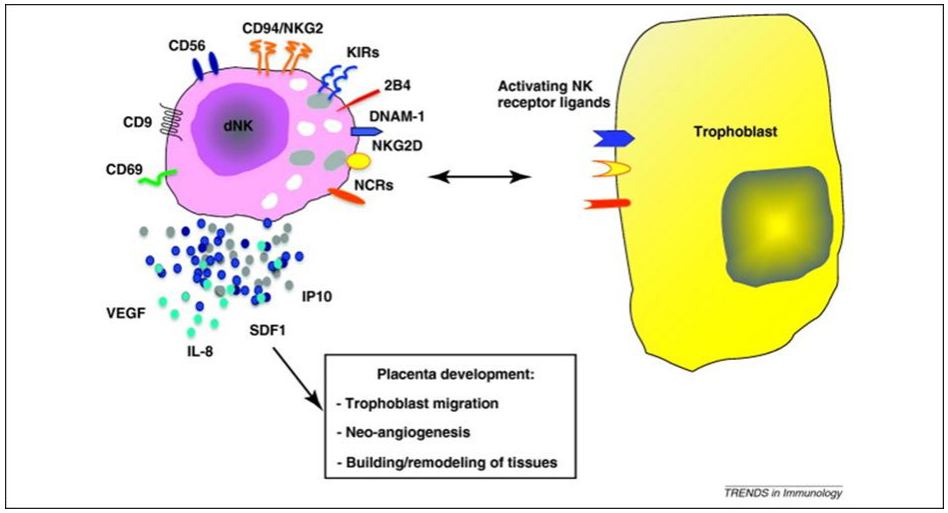

Figure 1.6: Activated NK cells express various surface glycoproteins and produce a variety of cytokines (VEGF, IL-8, SDF1 and IP10) that go on to perform function that are necessary in placental development and the progression of a normal pregnancy (Vacca, Moretta et al. 2011).

1.4. Hypothesis of the study

NK cells will be investigated via their cell-surface glycoproteins to review their involvement in HIV-associated PE. CD_{69}, CD_3, CD_{56} and CD_{16} receptors will be the main focus of this study, and will be used to determine the NK cell activation status and also uNK and pNK cell population. The study hypothesizes that the levels of CD_{69}-expressing NK cells will be higher in EOPE than LOPE women irrespective of immune status of women.

1.5. Aims and Objectives of the study

The study investigates two leading causes of foetal and maternal deaths in South Africa, mainly HIV and hypertension. South Africa is a suitable site for studying HIV-associated PE because statistical studies have shown that 30% of women attending the antenatal ward of South African clinics and hospitals are HIV-positive (Kalumba, Moodley et al. 2013).

The aim of the study is to determine the role of CD_{69} in HIV-associated PE among black South African, and also to evaluate clinical characteristics associated with PE. The objectives were to quantify NK cells in the normotensive HIV-negative, normotensive HIV-positive, EOPE HIV-negative, EOPE HIV-positive, LOPE HIV-negative and LOPE HIV-positive study groups; and also determining the levels of CD_{69} positive NK cells during pregnancy.

CHAPTER 2

MATERIALS AND METHODS

2.1. Study approval

This study was conducted at the Optics & Imaging Centre, Doris Duke Medical Research Institute (DDMRI), University of KwaZulu-Natal (UKZN) from February 2014 to November 2015. Approval from the Institutional postgraduate office and UKZN Biomedical Research Ethics Committee was obtained (Ref. no. BE 212/14; Appendix I, page 87).

2.2. Patient recruitment

Patients were recruited at the antenatal clinic and labor ward by a research midwife at Prince Mshiyeni Memorial Hospital, a regional hospital in the eThekwini region of KwaZulu-Natal, South Africa. The hospital is a 1200 bedded facility that serves the surrounding area, up to and including part of the Eastern Cape, South Africa. Informed consent was obtained from all patients by a trained research nurse (Appendix II, page 88). Patients' confidentiality was firmly maintained by keeping all information collected from the patients in a locked cupboard. Only personnel involved in the study had access to the cupboard.

2.3. Study population

A total of 180 patients were to be analyzed. The sample groups that were to be analyzed for the study are depicted in Figure 2.1. The study group included HIV-positive and HIV-

negative, pre-eclamptic and normotensive pregnant Black South Africans. Purposeful sampling strategy was used based on the inclusion and exclusion criteria outlined below.

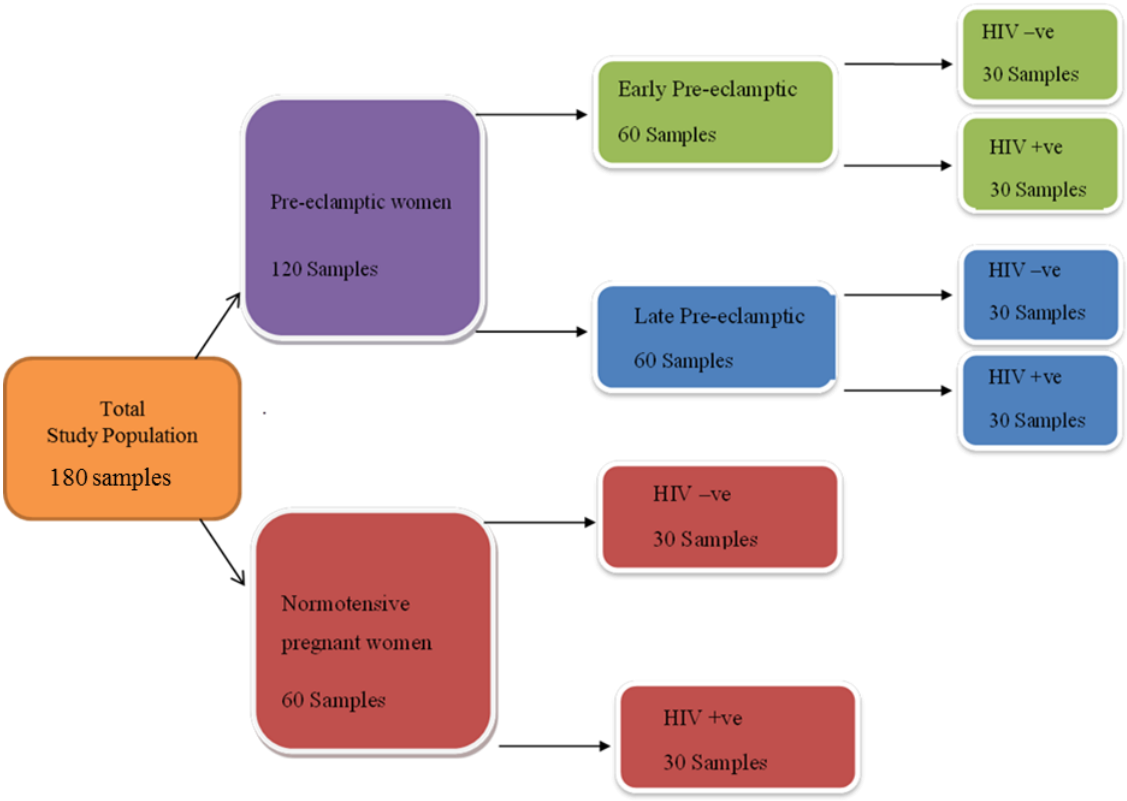

Figure 2.1: Study Groups with sub-stratification based on HIV status and onset of PE development.

2.4. Inclusion criteria

The study included primigravida and multigravida pre-eclamptic (≥140/90 mmHg and at least 1+ proteinuria on dipstick) and normotensive pregnant women. Only South African Black females ≥18 years were included in the study population. This sampling strategy was used to maintain a homogeneous population, and also because a greater percentage of woman visiting Prince Mshiyeni Memorial Hospital are Black South Africans. Both HIV-positive and HIV-

negative pregnant women participated in the study. HIV status was based on standard of health care protocol in KZN, SA.

2.5. Exclusion criteria

The exclusion criteria comprised of patients who were not booked into the antenatal and labor clinic of Prince Mshiyeni Memorial Hospital, non-black South Africans, patients with unknown HIV status, patients who are chronically using pharmaceutical drugs (recurrent antibiotic or steroid usage), and those who have been diagnosed with polycystic ovarian syndrome, chorioamnionitis, chronic hypertension, eclampsia, diabetes, chronic renal disease, systemic lupus erythematosus, sickle cell disease, antiphospholipid antibody syndrome, thyroid disease, cardiac disease, pre-existing seizure disorder, active asthma requiring medication during pregnancy, and patients who have suffered from abruption placentae and intrauterine death during previous pregnancies.

2.6. Data and blood collection methods and tools

The research midwife collated patient clinical information onto a patient's information sheet (Appendix III, page 92). Information was collected from the patient's case notes and verbal interviews. Clinical variables included maternal age, height and weight; parity; gestational age (GA); concentration of the proteinuria; blood pressure; HIV status; obstetric and neonatal outcomes and maternal and neonatal complications.

The research midwife also collected venous blood into a 4 ml K3EDTA anti-coagulant vacutainer blood collection tube (Greiner Bio-One, US). Blood samples tubes were labeled using a sample number unique for each patient and placed in an ice box and transported immediately to the Optics and Imaging Centre, DDMRI at UKZN Medical School Campus for multicolor flow cytometry analysis. The blood samples were analyzed within 6 hours of collection at K-RITH. Laboratory variables included CD_3; CD_{16}; CD_{56} and CD_{69} scatterplots.

2.7. Flow cytometry

2.7.1. Principle of flow cytometry

Analysis of the antigen-antibody complexes were prepared using the BD LSRFortessa™ multicolor flow cytometer (Becton, Dickinson and Company, San Jose, CA 95131 USA), which is able to analyze eleven different fluorochromes per analysis. A flow cytometer passes cells (or particles) in single file through a laser beam and then analyzes the interaction of the laser with the individual cells. The direction change of the laser provides information on size and shape of the cells (see Figure 2.2) (Ormerod 2000).

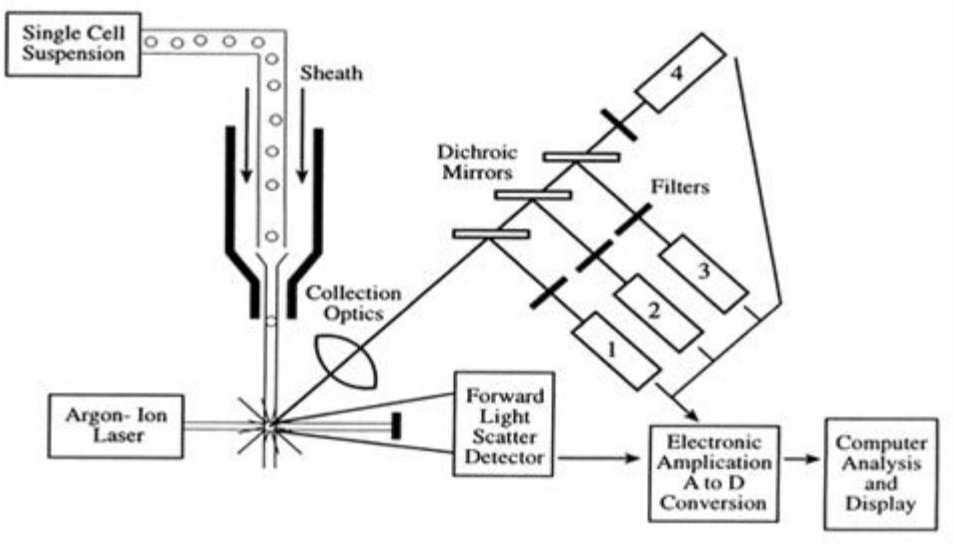

Figure 2.2: Cells move through the flow cytometer and are visualized with an aid of fluorochromes, laser beams, filters, deflectors and light detectors found within the apparatus. The structure of the cells are then determined by computer analysis, this helps determine the composition of the sample being analyzed (Brown and Wittwer 2000).

2.7.2. Flow cytometry analysis

CD_3, CD_{16}, CD_{56} and CD_{69} antibodies (Becton, Dickinson and Company, San Jose, CA 95131 USA) were conjugated to fluorescent chemical compounds known as fluorochromes. The fluorochromes re-emit light upon excitation and enable visualization of the antigen-antibodies complex (Ormerod 2000). Conjugated antibodies were: CD_3 attached to BD HV500 fluorochrome, CD_{16} to FITC, CD_{56} to PECy7 and CD_{69} to BV421. The wavelengths of fluorochromes were selected to ovoid spectral overlap during analysis of antigens on the BD LSRFortessa^{TM} multicolor flow cytometer apparatus. Care was taken to avoid non-specific binding and to eliminate background noise during acquisition.

2.7.3. Optimizing antibody dilutions for flow cytometry analysis

Six Falcon (Becton, Dickinson and Company, Franklin Lakes, NJ 07417 USA) sterile tubes were labeled 1 to 6. In all 6 tubes, 20 microliters (µl) of staining buffer (1x phosphate-buffered saline, LONZA BioWhittaker®, Belgium + 0.1% (w/v) sodium azide, Sigma-Aldrich®, Saint Louis, MO 63103, USA) (Appendix IV, page 98) was added followed by 10 µl of antibody and topped up to a final volume of 40 µl. Tube 1 was vortexed to mix the solution thoroughly and then 20 µl of the sample was transferred to tube 2, leaving 20 µl of sample in tube 1 and 40 µl of sample in tube 2. The same cycle was done for tube 2 up to tube 5. In tube 5, 20 µl of sample was discarded after vortexing, leaving all 6 tubes with a uniform amount of sample (see Table 2.1). Tube 6 only contained 20 µl of staining buffer and was used as a control tube, therefore containing no antibody solution. In all 6 tubes, 300 µl of whole blood was added. The tubes were vortexed to mix the antibody-staining buffer solution with the whole blood. All 6 tubes were then incubated at room temperature (20° to 25°C) in the dark for 30 minutes. The tubes were kept in the dark as they contained a light sensitive fluorochrome conjugated antibody. Post incubation, 3ml of 1X BD FACS Lysis solution (Cat. No. 349202) (Appendix IV, page 90) was added to all six tubes; the tubes were vortexed and incubated at room temperature in the dark for 10 minutes. The BD FACS Lysis solution was used for lysing the red blood cells (RBC), as the target NK cells are only found in the white blood cell (WBC) layer. Tubes were then centrifuged at 1500 revolutions per minute (rpm) (at 25°C) for 5 minutes to form a pellet of WBCs. The supernatant containing the lysed RBCs was decanted. The pellet was then washed twice with 3 ml of wash buffer (1x phosphate-buffered saline, LONZA BioWhittaker®, Belgium + 0.1% (w/v) sodium azide, Sigma-Aldrich®, Saint Louis, MO 63103, USA), centrifuging after every wash at 1500 rpm (at 25°C) for 5 minutes and discarding the supernatant after every wash. 300 µl of wash buffer was added into each tube

after the second wash. The tubes were stored at 2-8°C until they were analyzed. Before analysis all 6 tubes were vortexed thoroughly to mix the pellet with the wash buffer, tubes were then analyzed on the BD LSRFortessa^{TM} Flow Cytometer.

Table 2.1: Volume of antibodies utilized.

	CD_3	**CD_{16}**	**CD_{56}**	**CD_{69}**
Tube 1	5µl of CD_3	10µl of CD_{16} 3µl of CD_3	5µl of CD_{56} 3µl of CD_3	5µl of CD_{69} 3µl of CD_3
Tube 2	2.5µl of CD_3	5µl of CD_{16} 3µl of CD_3	2.5µl of CD_{56} 3µl of CD_3	2.5µl of CD_{69} 3µl of CD_3
Tube 3	1.25µl of CD_3	2.5µl of CD_{16} 3µl of CD_3	1.25µl of CD_{56} 3µl of CD_3	1.25µl of CD_{69} 3µl of CD_3
Tube 4	0.625µl of CD_3	1.25µl of CD_{16} 3µl of CD_3	0.625µl of CD_{56} 3µl of CD_3	0.625µl of CD_{69} 3µl of CD_3
Tube 5	0.3125µl of CD_3	0.625µl of CD_{16} 3µl of CD_3	0.3125µl of CD_{56} 3µl of CD_3	0.3125µl of CD_{69} 3µl of CD_3
Tube 6	No antibody	No antibody	No antibody	No antibody

2.7.4. Staining and lysis of whole blood

To analyze each blood sample the optimal concentrations of the fluorochrome conjugated antibodies CD_{16}, CD_3, CD_{69} and CD_{56} were added to the Falcon sterile tube and the antibody cocktail was topped up with distilled water to 50 µl. Blood (300 µl) was then added to the tube, and the mixture was vortexed gently and incubated in the dark at room temperature (20° to 25°C) for 30 minutes to allow for adequate attachment of the antibodies to the antigens

present on the WBCs. Following the incubation period, 3 ml of BD FACS Lysing Solution was added to the tube. The tube was vortexed and incubated for 10 minutes in the dark at room temperature. The tube was centrifuged at 1500 rpm for 5 minutes at 25°C to form a pellet of WBCs at the bottom of the tube. The supernatant containing the lysed RBCs was decanted from the tube, and the pellet was washed twice with 3 ml of wash buffer centrifuging after every wash at 1500 rmp (at 25°C), and discarding the supernatant after every wash. 300 µl of wash buffer was added to the pellet and the tube was stored at 4°C until analyzed. Before analysis, the tube was vortexed thoroughly to mix the pellet with the wash buffer, and the sample was analyzed on the BD LSRFortessa™ Flow Cytometer. Results for each blood sample were viewed and analyzed on the BD FACSDiva (Version 7, Becton, Dickinson and Company. © 2015 BD) and FlowJo (Version 10) software.

2.8. Data analysis techniques

2.8.1. Compensation set up

Compensation in multicolour flow cytometry is important for the accurate interpretation of data by making visualization of the different cell populations possible (Roederer 2001). Compensation set up is essential for matching antibodies to fluorochromes with colours of different wavelengths to prevent spill over and optimize visualization (Roederer 2001). In this study, the BD™ CompBeads Compensation Particles Set (Anti-Mouse Ig, κ/Negative Control) (Becton, Dickinson and Company, San Diego, CA 92121, USA) was used to optimize fluorescence compensation settings.

Compensation analysis was done for CD_3, CD_{16}, CD_{56} and CD_{69} fluorochrome-conjugated antibodies. For the analysis; 2.5µl of CD_3 antibody, 10µl of CD_{16} antibody, 2.5µl of CD_{56} antibody, and 2.5µl of CD_{69} antibody was used. The BD™ CompBeads were first vortexed to ensure that the microparticles within the sample were uniformly distributed. Four Falcon sterile tubes were labeled for each of the fluorochrome-conjugated antibodies. 100µl of staining buffer was added to all four tubes. One drop (approximately 60µl) of the BD™ CompBeads Anti-Mouse Ig,κ and one drop of the BD™ CompBeads Negative Control were both added to all four tubes, the tubes were slightly vortexed. The fluorochrome-conjugated antibodies were then added to the respective labelled tube (one antibody per tube). The BD™ CompBeads/Antibody mixture was vortexed and incubated at room temperature in the dark for 30 minutes. Following the incubation period, 2ml of staining buffer was added to all four tubes and the tubes were centrifuged at a speed 1500 rpm for 5 minutes at room temperature. The supernatant was discarded in all the tubes using a Pasteur pipette (LASEC, 2014), leaving bead pellets at the bottom of each tube. The pellets in all tubes were re-suspended in 0.5ml staining buffer. All tubes were vortexed thoroughly before analyzing on the BD LSRFortessa™ Flow Cytometer**.**

2.8.2. Fluorescence minus one (FMO)

FMO is a control used to properly interpret flow cytometry data. The FMO control is used for the accurate gating of cells. FMO controls were done for CD_3, CD_{16}, CD_{56} and CD_{69} fluorochrome-conjugated antibodies. Four Falcon sterile tubes were labeled for each of the fluorochrome-conjugated antibodies. In each tube the optimal concentrations of all the antibodies except for the antibody being tested were added (see Table 2.2). After adding the

antibodies, 300µl of whole blood was added to all the tubes. Tubes were then analyzed on the BD LSRFortessa^{TM} Flow Cytometer, and results were used for accurate gating of cells regarding that particular surface glycoprotein.

Table 2.2: Volume of FMO controls utilized in the study.

	Contents of the tube
Tube 1 (FMO-CD_3)	5µl CD_{16} 1.5µl CD_{56} 3µl CD_{69}
Tube 2 (FMO- CD_{16})	3 µl CD_3 1.5µl CD_{56} 3µl CD_{69}
Tube 3 (FMO- CD_{56})	3 µl CD_3 5µl CD_{16} 3µl CD_{69}
Tube 4 (FMO- CD_{69})	3 µl CD_3 5µl CD_{16} 1.5µl CD_{56}

2.8.3. Rainbow beads

Rainbow Beads are used for calibrating the flow cytometer apparatus before acquiring the cell sample. Rainbow Beads contain a combination of fluorochromes spectrally similar to the fluorochromes used in flow cytometry. For this study, the Sphero^{TM} Rainbow Fluorescent Particles (Becton, Dickinson and Company, San Diego, CA 92121, USA) that were used had a mid-range F_1 fluorescence of 3-3.4 micrometers.

Before acquiring samples on the BD LSRFortessa™ Flow Cytometer, a tube containing Rainbow Beads was first acquired to ensure that the voltages used on the BD LSRFortessa™ Flow Cytometer were accurate for the fluorochromes-conjugated antibodies analysis. The tube of Rainbow Beads was prepared by vortexing 3 drops of Sphero™ Rainbow Fluorescent Particles and 300 µl of sheath fluid (Becton, Dickinson and Company BD Biosciences 2350 Qume Drive San Jose, CA 95131 USA).

2.8.4. BD LSRFortessa™ multicolor flow cytometer

Samples from study participants were analyzed using the BD LSRFortessa™ multicolor flow cytometer. BD FACSDiva software in collaboration with FlowJo software was used to view and analyze the results. FACSDiva sorts the cells according to their different characteristics, therefore identifying the different cells and particles available in the mixture being analyzed. To perform analysis of the surface glycoproteins found on NK cells the gating strategy depicted in Figure 2.3 was used.

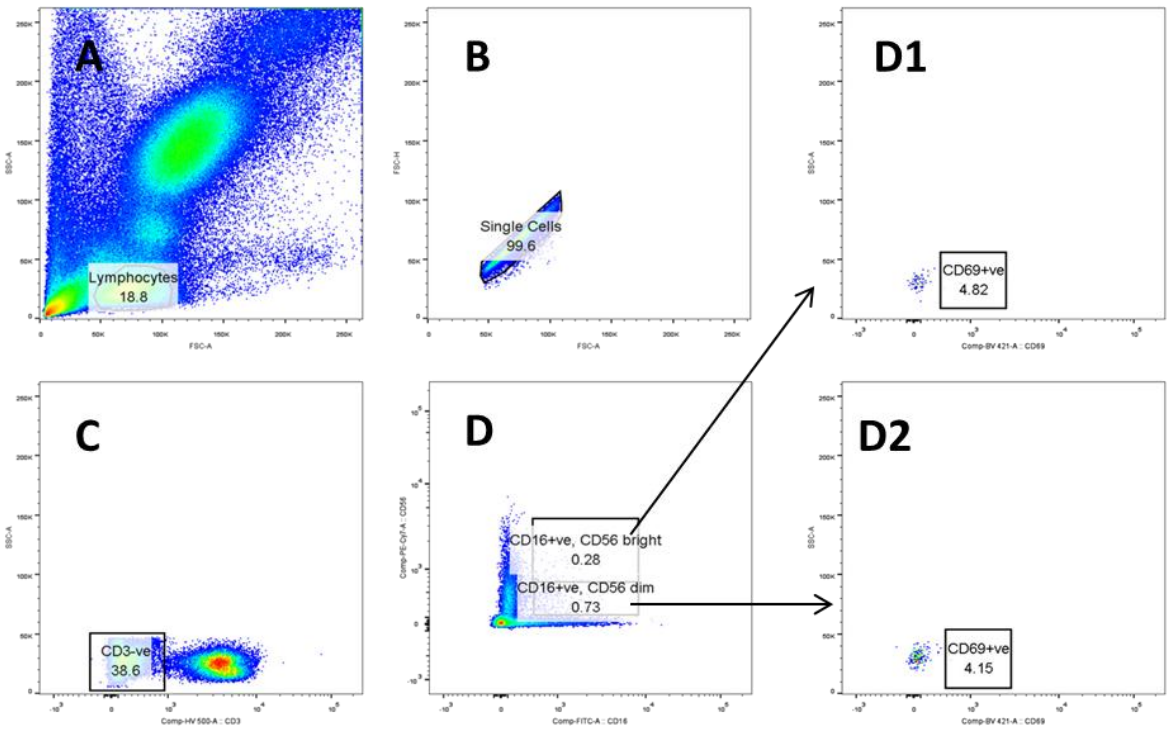

Figure 2.3: A: Lymphocytes were gated in the acquired cell population. B: From the lymphocyte population the single cell population was gated. C: CD_3-negative population was gated. D: From the CD_3-negative population, the CD_{16}-positive-CD_{56}^{Bright} and CD_{16}-positive-CD_{56}^{Dim} populations were gated. D1: From the uNK ($CD_{16}^{+}CD_{56}^{Bright}$) cells levels of CD_{69}-expressing cells were determined. D2: From the pNK ($CD_{16}^{+}CD_{56}^{Dim}$) cells levels of CD_{69}-expressing cells were determined.

2.9. Statistical analysis

SPSS (Version 22) was used to analyze patient clinical characteristics, quantity of the peripheral NK cells and to evaluate the difference in CD_{69} concentrations and levels (mean fluorescence intensity) in the different groups. To determine the statistical differences between study groups, firstly the normality of the sample distribution within the study groups was assessed using Shapiro–Wilk test. Based on the normality status of the data and the type of data to be analyzed, different statistical analyses were used to compare the proportions across

the study groups. Results from the flow cytometry and the statistical analysis were presented as tables containing significance of the results, means, standard deviations, quartiles and interquartile range.

The independent variables of the study were HIV-status, pregnancy type and study groups (HIV status and pregnancy type). The independent variables were classified as binary (HIV-status) or nominal (pregnancy type and study groups). All dependent variables (maternal age, maternal weight and BMI, systolic and diastolic pressure, dipstick, parity, gravidity, number of pregnancy losses, gestational age at birth, baby weight, and laboratory variables) were numerical data; these were classified on whether they had normally or non-normally distributed data. For comparing the effect of the binary independent variable (HIV-status) on dependent variables with normally distributed data the Independent samples T-test was used. For comparing the effect of the binary independent variable (HIV-status) on dependent variables with non-normally distributed data the Mann-Whitney test was used. For comparing the effects of the nominal independent variables (pregnancy type and study groups) on dependent variables with normally distributed data the one-way analysis of variance (ANOVA) test was used. For comparing the effects of the nominal independent variables (pregnancy type and study groups) on dependent variables with non-normally distributed data the Kruskal-Wallis test was used.

CHAPTER 3

RESULTS

3.1. Study groups

Cell surface glycoproteins of NK cells (CD_3, CD_{16}, CD_{56} and CD_{69}) were assessed in HIV-positive and HIV-negative pre-eclamptic and normotensive patients attending the antenatal and labour ward at the Prince Mshiyeni Memorial Hospital. A total of 160 pregnant Black South Africans were studied. Study groups were as follows:

1) Pre-eclamptic (n=102)
    (a) EOPE (n=73)
        i. HIV-positive (n=35)
        ii. HIV-negative (n=38)
    (b) LOPE (n=29)
        i. HIV-positive (n=11)
        ii. HIV-negative (n=18)
2) Normotensive (n=58)
    (a) HIV-positive (n=30)
    (b) HIV-negative (n=28)

The EOPE study group accounted for 45.63% of the study population. The EOPE study group was distributed between HIV-positive (21.88%) and HIV-negative (23.75%) subgroups. LOPE study group accounted for 18.13% of the study population. HIV infection within the LOPE group accounted for 6.88% of the study population whilst HIV-negative accounted for

11.25%. The normotensive study group was evenly distributed between HIV-positive and HIV-negative subgroups and accounted for 36.25% collectively.

3.2. Patient clinical characteristics

Table 3.1: Mean, standard deviation and skewness values of the study population's clinical characteristics.

	N		**Mean**	**SD**	**Skewness**	**Std. Error of Skewness**
	Valid	Not determined				
Maternal age (years)	158	2	27	6.93	0.61	0.19
Maternal weight (Kg)	158	2	74.63	17.75	0.62	0.19
BMI (Kg/m^2)	121	39	29.93	6.75	0.60	0.22
Systolic pressure (mmHg)	159	1	140.32	27.07	-0.15	0.19
Diastolic pressure (mmHg)	159	1	89.57	17.81	-0.31	0.19
Dipstick (mg/d)	151	9	1.07	0.88	0.69	0.19
Parity	159	1	1.06	1.15	1.96	0.19
Gravidity	159	1	2.33	1.63	2.99	0.19
No. of pregnancy losses	160	0	0.17	0.60	5.53	0.19
GA at birth (weeks)	117	43	35.74	4.34	-1.23	0.22
Birth weight (Kg)	117	43	2.62	0.95	-0.30	0.22

N= Study Population

Table 3.2: Clinical characteristics of all study groups

	HIV-negative normotensive	**HIV-positive normotensive**	**HIV-negative EOPE**	**HIV-positive EOPE**	**HIV-negative LOPE**	**HIV-positive LOPE**	***p*-value**
Maternal age (years)	23.64±4.42	26.87±6.36	27.58±7.36	27.88±7.14	26.24±7.38	31.64±8.27	*p=0.*062
Maternal weight (Kg)	73.96±18.88	68.76±16.58	77.81±18.04	77.76±17.50	71.34±17.90	76.85±16.06	*p*=0.346
BMI (Kg/m^2)	30.182±8.27	27.765±5.11	30.066±6.74	31.683±6.93	29.777±7.09	30.929±5.63	*p=0.*536
Systolic pressure (mmHg)	110.21±11.77	108.23±11.75	158.84±13.47	159.71±16.31	152.29±10.69	160.27±10.85	*p*<0.001
Diastolic pressure (mmHg)	68.86±9.87	68.7±5.91	102.71±8.51	101.8±8.85	98.76±7.24	100.73±7.67	*p*<0.001
Dipstick (mg/d)	0.5±0.95	0.15±0.37	1.57±0.69	1.51±0.74	1.25±0.58	1.27±0.47	*p*<0.001
Parity	0.61±0.69	1.03±0.85	1.08±1.10	1.26±1.54	1±1.06	1.64±1.5	*p*=0.233
Gravidity	1.79±0.83	2.37±1.35	2.32±1.44	2.69±2.29	2.41±1.81	2.45±1.7	*p*=0.454
GA at birth (weeks)	39.07±1.54	39.20±1.32	33.85±4.27	33.09±4.85	37.85±1.21	37.80±1.69	*p*<0.001
Birth weight (Kg)	3.389±0.36	3.321±0.49	2.149±0.83	2.056±0.90	3.029±0.50	3.346±1.04	*p*<0.001

Values are given as Mean ± Standard Deviation (SD)

3.2.1. Maternal age

Of the study population; 158 participants' maternal age was recorded. The mean ± SD of the maternal age of the 158 study participants was 27 ± 6.93 years. The maternal age ranged between 18 to 44 years. The raw data of the maternal age had a skewness value of 0.61 with

standard error of 0.19; this indicated that the data was not normally distributed across the study population.

The Mann-Whitney test was used to indicate the effect of HIV-statues on maternal age across the study population (U= 2562.50, p= 0.055). The analysis showed that there was no significant difference in maternal age between the HIV-negative group (mean rank= 72.87) and the HIV-positive group (mean rank= 86.83). The Kruskal-Wallis Test was used to show the correlation between pregnancy type (normotensive, early pre-eclamptic or late pre-eclamptic) and maternal age across the study population (n= 158). Analysis showed that there was no significant difference in maternal age between the pregnancy types (p= 0.165). The Kruskal-Wallis Test was also used to show the correlation between study groups and maternal age across the study population (n= 158). There was no significant difference in maternal age across the study groups (p= 0.062).

3.2.2. Maternal weight

Of the study population; 158 participants' maternal weight was recorded. The mean ± SD of the maternal weight was 74.63 ± 17.75 Kilograms (Kg). The minimum maternal weight was 41 kg whilst the maximum weight was 125 kg. The maternal weight raw data of the study population had a skewness value of 0.62 with standard error of 0.19; this indicated that the data was not normally distributed across the study population.

The Mann-Whitney test was used to indicate the effect of HIV-statues on maternal weight across the study population (U= 3112.00, p= 0.999). The analysis showed that there was no significant difference in maternal weight between the HIV-negative group (mean rank= 79.49) and the HIV-positive group (mean rank= 79.51). The Kruskal-Wallis Test was used to show the correlation between pregnancy type (normotensive, early pre-eclamptic or late pre-eclamptic) and maternal weight across the study population (n= 158). Analysis showed that there was no significant difference in maternal age between the pregnancy types (p= 0.117). The Kruskal-Wallis Test was also used to show the correlation between study groups and maternal weight across the study population (n= 158). There was no significant difference in maternal weight across the study groups (p= 0.346).

3.2.3. Body mass index (BMI)

Of the study population; only 121 participants' BMI was recorded. The mean ± SD of the BMI of the 121 study participants was 29.93 ± 6.75 Kilograms per square meter (Kg/m^2). BMI ranged between 18.2 - 49.7 Kg/m^2. The raw data of the BMI of the study population had a skewness value of 0.60 with standard error of 0.22; this indicated that the data was not normally distributed across the study population.

The Mann-Whitney test was used to indicate the effect of HIV-statues on the maternal BMI across the study population (U= 1799.50, p= 0.899). The analysis showed that there was no significant difference in the maternal BMI between the HIV-negative group (mean rank= 60.62) and the HIV-positive group (mean rank= 61.43). The Kruskal-Wallis Test was used to

show the correlation between pregnancy type (normotensive, early pre-eclamptic or late pre-eclamptic) and maternal BMI the study population (n= 121). Analysis showed that there was no significant difference in maternal BMI between the pregnancy types (p= 0.298). The Kruskal-Wallis Test was also used to show the correlation between study groups and maternal BMI across the study population (n= 121). There was no significant difference in maternal BMI across the study groups (p= 0.536).

3.2.4. Systolic pressure

Of the study population; 159 participants' systolic pressures were recorded. The mean systolic pressure of the 159 study participants was 140.32 mmHg with standard deviation of 27.07 mmHg. The systolic pressure ranged between 85 - 218 mmHg. The raw data of the systolic pressure had a skewness value of -0.148 with standard error of 0.192; this indicated that data for systolic pressure across the study population was normally distributed.

An Independent Samples T-test was used to indicate the effect of HIV-statues on systolic pressure across the study population [F (159) = 2.604, p= 0.109]. There was no significant difference in the systolic pressure of the HIV-negative group (mean ± SD: 141.1 ± 25.45 mmHg) compared to the HIV-positive group (mean ± SD: 139.47 ± 28.89 mmHg). The one-way ANOVA was used to show the effect of pregnancy type (normotensive, EOPE or LOPE) on systolic pressure [F (159) = 256.402, p< 0.001]. There was a difference in the systolic pressure between the pregnancy types (p< 0.001).

Normotensive study participants had the lowest mean systolic pressure (mean ± SD: 109.19 ± 11.69 mmHg, Confidence Interval (CI) 95%: 106.1–112.27 mmHg). LOPE study participants had a mean systolic pressure of 155.43 ± 11.27 mmHg (CI 95%: 151.06-159.80 mmHg), and EOPE study participants had the highest mean systolic pressure (mean ± SD: 159.26 ± 14.80 mmHg, CI 95%: 155.81– 162.71 mmHg).

A one-way ANOVA and post hoc Tukey B test indicated that systolic pressure was significantly different across all study groups (F (159)= 103.042, $p< 0.001$). The mean systolic blood pressure of the HIV-negative normotensive study group (mean ± SD: 110.21 ± 11.77 mmHg, CI 95%: 105.65-114.78 mmHg) was significantly lower than the HIV-negative EOPE group (mean ± SD: 158.84 ± 13.47 mmHg, CI 95%: 154.41-163.27 mmHg) and the HIV-negative LOPE group (mean ± SD: 152.29 ± 10.687 mmHg, CI 95%: 146.80-157.79 mmHg). Alternately, the systolic blood pressure of the HIV-positive normotensive group (mean ± SD: 108.23 ± 11.746 mmHg, CI 95%: 103.85-112.62 mmHg) was significantly lower than the mean systolic pressure of the HIV-positive EOPE group (mean ± SD: 159.71 ± 16.314 mmHg, CI 95%: 154.11-165.32 mmHg) and the HIV-positive LOPE group (mean ± SD: 160.27 ± 10.845 mmHg, CI 95%: 152.99-167.56 mmHg). Mean systolic blood pressure in HIV-negative EOPE, HIV-positive EOPE, HIV-negative LOPE, HIV-positive LOPE study groups were significantly higher than the systolic pressure of both the HIV-positive and HIV-negative normotensive study groups ($p< 0.001$). Additionally the systolic pressure of the HIV-negative normotensive study group was significantly higher than the systolic pressure of the normotensive HIV-positive group ($p< 0.001$).

3.2.5. Diastolic pressure

Of the study population; 159 participants' diastolic blood pressure was recorded. The mean diastolic pressure was 89.57 ± 17.81 mmHg. The diastolic blood pressure ranged between 54 - 130 mmHg. The raw data of the diastolic pressure had a skewness value of -0.306 with standard error of 0.192; this indicated that data for diastolic pressure across the study population was normally distributed.

An Independent Samples T-test was used to indicate the effect of HIV-statues on diastolic pressure across the study population [F (159) = 0.496, p= 0.483]. There was no significant difference in the diastolic pressure of the HIV-negative group (mean ± SD: 90.48 ± 17.84 mmHg) compared to the HIV-positive group (mean ± SD: 88.58 ± 17.84 mmHg). The one-way ANOVA was used to show the effect of pregnancy type (normotensive, EOPE or LOPE) on diastolic blood pressure across the study population [F (159) = 295.673, p< 0.001]. A significant difference in the mean diastolic blood pressure across the pregnancy types was demonstrated (p< 0.001). Normotensive study participants had the lowest mean diastolic pressure (mean ± SD: 68.78 ± 7.99 mmHg, CI 95%: 66.67 – 70.88 mmHg). LOPE study participants had a mean diastolic pressure of 99.54 ± 7.34 mmHg (CI 95%: 96.69– 102.38 mmHg). EOPE study participants had the highest mean diastolic pressure (mean ± SD: 102.27 ± 8.63 mmHg, CI 95%: 100.26– 104.29 mmHg).

The one-way ANOVA and post hoc Tukey B test indicated that diastolic pressure was significantly different between all study groups (F (159) = 116.575, p< 0.001). The mean

diastolic blood pressure of the HIV-negative normotensive study group (mean ± SD: 68.86 ± 9.87 mmHg, CI 95%: 65.03-72.69 mmHg) was significantly lower than that of the HIV-negative EOPE (mean ± SD: 102.71 ± 8.51 mmHg, CI 95%: 99.91-105.51mmHg) and HIV-negative LOPE study group (mean ± SD: 98.76 ± 7.242 mmHg, CI 95%: 95.04-102.49 mmHg). Alternately, the diastolic pressure of the HIV-positive normotensive study group (mean ± SD: 68.70 ± 5.91 mmHg, CI 95%: 66.49-70.91 mmHg) was significantly lower than that of the HIV-positive EOPE (mean ± SD: 101.80 ± 8.85 mmHg, CI 95%: 98.76-104.84 mmHg) and HIV-positive LOPE (mean ± SD: 100.73 mmHg ± 7.669 mmHg, CI 95%: 95.57-105.88 mmHg) study groups ($p< 0.001$). Mean diastolic blood pressure in HIV-negative and HIV-positive EOPE and LOPE study groups were significantly higher than the diastolic pressure of both the HIV-positive and HIV-negative normotensive study groups ($p< 0.001$).

3.2.6. Urine protein dipstick measurements

Of the study population; 151 participants' proteinuria (1+ being between 30-100mg/d) was recorded. The mean proteinuria of the 151 study participants was 1.07 ± 0.88, ranging from 0 to 4. The raw data of the proteinuria of the study population had a skewness value of 0.689 with standard error of 0.197; this indicated that the data was not normally distributed across the study population.

The Mann-Whitney test was used to indicate the effect of HIV-statues on the proteinuria across the study population ($U= 2543.00$, $p= 0.228$). The analysis showed that there was no significant difference in the proteinuria value between the HIV-negative group (mean rank=

79.81) and the HIV-positive group (mean rank= 71.82). The Kruskal-Wallis Test was used to show the correlate proteinuria on between pregnancy type (normotensive, EOPE or LOPE) and the urine protein dipstick measure within the study population (*n*= 151). Analysis showed that there was a difference in the mean proteinuria values between the pregnancy types ($p<$ 0.001). Normotensive group had the lowest dipstick values (mean rank= 36.78). The EOPE group (mean rank= 99.71) had higher proteinuria values than the LOPE group (mean rank= 88.31).

The Kruskal-Wallis Test was also used to show the correlation between study groups and the proteinuria within the study population (*n*= 151). Analysis showed that there was a difference in the proteinuria between the study groups ($p<$ 0.001). The normotensive HIV- positive study group had the lowest urine proteinuria (mean rank= 29.12) compared to all the study groups. The early pre-eclamptic HIV-negative study group (mean rank= 101.53) had highest dipstick values compared to all the study groups ($p<$ 0.001). Additionally, proteinuria for the HIV-positive EOPE (mean rank= 97.79) was higher than the HIV-positive LOPE (mean rank= 90.14).

3.2.7. Parity

Of the study population; the parity of 159 participants' was recorded. Mean parity of the 159 study participants was 1.06 ± 1.154. The parity range of the study population was 0 to 8. Raw data of the study population's parity had a skewness value of 1.964 with standard error of 0.192; this indicated that the data was not normally distributed.

The Kruskal-Wallis Test was used to show the correlation between pregnancy type (normotensive, EOPE or LOPE) and parity of the study population (*n*= 159). Analysis showed that there was no significant difference in parity between the pregnancy types (*p*= 0.342). The Kruskal-Wallis Test was also used to show the correlation between study groups and parity across the study population (*n*= 159). Analysis showed that there was no significant difference in parity between the study groups (*p*= 0.233).

3.2.8. Gravidity

Of the study population; the gravidity of 159 participants' was recorded. Mean gravidity of the 159 study participants was 2.33 ± 1.629, ranging from 0 to 14. Raw data of the study population's gravidity had a skewness value of 2.997 with standard error of 0.192; this indicated that the data was not normally distributed.

The Kruskal-Wallis Test was used to show the correlation between pregnancy type (normotensive, EOPE or LOPE) and gravidity of the study participants' (*n*= 159). Analysis showed that there was no significant difference in gravidity between the pregnancy types (*p*= 0.508). The Kruskal-Wallis Test was also used to show the correlation between study groups and gravidity across the study population (*n*= 159). Analysis showed that there was no significant difference in gravidity between the study groups (*p*= 0.454).

3.2.9. Number of pregnancy losses

The number of pregnancy losses of all the participants of the study was recorded. The mean number of pregnancy losses was 0.17 ± 0.60, ranging from 0 to 8. Raw data of the number of pregnancy losses had a skewness value of 5.53 with standard error of 0.192; this indicated that the data was not normally distributed.

The Mann-Whitney test was used to indicate the effect of HIV-statues on the number of pregnancy losses across the study population (U= 3126.50, p= 0.690). The analysis showed that there was no significant difference in the number of pregnancy losses between the HIV-negative group (mean rank= 81.28) and the HIV-positive group (mean rank= 79.64). The Kruskal-Wallis Test was used to show the correlation between pregnancy type (normotensive, EOPE or LOPE) and number of pregnancy losses of the study participants' (n= 160). Analysis showed that there was no significant difference in the number of pregnancy losses between the pregnancy types (p= 0.277). The Kruskal-Wallis Test was also used to show the correlation between study groups and and number of pregnancy losses of the study participants' (n= 160). There was no significant difference in the number of pregnancy losses between the study groups (p= 0.643).

3.2.10. GA at birth

The GA of 117 study participants was recorded. The mean GA was 35.74 ± 4.344 weeks. The minimum GA was 22 weeks whilst the maximum was 41 weeks. The raw data of the GA of

the infants had a skewness value of -1.233 with standard error of 0.224; this indicated that the data was not normally distributed.

The Mann-Whitney test was used to indicate the effect of HIV-statues on GA of infants of the study population (U= 1705.00, p= 0.978). The analysis showed that there was no significant difference in the GA of the infants between the HIV-negative group (mean rank= 59.08) and the HIV-positive group (mean rank= 58.91). The Kruskal-Wallis Test was used to show the correlation between pregnancy type (normotensive, EOPE or LOPE) and the GA at birth of infants of the study population (n= 117). Analysis showed that there was a difference in the GA of the infants between the pregnancy types (p< 0.001). Early pre-eclamptic study participants (mean rank= 39.25) had infants with the lowest GA at birth compared to the late pre-eclamptic (mean rank= 74.28) and the normotensive (mean rank= 91.16) study participants. Normotensive study participants had infants with the highest GA at birth.

The Kruskal-Wallis Test was also used to show the correlation between study groups and the GA at birth of infants of the study population (n= 117). Analysis showed that there was a difference in the GA of the infants between the study groups (p< 0.001). The early pre-eclamptic HIV-positive study group (mean rank= 38.13) had infants with the lowest GA at birth and the normotensive HIV-positive study groups (mean rank= 92.80) had infants with the highest GA at birth compared to all the other study groups.

3.2.11. Birth weight

The birth weight of 117 study participants was recorded. The mean birth weight was 2.62 Kg ± 0.95 Kg. The minimum birth weight was 0.43 Kg and the maximum was 5.8 Kg. The raw data of the birth weight had a skewness value of -0.295 with standard error of 0.224; this indicated that data for birth weight was normally distributed.

An Independent Samples T-test was used to indicate the effect of HIV-statues on birth weight across the study population [F (116) = 0.741, p = 0.391]. This analysis showed that there was no significant difference in the birth weight of the HIV-negative compared to the HIV-positive group (2.63 ± 0.87 Kg *versus* 2.63 ± 1.05 Kg; p= 0.391). The one-way ANOVA was used to show the effect of pregnancy type (normotensive, EOPE or LOPE) on birth weight across the study population [F (116) = 34.586, p< 0.001]. This analysis demonstrated a significant difference in the mean birth weight based on pregnancy type (p< 0.001). Babies of the normotensive group had the highest birth weight (mean ± SD: 3.35 ± 0.43 Kg, CI 95%: 3.19–3.52 Kg) compared to the babies of the LOPE group (mean ± SD: 3.17 ± 0.78 Kg; CI 95%: 2.83– 3.5 Kg) and EOPE group (mean ± SD: 2.1 ± 0.86 Kg, CI 95%: 1.87– 2.32 Kg).

The one-way ANOVA and post hoc Tukey B test indicated that the infant birth weight differed significantly across all study groups (F (116) = 13.878, p< 0.001). The birth weight of the HIV-negative EOPE study group (mean ± SD: 2.15 ± 0.83 Kg, CI 95%: 1.86-2.44 Kg) was significantly lower than the birth weight of the HIV-negative normotensive (mean ± SD: 3.39 ± 0.36 Kg, CI 95%: 3.18-3.60 Kg) and the HIV-negative LOPE (mean ± SD: 3.03 ± 0.50 Kg,

CI 95%: 2.73-3.33 Kg) study groups. Alternately, the birth weight within the HIV-positive EOPE study group (mean ± SD: 2.06 ± 0.90 Kg, CI 95%: 1.73-2.38 Kg) was significantly lower than that within the HIV-positive normotensive (mean ± SD: 3.32 ± 0.49 Kg, CI 95%: 3.05-3.59 Kg) and HIV-positive LOPE (mean ± SD: 3.35 ± 1.04 Kg, CI 95%: 2.61-4.09 Kg) study groups ($p< 0.001$). Mean birth weight in HIV-negative and HIV-positive normotensive and LOPE study groups were significantly higher than the birth weight of both the HIV-negative and HIV-positive EOPE study groups ($p< 0.001$).

3.3. Analysis of NK cells

Table 3.3: Mean, standard deviation and skewness values of the number of WBC cells in the study population.

	N		**Mean**	**SD**	**Skewness**	**Std. Error of Skewness**
	Valid	Not determined				
Lymphocyte cells	158	2	85556.32	67834.55	2.76	0.19
Single cells	158	2	83789.97	67280.21	2.74	0.19
CD_3^{-ve} cells	157	3	40536.85	55069.81	4.58	0.19
uNK cells	75	85	251.19	439.05	2.87	0.28
uNK CD_{69}^{+ve} cells	75	85	9.47	14.32	1.94	0.28
pNK cells	158	2	1119.27	1889.99	3.52	0.19
pNK CD_{69}^{+ve} cells	158	2	62.69	129.73	4.99	0.19

N= Study Population

Table 3.4: Number of WBC cells in all study groups

	HIV-negative normotensive	**HIV-positive normotensive**	**HIV-negative EOPE**	**HIV-positive EOPE**	**HIV-negative LOPE**	**HIV-positive LOPE**
Lymphocytes (*p*< 0.001)	38604.71±23362.06	96124.77±82324.44	76696.70±29272.51	109039.40±71860.64	86593.22±28360.30	134038.80±141523.66
Single cells (*p*< 0.001)	36348.75±23054.64	93944.20±81613.35	75504.65±29172.86	107282.60±71138.48	85265.56±28436.25	131938.20±139654.37
CD_3^{-ve} cells (*p*< 0.001)	13458.43±22268.64	47558.93±68112.03	33904.62±18475.49	54250.14±50493.62	33936.61±11326.74	84415.60±140809.96
uNK cells (*p*= 0.163)	224.79±299.19	207.77±396.2	324.71±614.68	59.60±75.27	748.2±931.8	0
CD_{69}^{+ve} UNK cells (*p*= 0.195)	11.04±15.41	9.63±14.35	2.00±3.7	3±4.24	16.6±20.68	0
pNK cells (*p*= 0.061)	904.75±749.72	768.63±1825.02	1672.76±2261.42	945.06±1235.63	599.11±669.24	2269.90±4345.32
CD_{69}^{+ve} pNK cells (*p*= 0.003)	40.46±52.32	17.90±26.28	60.38±90.57	106.97±171.82	35.22±48.37	162.30±325.55

Values are given as mean±SD

3.3.1. Total number of lymphocytes

The total number of lymphocytes of 158 study participants was recorded. Mean lymphocyte count was 85556.32 ± 67834.55 cells. The minimum lymphocyte count in the study participants was 7497 cells and the maximum was 509601. The raw data of the lymphocytes had a skewness value of 2.76 with standard error of 0.19; this indicated that the data was not normally distributed.

The Mann-Whitney test revealed a significant effect of HIV-statues on the lymphocyte count across the study population (U= 2230, p= 0.002). Additionally, the HIV-positive group (mean rank= 91.27) had significantly higher level of lymphocytes than the HIV-negative group (mean rank= 68.87; p= 0.002).

The Kruskal-Wallis Test showed a significant difference in the level of lymphocyte count based on pregnancy types (normotensive, EOPE or LOPE; p< 0.001). The lowest mean lymphocyte count was observed in the normotensive group (mean rank: 55.88) compared to the EOPE and LOPE group (mean rank: 91.74 and 96.96 respectively). Additionally, there was a higher lymphocyte count in the LOPE group compared to the EOPE group (p< 0.001).

The Kruskal-Wallis Test analysis (n= 158) demonstrated a significant difference in the level of lymphocyte count amongst the study groups. The HIV-positive LOPE study group had the highest levels of lymphocytes and the HIV-negative normotensive study group had the lowest levels (p< 0.001).

3.3.2. Lymphocyte single cell levels

The lymphocyte single cell count of 158 study participants was recorded. Mean single cell count was 83789.97 ± 67280.21 cells; range: 6785 - 502661 cells. The raw data of single cells had a skewness value of 2.74 with standard error of 0.19; this indicated that the data was not normally distributed.

The Mann-Whitney test showed a significant effect of HIV-statues on the level of lymphocyte single cells across the study population (U= 2214, p= 0.002). The HIV-positive group (mean rank= 91.48) had a significantly higher level of lymphocyte single cells compared to the HIV-negative group (mean rank= 68.67). The Kruskal-Wallis Test showed a significant difference in the level of lymphocyte single cells across the pregnancy types (n= 158; normotensive, EOPE or LOPE; p< 0.001). The normotensive group (mean rank: 55.47) had the lowest mean lymphatic cell count. Additionally, the LOPE group had a significantly higher lymphatic cell count compared to the EOPE group (mean rank: 97.07 *versus* 92.03, p< 0.001).

The Kruskal-Wallis Test showed a significant difference in the level of lymphocyte single cells amongst the study groups. The HIV-positive EOPE study group had the highest levels (mean rank: 100.80) and HIV-negative normotensive had the lowest levels (mean rank: 31.50) of single cells (p< 0.001).

3.3.3. CD_3 negative lymphocyte levels

The CD_3 negative cell count of 157 study participants was recorded. Mean CD_3 negative cell count was 40536.85 ± 55069.81 cells (range: 1977 - 478246 cells). The raw data of the CD_3 negative cells had a skewness value of 4.58 with standard error of 0.19; this indicated that data for the CD_3 negative cells was not normally distributed.

The Mann-Whitney test demonstrated a significant effect of HIV-statues on the level of CD_3 negative cells across the study population (U= 2350, p= 0.011). The HIV-positive group (mean rank= 88.74) had significantly higher levels of CD_3 negative cells than the HIV-negative group (mean rank= 70.31). The Kruskal-Wallis Test showed that there was a significant difference in the level of CD_3 negative cells based on pregnancy type (n= 157; normotensive, EOPE or LOPE; p< 0.001). Normotensive group (mean rank: 50) had the lowest mean CD_3 negative cell count compared to the EOPE and LOPE groups (mean rank: 95.99 and 94.36 respectively).

The Kruskal-Wallis Test demonstrated a significant difference in the level of CD_3 negative cells amongst the study groups (n= 157; p< 0.001). Additionally, the HIV-positive EOPE study group had the highest levels of CD_3 negative cells compared to all the other study groups (p< 0.001).

3.3.4. uNK Cell Levels

The uNK cell count of 75 study participants was recorded. Mean uNK cell count was 251.19 ± 439.05 cells (range: 0 - 2177 cells). The raw data of the uNK cells had a skewness value of 2.87 with standard error of 0.28; this indicated that the data was not normally distributed.

The Mann-Whitney test showed no effect of HIV-statues on the level of uNK cell count across the study population (U= 525, p= 0.063). The Kruskal-Wallis Test demonstrated no significant

difference in the levels of uNK cells based on the pregnancy types (*n*= 75; normotensive *versus* EOPE *versus* LOPE; *p*= 0.184). The Kruskal-Wallis Test was also used to show the correlation between study groups and uNK cells across the study population (*n*= 75). Analysis showed no significant difference in the levels of uNK cells between the study groups (*p*= 0.163).

3.3.5. CD_{69} positive-uNK Cell Levels

The CD_{69} positive-uNK cell count of 75 study participants was recorded. The mean CD_{69} positive-uNK cell count was 9.47 ± 14.32 cells (range: 0 - 54 cells). The raw data of the CD_{69} positive-uNK cells had a skewness value of 1.94 with standard error of 0.28; this indicated that the data was not normally distributed.

The Mann-Whitney test demonstrated no significant effect on levels of CD_{69} positive-uNK cells based on the HIV status (*U*= 662, *p*= 0.682). Similarly, there was no significant difference in the level of CD_{69} positive-uNK cells based on the pregnancy type (*p*= 0.06). Additionally the Kruskal-Wallis Test demonstrated no significant difference in the level of CD_{69} positive-uNK cells amongst the study groups (*p*= 0.195).

3.3.6. pNK Cell Levels

The pNK cell count of 158 study participants was recorded. The mean pNK cell count was 1119.27 ± 1889.99 cells (range: 0 - 14101 cells). The raw data of the pNK cells had a

skewness value of 3.52 with standard error of 0.19; this indicated that the data was not normally distributed.

The Mann-Whitney test showed a significant effect of HIV-statues on level of pNK cells across the study population (U= 2548.50, p= 0.05). The HIV-negative group (mean rank= 86.30) had significantly higher levels of pNK cells than the HIV-positive group (mean rank= 71.98). The Kruskal-Wallis Test no significant difference in the levels of pNK cells between the pregnancy types (n= 158; normotensive *versus* EOPE *versus* LOPE; p= 0.451). Additionally there was no significant difference in the levels of pNK cells between the study groups (n= 158; p= 0.061; Kruskal-Wallis Test).

3.3.7. CD_{69} positive-pNK Cell Levels

The CD_{69} positive-pNK cell count of 158 study participants was recorded. The mean cell count was recorded to be 62.69 ± 129.73 cells (range: 0 - 1075 cells). The raw data had a skewness value of 4.99 with standard error of 0.19; this indicated that the data was not normally distributed.

The Mann-Whitney test showed no significant difference on the level of CD_{69} positive-pNK cells based on HIV status (U= 3033.50, p= 0.783). The Kruskal-Wallis Test showed a significant difference in level of CD_{69} positive-pNK cells based on the pregnancy types (n= 158; normotensive *versus* EOPE *versus* LOPE; p= 0.009). The normotensive group (mean

rank: 64.85) had the lowest mean CD_{69} positive-pNK cell count compared to the EOPE and LOPE groups (mean rank: 88.77 and 86.00 respectively). Moreover, results showed that the HIV-positive LOPE study group had the highest levels in CD_{69} positive-pNK cells compared to all the study groups (p= 0.003).

CHAPTER 4

DISCUSSION

4.1. Maternal mortality

Maternal deaths in the world have significantly decreased over the past years, yet still about 800 women die every day due to pregnancy and childbirth related causes (WHO 2010). According to a report done by the World Health Organization (WHO) in 2010, almost all maternal deaths occur in developing countries due to low-resource settings and a lack of skilled care during and after childbirth. In a report done in South Africa to look at maternal deaths between 2011-2013, 50% of the maternal deaths were due to hypertension and non-pregnancy related infection mainly HIV, Tuberculosis (TB) and pneumonia (Pattinson 2014). Therefore studies focusing on decreasing maternal deaths in South Africa and the world still remain an important sector in the research domain.

Immunology studies focusing on NK cells are imperative in pregnancy and HIV-associated disorders. These cells are one of the early recruited and activated cells in the first phase of the immune system's fight against pathogens, making them early markers for a disturbed environment (Vacca, Moretta et al. 2011, Vivier, Raulet et al. 2011). In the maternal body, both uNK and pNK cells play a vital role in indicating whether there is a disruption in the normal progression of pregnancy. Analysis of the function and activation status of NK cells during pregnancy is very important, especially as the role of these cells is not fully recognized

post implantation in both normal and pathological pregnancies (Mosimann, Wagner et al. 2013).

4.2. Maternal characteristics

The aetiology of PE remains elusive, yet various maternal factors have been associated with increasing the risk of developing this disorder (Rebelo, Schlüssel et al. 2013). Numerous studies have investigated some of these factors and yielded results that contribute to the better understanding and management of PE. In this study various maternal characteristics presented at antenatal admission, that may indicate a higher probability of developing PE, were investigated.

4.2.1. Maternal age on PE development

The results of this study yielded no significant difference when looking at maternal age *versus* pregnancy type and HIV-associated pregnancy. This was contradictory to other studies that have been performed on larger study populations. In this study population the maternal age ranged from 18 to 44 years, which is a range that is not considered to greatly affect PE development.

A study by Ananth *et al* (2013) indicated that women of younger (<18 years) and mature (>40 years) age had a greater risk of developing PE. Additionally, Duckitt and Harrington (2005) reported that the risk of developing PE was almost double in women aged ≥ 40 years, and that

women also experienced a 30% risk increase for every additional year over the age of 34 years. In contrary, de Vienne *et al* (2009) reported that women of younger (<18 years) child barring age were associated with a lower risk of developing PE and having delivery complications. The myocardial function and connective tissue elasticity in younger women is in better condition, making the maternal body more suited for pregnancy development (de Vienne, Creveuil et al. 2009). Additionally, pregnancy complications and illness like gestational diabetes and PE are higher in mature women over the age of 40 years, predisposing these women to an increased risk of developing PE (Jacobsson, Ladfors et al. 2004).

Unsuccessful pregnancies, foetal mortalities and premature births are therefore higher in women of mature age (≥ 40 years) compared to younger women of child barring age (20-29 years) (Cleary-Goldman, Malone et al. 2005). In most studies the negative effects of age on pregnancy is seen to be significantly reduced in cases where good prenatal and postnatal care was given to both the mother and child (Jacobsson, Ladfors et al. 2004).

4.2.2. Weight and BMI on PE development

The results of this study yielded no significant difference when looking at maternal weight and BMI *versus* pregnancy type and HIV-associated pregnancy. This was inconsistent with other studies conducted in other countries associating weight with pregnancy complications. It is plausible to suggest that we didn't get a significant difference in the study because South African women are normally overweight even pre-pregnancy (Katz, O'Connell et al. 2005). Additionally, in 2005, Obesity Task Force reported that South Africa is among the top 3

countries with highest incidences of obesity globally, especially among women of reproductive age.

Many studies have associated maternal weight and the biochemical factors of being over weight with pregnancy complications and birth outcomes. A study by Rebelo *et al* (2013) showed that in pre-eclamptic women the inflammatory cytokines and maternal weight was higher compared to normotensive pregnant women. Increase in weight and BMI ultimately increases inflammatory cytokines like C-reactive protein, in this way increasing the risk of developing PE (Rebelo, Schlüssel et al. 2013). This evidence further implicates the immune system in the pathogenesis of PE. Haugen *et al* (2014) not only implicated maternal weight pre-pregnancy to adverse pregnancy and birth outcomes, but also associated maternal gestational weight gain (GWG) to the PE epidemic. Haugen *et al* (2014) also concluded that excessive weight gain during pregnancy negatively affect pregnancy and delivery outcomes in all women irrespective of their parity and gravidity states. Excessive GWG is also seen to increase the risk of maternal post-partum weight retention (PPWR) in women (Haugen, Brantsæter et al. 2014). Weight retention post-pregnancy may also have clinical complications associated with obesity and weight gain, causing maternal morbidity.

In contrary, a study by Heude *et al* (2012) identified that women who were over weight or obese gained less weight during their pregnancy. In this way over weight and/or obese women were at lower risk of developing PPWR. Weight gain in over weight and/or obese women has been speculated to be physiologically unnecessary due to the fact that fat stores to facilitate

pregnancy and lactation are already available, causing limited weight gain (Heude, Thiébaugeorges et al. 2012).

4.2.3. Blood pressure as one of the diagnostic tools for PE

Significant difference was shown in blood pressure between the pregnancy types. From the results of this study it was observed that both systolic and diastolic pressures were higher in the EOPE and LOPE women compared to normotensive women. Minimum raise in blood pressure was seen in normal pregnancies due to the natural increase of demand of oxygen and nutrients to supply both mother and child.

EOPE women had the highest blood pressure compared to both normotensive and LOPE women. EOPE develops early in pregnancy (<34 weeks of gestation) causing severe restrictions to the development and progression of the pregnancy, while LOPE develops later in the pregnancy (≥34 weeks of gestation) causing limited effects on both the mother and the child (Raymond and Peterson 2011, Li, Chen et al. 2014). EOPE is highly associated with abnormal placentation leading to constriction of maternal blood vessels that perfuse the placenta and supply the growing foetus. The early restriction of blood vessel dilation in EOPE is responsible for the increased in blood pressure that is seen in this type of PE.

Pregnancy-related hypertension may also affect the mother post-pregnancy due to the mobilization of fluid accumulated outside the vascular vessels during pregnancy into the

vascular vessels (Moodley 2013). The increase of fluid within the vascular vessels will raise the pressure, causing high blood pressure in these women. Continued hypertension post-partum may also cause pulmonary oedema, which could be detrimental for vital organs including the lungs, liver and the heart (Moodley 2013).

4.2.4. Parity and gravidity on PE development

In this study, there was no significant difference observed in both parity and gravidity based on pregnancy type and HIV-associated pregnancy. PE occurs most frequently in young primiparous and adult multiparous women (Jasovic-Siveska and Jasovic 2015). Primiparity was not an automatic inclusion criterion in this study. Only women 18 years or older were included, meaning primiparous women who were ≤18 years old and were admitted into the antenatal ward were not sampled. Women aged above 40 years have twice higher the risk of developing PE compared to younger women, irrespective whether they are primiparous or multiparous (Jasovic-Siveska and Jasovic 2015). According to Duckitt and Harrington (2005) being nulliparous almost triples the risk of developing PE. While the lower overall risk of PE among parous women is not explained by fewer pregnancies among women who experienced PE in a previous gestation (Hernández-Díaz, Toh et al. 2009).

4.3. Foetal characteristics

4.3.1. HIV and PE on Birth weight

In this study, there was no significant difference in the birth weight of the HIV-negative compared to the HIV-positive group ($p = 0.391$). Maternal nutritional status before and during pregnancy is a significant predictor of poor pregnancy outcome; however, its association in the presence of HIV infection remains contradictory (Dreyfuss, Msamanga et al. 2001). Low birth weight is significantly associated with maternal HIV status, GA, antenatal care, maternal age less than 20 years and > 35 years (Mitgitti, Seanchaisuriya et al. 2008).

The results of this study noted significantly higher birth weight babies in the normotensive compared to those from the LOPE and EOPE group ($p < 0.001$). PE/eclampsia is a significant risk factor for perinatal death, preterm birth and low birth weight (Ota, Ganchimeg et al. 2014). PE is a disorder subsequently caused by failure of the utero-placental blood vessels to stretch into larger, more efficient blood vessels. This decrease in the utero-placental blood flow results in a hypoxic placenta which may alternately lead to a low birth weight, this is due to the lack of oxygen reaching the growing foetus (Powe, Levine et al. 2011). Various studies have revealed that not all pre-eclamptic women deliver small babies. Opposing the classical viewpoint is the recent work by Xiong *et al* (2001), who concluded that babies born to mothers with PE at term have fetal growth similar to that of babies born to normotensive mothers. The birth weight is also determined by other maternal factors aside from PE. One of

these is the excessive maternal gestational weight gain which according to Haugen *et al* (2014) may lead to an increased foetal birth weight.

We observed the lowest birth weight in the EOPE group compared to the LOPE and normotensive groups. This finding is supported by the fact that EOPE is characterized by defective cytotrophoblast placentation, defective spiral artery conversion with resultant poor blood flow (Naicker, Khedun et al. 2003). Our results also reflect that PE is an etiologically heterogeneous disorder as our EOPE birth weight differed from the LOPE group. Birth weight may also be affected by multiple pregnancies, maternal obesity and under weight, inter-current chronic medical conditions in pregnancy, anaemia, cigarette smoking, alcohol and other substance abuse (England, Levine et al. 2002, Xiong, Buekens et al. 2011). Confounding conditions such as maternal obesity and under weight, anaemia, cigarette smoking, alcohol and other substance abuse were not exclusion criteria in this study.

4.3.2. HIV and PE on GA

In this study, there was no significant difference in GA between the HIV-negative group and the HIV-positive group (p= 0.978). Significant difference was observed in the GA of the infants between the pregnancy types (p< 0.001). As expected, GA was lowest in the EOPE compared to normotensive and LOPE groups.

Our findings support the hypothesis of placental hypo-perfusion caused by shallow invasion of cytotrophoblast in early pregnancy leading to foetal growth restriction in PE (Naicker, Khedun et al. 2003). EOPE (before 34weeks) is frequently linked with abnormal spiral arteries, foetal growth restriction, and adverse maternal and neonatal outcomes (Ness and Sibai 2006, Huppertz 2008). In contrast, LOPE (after 34 weeks) is predominantly correlated with normal or slightly elevated uterine resistance index, a low rate of foetal involvement, and better perinatal outcomes (Sibai, Dekker et al. 2005).

4.4. Lymphocytes in HIV-associated PE

The immune system is composed of various cells that act to protect the body against a variety of foreign pathogens. Lymphocytes are one of the five types of cells that form part of the immune system. T-cells, B-cells and NK cells are all subtypes of lymphocytes that have different functions in the fight against foreign pathogens. In this study lymphocytes were quantified in an aim to evaluate the levels of these cells in study participants of different HIV status, pregnancy type and study groups.

In this study, a significant effect was observed in the lymphocyte count based on the HIV-status (p= 0.002). Results revealed that there were significantly higher levels of lymphocytes in HIV-positive participants compared to HIV-negative participants. Increase in the levels of lymphocytes indicates that there is a disease or foreign pathogen present, in this case HIV and the paternal antigens. Levels of lymphocytes are increased in the early stages of HIV infection and thereafter decrease as the virus over-powers the attempt of the immune system to eradicate

it (Lavu, Kutson et al. 2004). The high levels of lymphocytes in could be present in an attempt to combat the HIV in the body. Lavu *et al* (2004) found that the lower the total lymphocyte count, the more clinically advanced was the HIV disease state. Increased levels of CD_4-expressing lymphocytes may also be due to the intake of Antiretroviral (ARV) drugs. In this study, all the HIV-positive women were given the ARV drugs to strengthen their immune system for the survival of both the mother and the child, and also to decrease the viral load to prevent mother-to-child transmission. WHO has submitted that, absolute lymphocyte count (ALC) of $\leq$1200/μL can constitute CD_4 cell count of $\leq$200/μL in resource-constrained countries throughout the world (Kakar, Beri et al. 2011). Indicating that lowered lymphocyte count is less able to mount an inflammation response and in turn eliminate foreign pathogens.

The study by Boyajian *et al* (2012) found that there was no difference in the prevalence of PE between women on highly active anti-retroviral therapy (HAART) and HIV-negative women. While further studies has revealed that HIV-positive pre-eclamptic women who are on HAART develop severe cases of this disorder (Wimalasundera, Larbalestier et al. 2002). This may be associated to the fact that HAART drugs predispose women to symptoms similar to those of severe PE (HELLP and thrombocytopenia), making diagnosis difficult (Boyajian, Shah et al. 2012).

Results of this study also revealed lower levels of lymphocytes in normotensive participants; LOPE participants had the highest levels of lymphocytes ($p< 0.001$). PE has been intensively identified as an immune triggered disorder. Increase in lymphocytes indicates an activated

immune system, which could alternately lead to the development of PE. Lower levels of lymphocytes are therefore predicted in normotensive pregnant women due to the absence of PE. The levels of T-cell lymphocytes are elevated in normal pregnancies compared to non-pregnant women due to the role these lymphocytes play in the success of pregnancy (Aluvihare, Kallikourdis et al. 2004). Lymphocytes play a vital role in creating a more accepting environment in the maternal body, making the presence of these cells important (Darmochwal-Kolarz, Saito et al. 2007).

Additionally, the highest levels of lymphocytes were seen in the HIV-positive LOPE study group; HIV-negative normotensive had the lowest levels of lymphocytes ($p< 0.001$). Results of this study contradicted results from a study done by Hall *et al* (2013) which concluded that due to the weak immune system existing in HIV-positive women, the prevalence of PE would be lower compared to the general population. Various studies have also stated that presence of HIV in pregnant women prevents other aspects involved in the development of PE (Boyajian, Shah et al. 2012).

4.4.1. NK cells in HIV-associated PE

NK cells have been extensively associated with the succession of pregnancy. These cells are said to play an important role in preparing a more suitable environment for the growing foetus within the maternal body. In this study the NK cells were evaluated through their cell surface glycoproteins to validate their role in pathological pregnancies, more specifically PE. Levels

of NK cells were evaluated to establish their relation in HIV status, pregnancy type and study group.

In this study the NK cells were meticulously differentiated from other lymphocytes within the lymphocyte pool as CD_3 negative. Levels of CD3 negative lymphocytes were counted and our results yielded significant difference in the amount of these cells between the HIV-positive and HIV-negative groups (p= 0.011). Our results showed a higher amount of CD_3 negative cells in the HIV-positive group compared to the HIV-negative.

Normally NK cells constitute approximately 15% of the total peripheral lymphocyte count, they are a first line of defence against viral infection and tumour development (Fauci, Mavilio et al. 2005). It is therefore anticipated that these cells would be increased in the presence of a virus, in this case HIV. Although NK cells are increased in presence of HIV to mount an inflammatory response against the virus, various studies have determined that HIV is able to compromise the functionality and mechanism of action of these cells (Brunetta, Hudspeth et al. 2010).

With regard to the pregnancy type, this study found that there were higher amounts of CD_3 negative NK cells in the pre-eclamptic group (p< 0.001). Additionally, results showed high amounts of NK cells in the EOPE compared to the LOPE. In EOPE, the rejection of the growing foetus triggers the immune system and the production of increased levels of immune

cells (Huppertz 2008). These high amounts of immune cells and the exaggerated inflammatory response contributes to the inadequate and incomplete trophoblast invasion of maternal spiral arteries and abnormal blood flow of the umbilical arteries (Raymond and Peterson 2011).

This study also established high levels of the CD_3 negative NK cells in HIV-positive EOPE study group ($p< 0.001$). EOPE develops early in pregnancy and is associated with and increased inflammatory response (Brunetta, Hudspeth et al. 2010). The increased CD_3 negative cell count is an indication of amplified production of inflammatory cells.

4.4.2. Effects of different NK cell sub-groups on HIV-associated PE

NK cells may be divided into different sub-groups based on the location within the body, the surface glycoproteins they express, and their cytotoxicity (Brunetta, Hudspeth et al. 2010). In this study the amount of uNK and pNK cells were determined within the CD_3 negative NK cell population based on the amount of glycoproteins on their surface. The uNK cells were defined as CD_{16} positive and CD_{56}^{Bright}, while pNK cells were predominately defined as CD_{16} positive and CD_{56}^{Dim}. Levels of both NK cell subsets were evaluated based on the HIV status, pregnancy type and the study groups.

No significant difference was seen in the peripheral blood with regard to the uNK cell population. This was due to that peripheral blood, used in this study, possesses low amounts of

uNK cells. uNK cells migrate into the uterus during pregnancy were they are needed and more suitable to perform their function (Mosimann, Wagner et al. 2013, Vacca, Mingari et al. 2013).

This study showed significant difference in the amount of pNK cells between the HIV groups irrespective of pregnancy type (p=0.005). A higher amount of pNK cells were observed in the HIV-negative group compared to the HIV-positive group irrespective of pregnancy type. pNK cells are more cytotoxic and possess more CD_{56} and CD_{16} glycoproteins which are involved in Antibody Dependent Cell Cytotoxicity (ADCC) (Brunetta, Hudspeth et al. 2010, Vacca, Moretta et al. 2011). NK cells in HIV-positive individuals are less cytotoxic, they possess dysfunctional surface glycoproteins; this is a defence mechanism of the virus to override the immune response (Brunetta, Hudspeth et al. 2010). Less pNK cells were therefore observed in the HIV-positive group due to the lack of characteristic surface glycoproteins. NK cells in HIV-positive individuals are also functionally disabled, meaning the NK cells will not be able to perform their function during pregnancy- leading to a the development of PE (Brunetta, Hudspeth et al. 2010).

There were no significant differences observed between the pregnancy types and study groups with regard to both NK cell subsets. Other studies have implicated more cytotoxic pNK cells with the EOPE study group. This is as EOPE is associated with higher organ and placental dysfunctions (Huppertz 2008, Raymond and Peterson 2011).

4.4.3. Effects of CD_{69}-expressing NK cells on HIV-associated PE

CD_{69} is one of the early expressed receptors on NK cells in inflammatory sites (Mosimann, Wagner et al. 2013). Expression of this glycoprotein means cells have been activated by inflammatory cytokines (Sancho, Gómez et al. 2003). The aim of this study was to quantify CD_{69}-expressing NK cells in women of different HIV status, pregnancy type (normotensive *versus* EOPE *versus* LOPE) and HIV-associated pregnancy. This was done to get a clear understanding of the role profile of this surface glycoprotein, and also to see if it may be used as an early diagnostic biomarker in pre-eclamptic women.

This study revealed no significant difference in the levels of CD_{69}-expressing NK cells in both the NK cell subgroups. This could be related to that CD_{69} is an early expressed receptor, that has shown to be present in high levels in acute infections (Hosono, de Boer et al. 2003). CD_{69} levels are said to be rapidly decreased when the antigen stimulus is not present (Lund, Boysen et al. 2012), referring to that CD_{69}-expression on NK cells may be decreased as the HIV is able to override mechanisms of the immune system to eliminate it. In this study the period when the women were first diagnosed with HIV was not documented, meaning we were unable to determine whether these women were in the acute or chronic stages of infection.

With regard to pregnancy type, levels of CD_{69}-expressing pNK cells were shown to be significantly different (p= 0.009). Results of this study showed higher levels of CD_{69} positive-pNK cells in women diagnosed with EOPE. A study by Thum *et al* (2004) also showed that levels of CD_{69} positive-pNK cells were higher in women who had failed pregnancy. EOPE is

regarded as one of the severe type of PE associated with detrimental effects on both mother and child (Huppertz 2008). A study by Roussev *et al* (2013) found that factors released by the placenta to render NK cells less cytotoxic decreased the levels of CD_{69} in the maternal body. This suggests that high levels of this glycoprotein are associated with increased cytotoxic levels which could lead to severe cases of PE. CD_{69} expression on T-lymphocytes has also been observed to be up-regulated with increased severity of clinical symptoms of cardiac diseases (Hosono, de Boer et al. 2003). The role of this glycoprotein is not fully understood, yet several studies have involved CD_{69}-expression to the down regulation of the immune response by promoting production of TGF-β within NK cells (Sancho, Gómez et al. 2005). Indicating that increase in CD_{69} in severe cases in the body's way to control pathological condition.

4.5. Limitations of the study

One should note that a small amount of CD_{69} positive-NK cells has been isolated in healthy donors in the absent of any inflammation or disease (Lund, Boysen et al. 2012). This has been associated with the vaccination history and exercise program of the individual (Lund, Boysen et al. 2012). The CD_{69}-expression on NK cells may also be influenced by the HIV treatment regimen. In this study all pregnant women received HAART and nevirapine. Therefore the use of CD_{69}-expression on NK cells before the onset of maternal symptoms may lead to inaccurate diagnosis.

This study employed a one-time point sample collection. It is important to establish a base-line for the CD_{69}-expression on NK cells across all three gestational trimesters of normotensive uninfected pregnancies. This profile would enable comparisons between the aforementioned baselines with pathological pregnancies such as pre-eclamptic and HIV-associated pregnancies.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

This study demonstrates an elevation in peripheral blood lymphocytes in patients with HIV-associated PE. A neutralization of the immune response in HIV-associated PE has been previously suggested in other studies. However, the up-regulation in this study may be due to a modification of the functional profile of these cells, where the NK cell population augments cytotoxic amplification. More specifically, CD_{69}-expressing pNK cells were elevated in PE compared to normotensive pregnancies. In PE the placenta is dysfunctional; therefore it is plausible to assume there is an inadequate release of factors that renders NK cells less cytotoxic. This suggests that high levels of the CD_{69}-expressing NK cells are associated with increased cytotoxic levels which could lead to PE development.

We propose that CD_{69}-expressing NK cells have potential as a biomarker as an early diagnostic tool. Early identification of PE development is vital as PE is associated with diminished maternal and foetal morbidity and mortality. Additionally, being pre-eclamptic during pregnancy affects the quality of life post-partum. We also recommend better surveillance of maternal characteristics at antenatal booking to assess the risk of developing PE so that better management can be implemented in an attempt to save the lives of both the mother and baby.

The actual physiological role of CD_{69} in immunity responses is still elusive and more research still needs to be done. Evaluating CD_{69}-expressing NK cells across the three trimesters of gestation as well as a sub-stratification of lymphocyte count would be beneficial in understanding the role of CD_{69} in pregnancy. More importantly, an evaluation of the CD_{69} glycoprotein in women <20 weeks gestation would validate the use of CD_{69} as risk indicator/biomarker for early identification of PE development. CD_{69} expression is triggered by several mechanisms, including other lymphocyte receptor and cytokine stimulation. Future studies should also concurrently evaluate CD_{69}-expression on NK cells with T-cell receptors and other cytokines.

CHAPTER 6

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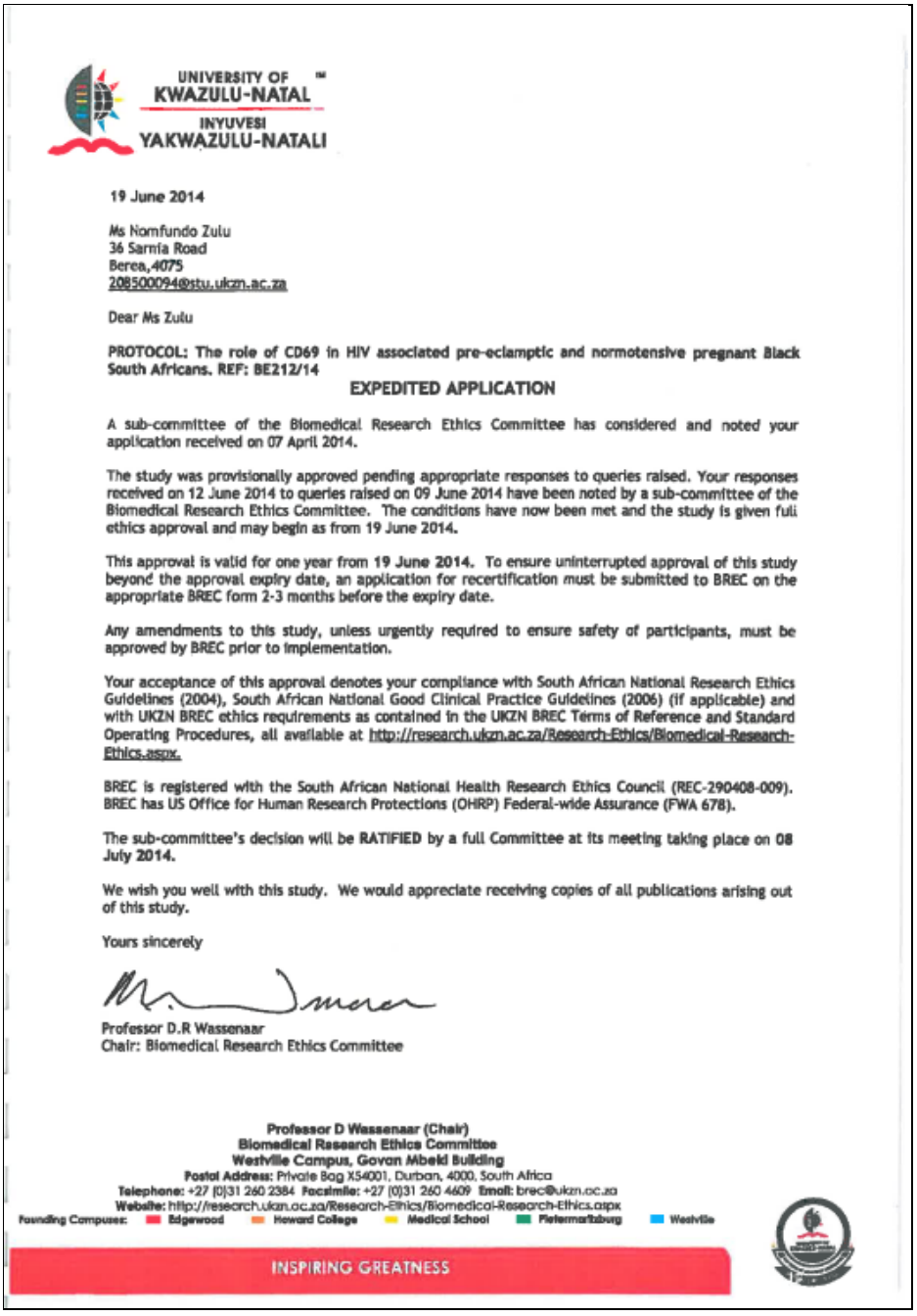
CHAPTER 7

APPENDICES

Appendix I: Institutional postgraduate office and UKZN Biomedical Research Ethics Committee ethical approval



UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

19 June 2014

Ms Nomfundo Zulu
36 Sarnia Road
Berea,4075
208500094@stu.ukzn.ac.za

Dear Ms Zulu

PROTOCOL: The role of CD69 in HIV associated pre-eclamptic and normotensive pregnant Black South Africans. REF: BE212/14

EXPEDITED APPLICATION

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 07 April 2014.

The study was provisionally approved pending appropriate responses to queries raised. Your responses received on 12 June 2014 to queries raised on 09 June 2014 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 19 June 2014.

This approval is valid for one year from **19 June 2014.** 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 meeting taking place on **08 July 2014.**

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

Professor D Wassenaar (Chair)
Biomedical Research Ethics Committee
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban, 4000, South Africa
Telephone: +27 (0)31 260 2384 **Facsimile:** +27 (0)31 260 4609 **Email:** brec@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx
Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

INSPIRING GREATNESS

Appendix II: Consent form

STUDY NO.

Ikhasi *nemvume* enolwazi yokuhlanganyela ocwaningweni

Usuku:

Nkosazane/Nkosikazi

Sawubona, nginguNkosazane Nomfundo Nokuthula Simlindile Zulu, umcwaningi kanye nesitshudeni se-Masters 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 nganoma 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 BE212/14).

Uma kwenzeka uba nezinkinga noma okukukhazathayo/ imibuzo ungathintana nami kunombolo yeselula: 073 743 1278, Emsebenzini: 031 260 4750 nakwi-imeyili: 208500094@stu.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: 073 743 1278, Emsebenzini: 031 260 4750 nakwi-imeyili: 208500094@stu.ukzn.ac.za

Uma nginemibuzo noma ukukhathazeka mayelana namalungelo ami njengomhlanganyeli, noma uma nginokukhathazeka mayelana nengxenye yalolu cwaningo noma ngabacwaningi ngingathintana ne-:

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

_____ _____

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 III: Patient's information sheet

THE ROLE OF CD69 IN HIV ASSOCIATED PRE-ECLAMPTIC 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** ☐

1

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

Parity	P: G:		Weeks gestation on admission			
Reason for previous pregnancy loss (If any)						
Highest BP	Systolic: Diastolic:					
Maternal weight		Maternal height		BMI		
Oedema (tick)	ankle	Up to knee	Up to groin	Generalised (facial)		
Lab results (or attach copy of results)	Proteinuria	Dipstick				
		Lab 24hr protein (if done)				
		Creatinine clearance (if done)				
	Full blood count	Red cell count		White cell count		
		Haemoglobin		Neutrophils		
		Haematocrit		Lymphocytes		
		Mean cell volume		Monocytes		
		Mean cell Hb		Eosinophils		
		Platelets		Basophils		

	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 fetal investigations

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

Birth details

Weeks of gestation at time of birth			
Date of birth		**Time of birth**	
Sex of baby			
Indication for delivery (tick one)	Maternal interest	Fetal Distress	Combination of Maternal and fetal 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	Normal vaginal		Caesarean	
	Spontaneous		Elective	

one)	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 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	

Appendix IV: Preparation of reagents

PBS + 0.1% Sodium Azide (Staining Buffer)

PBS + 0.1% Sodium Azide buffer was prepared and kept at room temperature (± 24°C). 50ml of 10 X PBS solutions (Lanza BioWhittaker® PBS without Ca and Mg) and 0.5g of Sodium Azide (Whit eSa` sigma) were added into a measuring cylinder. The solution was then topped up to 500ml with de-ionized water (dH_2O) and mixed thoroughly. In the final solution the concentration of the PBS was 1X and Sodium Azide was 0.1%.

1X FACS Lysis solution

1X FACS Lysis solution was prepared and kept at room temperature (± 24°C). 25ml of 10X BD FACS Lysis solution was added into a measuring cylinder, the solution was then topped up to 250ml with de-ionized water (dH2O).

Appendix V: Microscopy Society of Southern Africa Proceedings

THE ROLE OF CD69 IN NORMOTENSIVE BLACK SOUTH AFRICANS

N.N.Zulu[1], T. Naicker[1], J. Moodley[2] and A. Ajith[1]

[1]Optics and Imaging Unit and [2]Women's Health and HIV Research Group, University of KwaZulu-Natal, Durban, South Africa

Normal pregnancy can only take place if the necessary changes have occurred in the maternal body to accommodate the growth of the new foetus[1]. The role of natural killer (NK) cells in the progression of pregnancies after implantation is said to be inconclusive[1]. Even though these lymphatic cells are said to play a major role in rendering the maternal immune system more tolerant towards the growing foetus, it is also seen that increased activation of NK cells in peripheral blood may result in failed pregnancies[1]. CD69 is a cell surface glycoprotein and is an established early specific marker for the activation of NK cells[1]. CD69 can therefore be used as a biomarker for activated NK cells which can assist in the early diagnosis of pathological pregnancies which are normally detected late. The aim of this study was to determine CD69 expression in uterine and peripheral NK cells innormal pregnancies. This study will also provide a platform towards understanding the role of NK cells in pathological pregnancies.

Whole blood was drawn from 60 normotensive pregnant Black South African women at Prince Mshiyeni Memorial Hospital following institutional ethical approval and informed consent. Samples were lysed and then stained with fluorochrome-conjugated CD3, CD56, CD16 and CD69 and analysed on the BD LSRFortessa™ Flow Cytometer. NK cells are CD3 negative and are further subdivided into CD16 positive, CD16 negative and the intensity of the CD56 (bright or dim)[1]. Uterine NK (uNK) cells are predominantly $CD16^{-}+CD56^{bright}$, while peripheral NK (pNK) cells are predominantly $CD16^{+}+CD56^{dim}$ [1].Results were assessed on the FlowJo software package and exported to Microsoft Excel for statistical analysis.

Significant differences were observed between the levels of uterine and peripheral NK cells in normotensive pregnant women. A higher percentage of peripheral NK cells compared to uterine NK cells were observed. The levels of CD69 expression were also different between the uterine and peripheral NK cell populations.A higher percentage of CD69 expression in the peripheral NK cell population compared to the uterine NK cell population was observed.

Presence of CD69 on NK cells indicate that these cells are activated. CD69-expressing NK cells are cytotoxic and are important for preventing foreign substances from affecting the human body[2]. Activated NK cells are also involved in the dilation of maternal blood vessels, a process that is important for the progression of the pregnancy [2]. The dilated blood vessels are more capable of supplying the developing infant with life sustaining requirements such as oxygen and nutrients[2].

Future experiments in this study would be to compare the levels of CD69 in pre-eclamptic and normotensive pregnancies and to determine to the role this surface glycoprotein plays in pathological pregnancies. Since KwaZulu-Natal is the epicenter for HIV infection in South Africa, it will also be interesting to determine the role of NK cells in HIV associated preeclampsia and normotensive pregnancies.

References

1. Mosimann, B. *et al.* (2013) J Reprod Med.2013, 1-8.
2. Vacca, P. *et al.* (2011) Trends Immunol.32(11), 517-523.

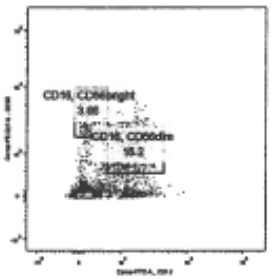


Figure 1.Uterine and peripheral NK cell populations.

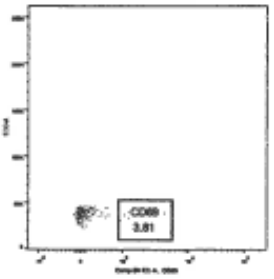


Figure 2.CD69 expression of the CD56 bright population.

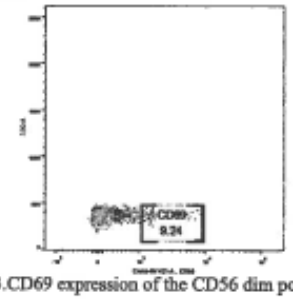


Figure 3.CD69 expression of the CD56 dim population.

Corresponding author: *208500094@stu.ukzn.ac.za*

Appendix VI: College of Health Science Research Symposium Proceedings

THE ROLE OF NK CELLS IN HIV ASSOCIATED PRE-ECLAMPTIC AND NORMOTENSIVE PREGNANCIES

Zulu, N.N.S.[*], Ajith, A.[*], Naicker, T.[*]and Moodley, J.[#]

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The role of Natural Killer (NK) cells in the progression of pregnancies after implantation is said to be inconclusive. These lymphatic cells play a major role in rendering the maternal immune system more tolerant towards the growing fetus, yet their extensive activation in maternal blood may also result in failed pregnancies. HIV affects the human immune system, particularly the expression and function of the NK cells. The aim of this study is to determine the levels and effects of NK cells in HIV associated pre-eclamptic and normotensive pregnant Black South Africans.

Whole blood and clinical demographics were collected from 160 pre-eclamptic and normotensive pregnant Black South African women at Prince Mshiyeni Memorial Hospital following institutional ethical approval and informed consent. Purposeful sampling strategy was used based on the inclusion and exclusion criteria. Samples were lysed and then stained with fluorochrome-conjugated CD3, CD56, CD16 and CD69 and analysed on the BD LSRFortessa™ Flow Cytometer. NK cells are CD3 negative, CD16 positive and CD56 positive. Results were assessed on the FlowJo (Version 10) software and exported to SPSS (Version 22) for statistical analysis.

Results revealed higher levels of NK cells in the HIV positive group (p= 0.011) and the pre-eclamptic group ($p\leq$ 0.001). Additionally, the results showed that presence of HIV, pre-eclampsia and the high levels of NK cells had detrimental effects on the maternal and neonatal health. Further studies will compare the levels of NK cells between late-onset and early-onset pre-eclamptic patients.