THE ROLE OF TROPHOBLAST CELLS IN HIV-1 PASSAGE ACROSS THE PLACENTA

by

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submitted in partial fulfilment of the requirements for the degree of

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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, Durban, South Africa under the supervision of Professors T. Naicker and J. Moodley.
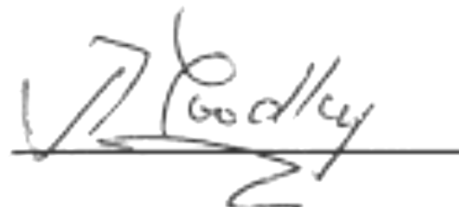
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DECLARATION

I, Vinogrin Dorsamy, declare that:

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

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   Date: _31 March 2015_____

DEDICATION

To all mothers and their babies.

To their happiness.

PEER REVIEWED CONFERENCE PRESENTATIONS

International:

1. V. Dorsamy, C. Vallen, J. Moodley and T. Naicker (2015) Transcytosis of HIV across pre-eclamptic pregnancies. *Malaysian Society of Hypertension*. Kuala Lumpur Abstract: Accepted November 2014.

2. V. Dorsamy, T. Naicker, R. Khan, J. Moodley and A. Ajith (2014) The differential expression of complement regulatory factors CR1 and DAF in Pre-eclamptic and HIV affected pregnancies. Placenta. 35(9):A85.

National:

3. V. Dorsamy, C. Vallen J. Moodley and T. Naicker (2014) Immunolocalisation of HIV and CD4 in normotensive and pre-eclamptic pregnancies. *Proceedings of Microscopy Society of Southern Africa* 44, Stellenbosch, South Africa. ISSN 0250-0418:ISBN 0-620-35056-3

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LIST OF ABBREVIATIONS

°C	degrees Celsius
µg	micrograms
µl	microliters
µm	micrometre
ANOVA	analyses of variance
AV	anchoring villi
BM	thickened basement membrane
BP	blood pressure
BSA	bovine serum albumin
C	fetal capillaries
CI	confidence interval
cm	centimetre
CT	cytotrophoblast
DAB	diamino-benzidine
dH$_2$O	distilled water
DOH	Department of Health South Africa
DNA	deoxyribonucleic acid
DPX	dibutylphthalate
EC	endothelial cells
ER	endoplasmic reticulum
EVT	extravillous trophoblast cells
F	intravillous and perivillous fibrin
g	grams
H&E	haematoxylin and eosin
HIV	human immunodeficiency virus
hrs	hours
IgG	immunoglobulin G
IHC	immunohistochemistry
KZN	KwaZulu-Natal

L	lumen
LC	Langhans cells
M	mitochondria
min	minute
ml	millilitres
mM	millimolar
mmHg	millimetres mercury
mv	microvilli
N	nuclei
N-	HIV negative normotensive pregnant women
N+	HIV positive normotensive pregnant women
nm	nanometres
ns	non-significant
P	platelets
P-	HIV negative pre-eclamptic pregnant women
P+	HIV positive pre-eclamptic pregnant women
PBS	phosphate buffered saline
RBC	red blood cells
RNA	ribonucleic acid
RT	room temperature
SB	syncytial bridges
SD	standard deviation
sec	seconds
SK	syncytial knots
SL	spongy layer
ST	syncytiotrophoblast
TB	tuberculosis
TGF-β_1	transforming growth factor-beta 1
USA	United States of America
wks	weeks
yrs	years

ABSTRACT

Background

The combination of pre-eclampsia and human immunodeficiency virus (HIV) remains a major obstetric dilemma in South Africa and, consequently, mother to child transmission of HIV remains a burden in our community.

The human placenta is in direct contact with maternal blood and is therefore susceptible to HIV infection or transmission. Cluster of differentiation (CD) 4 (CD4) and C-C chemokine receptor type 5 (CCR5 or CD195) are known to be important receptors for HIV transmission. Maternal in utero transmission is thought to mainly rely on R5-trophic viruses. Intercellular adhesion molecule 2 (ICAM-2 or CD102) is a member of intercellular receptor family found on endothelium and leucocytes that is involved in cell mediated immune recruitment. ICAM-2 is implicated in HIV transmission.

The aim of this study was to immunohistochemically localise HIV (p24 viral core) and the HIV receptor (CD4) within the placenta of HIV associated pre-eclamptic pregnancies to elicit the mechanism of vertical transmission of HIV. A further aim was to immuno-localise the HIV co-receptor (CCR5) and ICAM-2 within these placentae.

Method

Post institutional ethics approval, a retrospective cohort of 80 out 180 archived paraffin embedded placental blocks was sourced from mothers who delivered at Prince Mshiyeni Memorial district hospital in KwaZulu-Natal. Four groups (n=20/group) were categorised according to pregnancy status (normotensive and pre-eclamptic) and HIV status (positive and negative). Pre-eclampsia was defined as new onset hypertension (≥140/90mmHg) with proteinuria. Immunohistochemistry was performed using the Envision kit (DAKO, Denmark). Anti-human mouse CD4, and p24 antibodies were used to identify presence of HIV and CD4 receptors. The antihuman mouse antibodies CCR5 and ICAM-2 were used to detect these receptors within placentae. An Axiovision A1 light microscope and Axiovision (Zeiss) software was used for image acquisition and analysis where percentage staining in microns per field area (20x objective) was measured.

Results

Eight out of 180 HIV positive mothers in both pre-eclampsia and normotensive groups transmitted HIV to their babies (4%) despite receiving antiretroviral therapy. CD4 positive maternal immune cells and endothelial cells lining fetal vessels were found. CD4 was very rarely seen on syncytiotrophoblast. p24, however, was present in both the maternal and fetal blood circulation, as well as within Hofbauer cells.

CCR5 showed diffuse staining of all exchange villi within all four cohorts. A factorial univariate ANOVA was then performed and both HIV and pregnancy status had a significant main effect on expression of CCR5 in placental tissue. There was greater expression of CCR5 in the HIV positive groups compared to the HIV negative groups [$F_{(1,169)}$ =6.979, p=0.009] and the pre-eclamptic compared to the normotensive groups [$F_{(1,169)}$ =8.803, p=0.003].

ICAM-2 was sparse and found in areas around syncytial knots as well as lining an arterial supply in a stem villus. An independent samples Mann-Whitney U test showed a significant difference in the distribution of ICAM-2 expression between the pre-eclamptic and normotensive groups (p<0.001) and the HIV positive groups. The HIV negative pre-eclamptic group showed the greatest expression of ICAM-2 compared to the 3 other groups.

Discussion and Conclusion

The immunolocalisation of p24 provides evidence of HIV successfully traversing the fetomaternal barrier. Interestingly, HIV was found to be present in the placentas of babies that were HIV positive as well as those who were HIV negative 6 weeks later. This suggests an unknown mechanism that protects the fetus from viral insult.

All receptors and co-receptors used for HIV infection are found on the trophoblast, suggesting that this barrier is susceptible to HIV infection. Pre-eclampsia

increases expression of both cytokine receptors and inflammatory markers which may increase susceptibility to infection. Further ultrastructural and biomolecular work to identify the immune cells and expression of receptors involved will corroborate our findings.

CHAPTER ONE

INTRODUCTION

Successful pregnancy is a veritable battle between two opposing systems: a maternal system engaged in protection of self, and a fetal system that leaches from the maternal system and in so doing breaches maternal defence. The placenta lies at the interface of this conflict and if the baby is to survive, and the species requires it, favour needs to lie with the fetal system successfully invading the maternal system. Successful pregnancy depends on immunoregulation of the mother and an inadequate invasion of the mother manifests from as dire as total loss of the fetus to morbid conditions such as pre-eclampsia (Redman & Sargent, 2010). In fact, more pregnancies are unsuccessful than successful and most of these are related to immune maladaptation during the fetomaternal union (Raghupathy, 1997). The process of immune maladaptation is thought to begin early during pregnancy, leading to insufficient trophoblast invasion (Naicker *et al.*, 2013) leading to pre-eclampsia and the more life threatening eclampsia.

In Sub-Saharan Africa, that proportion increases significantly to almost 16 percent of all pregnancies (Moodley *et al.*, 2010). In South Africa, according to the latest Saving Mothers: Fifth National Confidential Enquiries into Maternal Deaths (Department of Health, 2012) forty one percent of all maternal deaths is HIV related, whilst hypertension (14%) is the commonest direct cause of maternal deaths in pregnancy. With these two conditions afflicting pregnancies to such orders of magnitude as experienced in South Africa, it is incumbent that valuable research

targeting these conditions is prioritised. This is directly in keeping with the DOH measures to reduce maternal and fetal mortality and morbidity. Additionally, it is in line with the global millennium development goals 4, 5 and 6 to reduce child mortality, improve maternal health and combat HIV/AIDS and other diseases respectively ("United Nations Millennium Development Goals", 2008).

HIV has been shown to cross the placenta. It has been found in both the placenta and the fetal tissue early in pregnancy (Anderson, 1997; Sheikh, Polliotti & Miller, 2000; Douglas & Thirkill, 2001), while others suggest that passage is relegated to the peripartum stage where an admixture of HIV positive maternal blood with fetal blood is responsible for infection during delivery (Al-husaini, 2009). Other studies found that breastfeeding significantly influences transmission rates and caesarean sections are safer for the baby (Coovadia *et al.*, 2007; Kourtis & Bulterys, 2010). What remains undeniable is that even with the advent of ARVs and all PMTCT puerperal interventions; the rate of mother to child infection is still significant. *In utero* transmission is therefore still a considerable factor in childhood HIV and nowhere is there more burden than sub-Saharan Africa, where access to interventions is limited and rates of mother to child transmission is much higher than that boasted for developed countries.

In utero transmission may involve many mechanisms, and many paradigms have been purported. Transcytosis of the HIV across the placenta (Bomsel, 1997; Vidricaire & Tremblay, 2007), placental inflammation and co-infection (Mwanyumba *et al.*, 2002; Bhoopat *et al.*, 2005), infection of the amniotic fluid (Lobato *et al.*, 2010),

placental detachment and loss of membrane integrity (Burton *et al.*, 1996) and HLA concordance (Mackelprang *et al.*, 2008) are among the popular mechanisms. In this study, we focus our interest on infection of the placenta and transcytosis from the maternal to fetal circulation.

There are two popular mechanisms that HIV uses to infects cells: the classical route which requires the use of CD4 and co-receptors to actively infect the syncytiotrophoblast (David *et al.*, 1992; Behbahani *et al.*, 2000); and alternate cell mediated routes that use alternate inflammatory signalling mechanisms to recruit HIV infected cells to the trophoblast barrier (Lairmore *et al.*, 1993; Arias, Muñoz & Muñoz-Fernández, 2003; Vidricaire & Tremblay, 2007; Tremblay & Vidricaire, 2008).

The epithelial-like placental lining that separates the two circulations may play a role in either or both. Expression of CD4 and CCR5 on the placental barrier will allow cell free virus to infect the trophoblast (Shen *et al.*, 2010). Expression of other co-receptors or receptors involved in inflammation or suppression of inflammation may also facilitate a cell mediated mechanism of HIV transfer (Bomsel, 1997; Lagaye *et al.*, 2001; Arias, Muñoz & Muñoz-Fernández, 2003).

Furthermore, the virus may take advantage of the endocytic pathway to pass the placental barrier. Does the placenta express receptors that are implicated in the passage of HIV? How does expression change during conditions such as pre-eclampsia?

1.1 Aims

In the present study, we aim to:

- Localise CD4 receptors and HIV co-receptor CCR5 in trophoblasts and associated Hofbauer cells within the placenta.

- Quantify the expression of these receptors within the tissue, as well as to compare and contrast such expression between the normotensive and pre-eclamptic HIV affected pregnancies.

- Locate the presence of p24 capsid proteins as well as expression of membrane receptors (ICAM-2) that may be associated with recruitment of immune cells to the trophoblastic barrier between mother and fetus.

Such association will provide valuable insight into the mechanism of *in utero* transmission of HIV. Furthermore, by comparing cohorts of normotensive to pre-eclamptic placentae, we can evaluate any synergy or comorbidity between these geographically common conditions burdening the South African population.

CHAPTER TWO

LITERATURE REVIEW

2.1 HIV

Globally, Sub-Saharan Africa accounts for two thirds (67%) of the 33 million people living with the Human Immunodeficiency Virus (HIV). Of this, almost half are women in their reproductive years (UNAIDS, 2012). South Africa's HIV epidemic is defined as being a hyper-endemic epidemic due to greater than 15% of its population within the age range 15–49 years being HIV infected (UNAIDS, 2012).

In South Africa (SA), HIV infection accounts for 40.5% of maternal deaths. Almost 4 out of 5 HIV infected women (70.4%) die in childbirth or the puerperium (Department of Health, 2012). Additionally, HIV prevalence is higher in females than males and peaks at an earlier age than in males (Department of Health, 2012). The province of KwaZulu-Natal (KZN) on the eastern coast of South Africa is considered the epicentre of the global HIV pandemic with 23% of maternal deaths occurring in this region (Department of Health, 2012).

HIV is a major cause of maternal deaths caused by infections not originating in the genital tract, that is, non-pregnancy related infections (NPRI) (Moran & Moodley, 2012). Pneumonia, meningitis and tuberculosis are examples of HIV associated indirect maternal deaths. Prior to HIV becoming so prevalent in our communities, hypertensive disorders of pregnancy was the leading cause of maternal death.

The recent report of the National Committee on Confidential Enquiries into Maternal Deaths in South Africa, highlights hypertensive disorders of pregnancy and haemorrhage as the two commonest direct causes of maternal death contributing to 14% and 14.1% of deaths respectively (Department of Health, 2012). This maternal mortality surveillance shows an elevation in maternal deaths over preceding years, emanating from the increase in HIV infection. Hypertension in pregnancy, however, has decreased from 19.1% to 15.7% to 14.0% over the last three triennum periods viz., 2002-2004, 2005-2007 and 2008-2010 respectively.
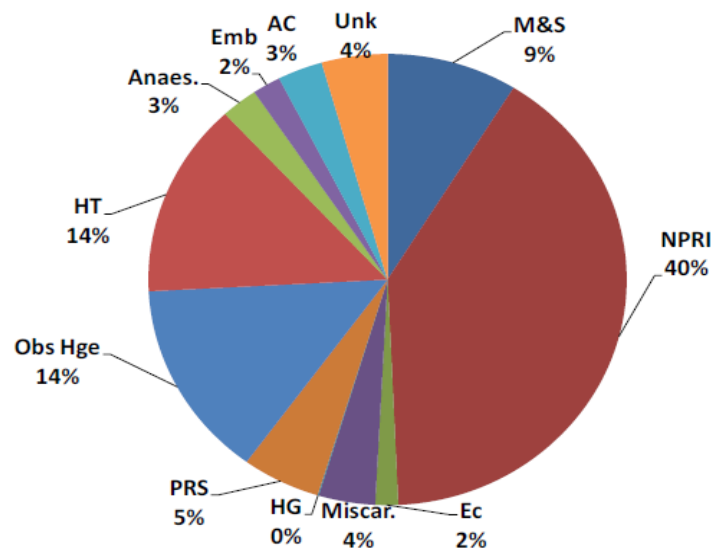
Figure 2.1: Distribution of underlying causes of maternal death for period 2008-2010 in South Africa. Non pregnancy related infections (NPRI) such as TB and HIV account for the highest proportion of maternal mortality, followed by hypertensive disorders of pregnancy and obstetric haemorrhage. With permission: (Department of Health, 2012).

2.2 Pre-eclampsia

As a hypertensive disorder of pregnancy, pre-eclampsia is a multisystem syndrome that is characterised by a systolic blood pressure of greater than or equal to 140mmHg and diastolic blood pressure of greater than or equal to 90mmHg with a 24 hour sustained proteinuria of greater than 0.3g/dl (Moodley *et al.*, 2010). In resource poor settings where intensive care facilities are limited, pre-eclampsia often progresses to eclampsia, where it is responsible for significant fetal and maternal mortality and morbidity (Duley, 2009).

Pre-eclampsia can lead to the development of chronic hypertension, heart disease and stroke prematurely in the mother whereas their children may be growth restricted at birth and suffer greater risk of developing stroke, coronary heart disease and metabolic syndrome as adults (Uzan *et al.*, 2011). There is no definitive curative treatment for pre-eclampsia except for delivery of the placenta, therefore managing the condition remains a balancing act between induced preterm delivery and the risk of continuing with pregnancy and its complications (Steegers *et al.*, 2010). Delivery of the baby to resolve the condition indicates that the placenta may be the major contributing factor to the cause of the syndrome.

2.3 Pathophysiology of pre-eclampsia

In normal pregnancy, cytotrophoblast cells from the basal plate and anchoring villi infiltrate into the decidua and inner myometrium during placentation (Pijnenborg, Vercruysse & Hanssens, 2006). This invasion also occurs within the lumen of the spiral arteries where the internal elastic lamina and musculo-elastic media of the

arteries are replaced by a fibrinoid material in which trophoblast cells are embedded (Naicker *et al*., 2003). This physiological change occurs early in pregnancy and is complete by 20 weeks gestation. This change in the spiral arteries causes them to become flaccid low resistance vessels with a dilated lumen that enables high flow of blood to the fetus (Naicker *et al*., 2013).

In pre-eclampsia, abnormal invasion of the spiral arteries by cytotrophoblasts causes the spiral arteries to inhibit nitric oxide synthesis and maintain vascular tone with inadequate blood flow to the baby. This leads to increased uterine artery resistance with greater sensitivity to vasoconstrictive promoters with resultant placental ischaemia and oxidative stress. Fetal intrauterine growth retardation and/or death may ensue (Burton & Jauniaux, 2011).

Oxidative stress causes the release of free radicals, oxidised lipids, vascular endothelial growth factor 1, as well as other Th1 type cytokines into the maternal circulation. These contribute to endothelial dysfunction, hypertension, thrombophilia and vascular hyperpermeability as a compensatory mechanism for reduced uterine blood flow in pre-eclampsia (Burton & Jauniaux, 2011; Govender, Naicker & Moodley, 2013). Moreover, haemolysis, elevated liver enzymes and low platelet count (HELLP syndrome), cerebral endothelial dysfunction and reduced vascular endothelial growth factor as well as podocyte effacement diminishes glomerular filtration that leads to proteinuria (Uzan *et al*., 2011).

The incidence of pre-eclampsia is 1-3% in multiparous women and 3-7% in nulliparous women (Redman & Sargent, 2010). Primigravidae have an increased risk of pre-eclampsia development, and women under 24 years in this group are more likely to suffer hypertensive disorders of pregnancy (Department of Health, 2012). The propensity for pre-eclampsia to occur during first pregnancies may be related to the semi-allogeneic fetus being rejected by the maternal immune system (Dekker & Sibai, 2001; Saito, Sakai, *et al.*, 2007; Saito, Shiozaki, *et al.*, 2007). This is especially true when there is inadequate sperm exposure that results in insufficient maternal tolerance. In cases where sperm exposure is low, such as in teenage pregnancies as well as in artificial insemination, the risk of pre-eclampsia occurrence is high (Dekker & Sibai, 2001). This indicates an inadequate maternal immune response to the fetal allograft.

2.4 Mother to Child Transmission (MTCT) of HIV

The high prevalence of HIV amongst primigravid pregnant women in South Africa is associated with a concomitant increase in HIV vertical transmission to their infants (Al-husaini, 2009). Prior to both antiretroviral drug rollout and public health initiatives, mother to child transmission (MTCT) rates of HIV-1 was responsible for up to 42% transmission in breastfed infants (Kourtis & Bulterys, 2010; Mofenson, 2010). There has been a significant increase in the global coverage of antiretroviral (ARV) therapy to prevent mother to child transmission. There was a 30% global increase in the number of women accessing ARV treatment regimens in 2012 compared to 600 000 women in 2009 (Moodley et al., 2010). In Africa, according to the *Countdown to Zero: Global Plan towards the elimination of new HIV infections among children by*

2015 and keeping their mothers alive (Joint United Nations Programme on HIV/AIDS, 2011), the number of women receiving ARVs has steadily increased from 49% in 2009 to 65% in 2012.   However, there are still major hurdles for the successful provision of ARV treatment regimens to the targeted 90% of all pregnant women living with HIV in Africa. Major obstacles include limited access to healthcare facilities, social stigma, economic insecurity and lack of HIV testing (*WHO | Global update on HIV treatment 2013*, 2013).

Where therapy is not accessed, there is subsequent restriction of HIV prophylaxis and therapy to their infants as well. South Africa has the highest burden of HIV yet there is still only 67% coverage of eligible children (0-14years) receiving ARVs (*WHO | Global update on HIV treatment 2013*, 2013). As most of these children become infected vertically, it is important to gain further insight into the mechanism by which they become infected in order to more effectively direct resources towards achieving global targets.

2.5 Types of Mother to Child transmission

Vertical transmission of HIV-1 occurs when the fetus is exposed to cell free virus or infected maternal cells. This may occur during gestation, delivery or breastfeeding (Arias, Muñoz & Muñoz-Fernández, 2003). The timing and circumstance of transmission is difficult to establish due to several confounding factors. This becomes obvious when you remove one of the transmission pathways, for example, breastfeeding. Although there is a 33 - 50% reduction in transmission of HIV-1 reported in non-breast fed infants of HIV positive mothers, the balance of

transmission still occurs either *in utero* or intrapartum (Kourtis *et al.*, 2010). Thus in non-breast fed infants, deciphering when such transmission occurs is unclear. Whilst testing HIV positive infants immediately postpartum only differentiates between *in-utero* and intrapartum mode of transmission, the precise moment of HIV infection remains unknown. For clarity it is important to define the three methods of MTCT of HIV-1 (Figure 2.2) (Lehman & Farquhar, 2007).
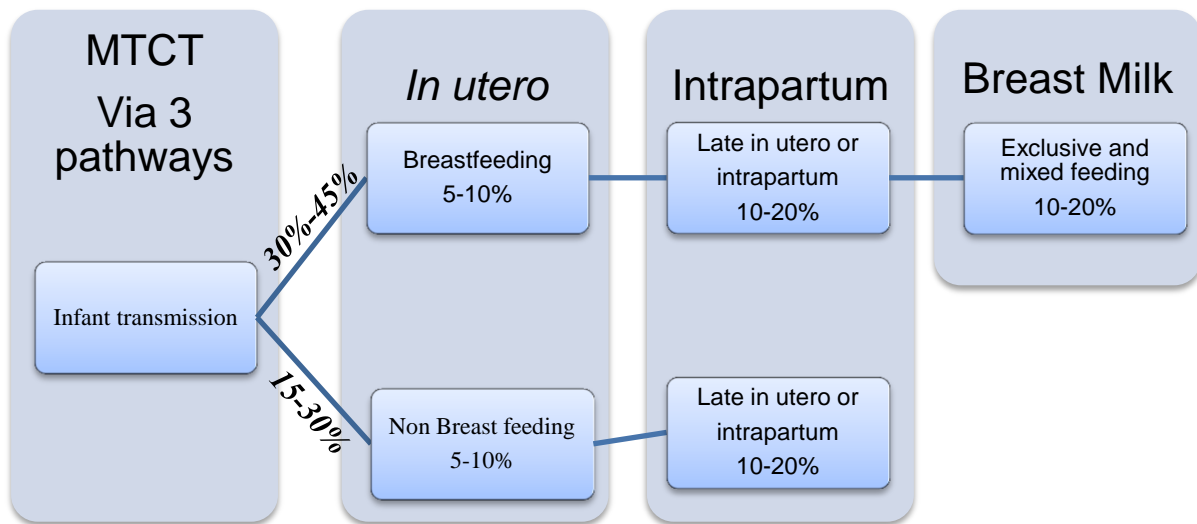
Figure 2.2: Three pathways of Mother to Child Transmission of HIV including the rates of infection of each. In mothers that breastfeed, 30-45% overall rate of transmission compared to mothers that do not breastfeed (15-30%). The exact timing of transmission is unclear and HIV testing at delivery or after 6 weeks may indicate mode of transmission, but may not be reliable when differentiating late *in utero* infection from intrapartum (exposure to maternal blood and vaginal secretions) infection. Adapted from Lehman and Farquhar (2007).

2.6 *In utero* transmission

In utero transmission occurs during the gestational period via ascending infection of the amniotic membranes or fluid;   or transfusion of maternal blood into the fetal bloodstream via different mechanisms and time (Lehman & Farquhar, 2007; Kourtis & Bulterys, 2010). The majority of HIV-1 infections occur during the third trimester, although there are reports of HIV-1 infection occurring as early as 8 weeks (Lehman & Farquhar, 2007). The presence of HIV in early pregnancy suggests that the virus has crossed the placenta. This hypothesis is, however, controversial as many studies dispute the presence of HIV in the amniotic fluid or its ability to infect the fetus (Mohlala *et al.*, 2005; Lobato *et al.*, 2010). Indeed, in maternal fetal sample pairs, Lobato *et al* (2010) proposed that although HIV was found in the amniotic fluid of 3 cases, it occurred in a cell free form. This finding suggests that there is a likelihood that *in utero* infection does occur via the transplacental route. Burton *et al* (1996) suggests that the loss of patency of the placental barrier may mediate infection of the placenta.

Importantly, the virus has been found within the placenta of HIV infected mothers (Mattern *et al.*, 1992; McGann *et al.*, 1994; Menu *et al.*, 1997; Kourtis *et al.*, 2010). However, the transmission of HIV to the baby does not correlate with its presence within the placenta as some babies are born HIV negative even though HIV was documented within the placenta (Bhoopat *et al.*, 2005). This means that the virus is either unable to infect the baby or the placenta itself is resistant to productive infection or is somehow able to evade viral entry. Fetal cells may evade infection due to lack of HIV receptors or co-receptors necessary for infection within placental or fetal tissue, or fetal bloodstream. The baby or placenta may also produce antibodies

that fight off infection, or selectively permit only certain subspecies of virus to enter the fetal system (Lehman & Farquhar, 2007). As the infection of the placenta can occur from early pregnancy right up until detachment from the uterine wall, the precise moment of *in utero* infection remains an enigma.

2.7 Intrapartum infection

If infection occurs closer to the time of delivery, it is very difficult to distinguish it from intrapartum infection, which occurs during parturition. The intimate contact of the infants mucous membranes with maternal blood, amniotic fluid or cervico-vaginal secretions in the birth canal, as well as during extended exposure to ruptured membranes makes it susceptible to infection (Mofenson, 2010). Placental microtransfusions of maternal blood forced during contractions (Lin *et al.*, 1996), the act of labour as well as detachment of the placenta before clamping of the umbilical cord may all contribute to intrapartum infection. Extended rupture of membranes as well as increased swallowing and direct fetal skin exposure to maternal amniotic fluid, blood and vaginal secretions during birth result in increased risk of infection, especially in cases where there is fetal microtrauma or viral entry into the fetal gut (Mohlala *et al.*, 2005).

2.8 Breastfeeding

Another mechanism of MTCT occurs during breastfeeding and although avoidable, it accounts for a significant increase in transmission rates in middle to lower income countries where access to alternative feed, potable water, hygiene and sanitation are

inaccessible or unaffordable or where there is stigma associated with not breastfeeding (Mofenson, 2010).

Breast milk has been shown to contain HIV in both free and cell associated form and the amount of virus present is dependent on maternal antiretroviral treatment, maternal plasma viral load as well as breast health (Coovadia *et al*., 2007; Becquet *et al*., 2009). Such virus has been shown to infect the infant postnatally (Becquet *et al*., 2009). This leads to a 15% increase of MTCT rate in breastfeeding mother-infant pairs (Lehman & Farquhar, 2007).

Exclusive breastfeeding (EBF) has been shown to be more beneficial and improves survival rates of children under 5 years of age, regardless of HIV status of the mother (Rollins *et al*., 2013). The WHO recommends EBF up until 6 months, to reduce infant diarrhoeal morbidity and all-cause mortality ("WHO", 2013). In recent years, EBF coupled with both maternal and infant ARV prophylaxis of HIV mother-infant pairs has shown a significant reduction in post-natal infection and increased survival rates (Coovadia *et al*., 2007; Becquet *et al*., 2009; Mofenson, 2010).

In South Africa, where ideal targets of ARV coverage have not been met, the possibility of post-natal MTCT is unfortunately still a significant problem.

2.9 Timing of transmission

It has been proposed that HIV testing immediately after birth and at subsequent follow-up visits would elucidate the timing of MTCT (Bryson *et al.*, 1992). The attempt to define this timing was borne out of the need for a protocol to reduce post-natal infection by antiretroviral treatment regimens and perinatal interventions such as caesarean delivery before the onset of labour (Siegfried *et al.*, 1996; Kourtis *et al.*, 2001; Nightingale & Dabis, 2006; Kourtis & Bulterys, 2010).

In certain parts of Sub-Saharan Africa the coverage rates of Prevention of Mother to Child Treatment (PMTCT) strategies have improved to as much as 59% (UNAIDS, 2012). This has contributed to a decline in the rate of newly HIV infected children (24% decrease in period 2009-2011).

In places where access to healthcare, HIV testing, provision of antiretroviral (ARV) therapies, elective caesarean delivery and discontinuance of breastfeeding is possible, there has been a dramatic decrease in MTCT rates to less than 2% (Kourtis & Bulterys, 2010). However, in areas of high HIV prevalence, a similar decline of transmission rates is not immediately achievable. In Sub-Saharan Africa, access to ARV rollout programmes is still below par and breastfeeding is unavoidable. Family planning and adequate maternal antenatal monitoring also remain a challenge (Lehman & Farquhar, 2007).

Even though a significant reduction in transmission has been achieved, there are still greater than 1000 children infected with HIV each day. Real number values of vertical transmission of HIV to neonates is considerable in areas where access to antiretroviral therapy and antenatal care is limited, hence further intervention strategies or research into understanding of transmission pathways is urgently warranted (Department of Health, 2012).

2.10 Structure of the placenta

Humans possess a villous haemochorial placenta which has a large surface area of fetal villi in direct contact with maternal blood. The placenta is divided into two main functional zones: the chorionic villi which contain the fetal blood vessels and stroma; and the exchange villi (syncytiotrophoblast and cytotrophoblasts) (Benirschke & Kaufmann, 2006).

The development of the placenta starts with blastocyst attachment to the uterine wall and development and infiltration of the chorionic villi to enable fetal access to maternal blood. Further development leads to the formation of a placental barrier which separates the maternal blood from the fetal blood. This interface between the mother and the fetus is composed of a double layer of cytotrophoblast and syncytiotrophoblast that separate maternal and fetal circulation. The outermost layer, the syncytiotrophoblast, is a multinucleated syncytium which covers a mononuclear cytotrophoblastic layer. This barrier selectively permits food, nutrients, gases and waste to travel between maternal and fetal circulation (Benirschke & Kaufmann, 2006).

Within the chorionic villi are found fetal vascular endothelium, connective tissue stroma, and circulating macrophages called Hofbauer cells. As pregnancy progresses, the barrier becomes thinner. Cytotrophoblasts differentiate into supporting structures that anchor the villi within the placenta. These anchoring villi attach the placenta to the uterine wall. Other cytotrophoblast cells fuse to form a multinucleated syncytiotrophoblast which occurs after 12 weeks of gestation (Benirschke & Kaufmann, 2006). By virtue of its structure and its immediate and constant exposure to maternal blood, the placenta is susceptible to and implicated in the transmission of viruses (Arechavaleta-Velasco *et al*., 2002; Berencsi, 2012).

2.11 HIV

HIV infection ultimately causes acquired immunodeficiency syndrome (AIDS), which in humans is characterised by the destruction of immunocompetent cells. Loss of these immune cells renders the body unable to fight off infection from invading organisms, resulting in inadequate removal of cancerous cells or the inability to arrest pathologic proliferation of commensal organisms. This ultimately makes an infected person prone to opportunistic infections (Salemi *et al*., 2009). Infection occurs via bodily fluids. An infected person can transfer the virus to an uninfected person via blood transfer, vaginal and seminal fluids, and breast milk.

When HIV, either in free form or within an infected cell, is transferred to an uninfected individual, it targets certain cells of the immune system ("HIV/AIDS",

2014). Typically frontline immune cells exposed to the environment such as macrophages and dendritic cells are the first to be infected and these go on to infect CD4 positive T cells resulting in further T cell loss either directly by viral destruction or indirectly by host immunity targeting the infected cell.

The human immunodeficiency virus belongs to the family Retroviridae (Keays & Soni, 2004). As a lentivirus the genetic material is stored in RNA which is reverse transcribed into double stranded DNA by a reverse transcriptase enzyme. This DNA is subsequently integrated into the host genome (Craigie & Bushman, 2012). Before infection this genetic material, as well as enzymes necessary for transcription and integration, is encased in a conical capsid. This capsid is made of p24 protein, which is covered by a matrix protein p17, which in turn is enveloped by a lipid bilayer (Nielsen, Pedersen & Kjems, 2005).

The lipid bilayer complexes with integral protein complexes called Env that contains a triplet of glycoprotein (gp120) that form externally protruding caps (Nielsen, Pedersen & Kjems, 2005; Craigie & Bushman, 2012). Also part of this protein is a stem region which is made up of gp41. This stem region anchors the Env complex to the envelope matrix (Figure 2.3). This protein complex is responsible for the recognition and attachment to receptors on host cells in order to initiate infection.

HLA DR and other epitopes from host cell

gp120 surface protein

Gp41transmembrane protein

Reverse transcriptase

Integrase (In)

Protease (Pr)

Matrix protein (MA/p17)

Viral RNA

p24 capsid (CA)

Lipid bilayer
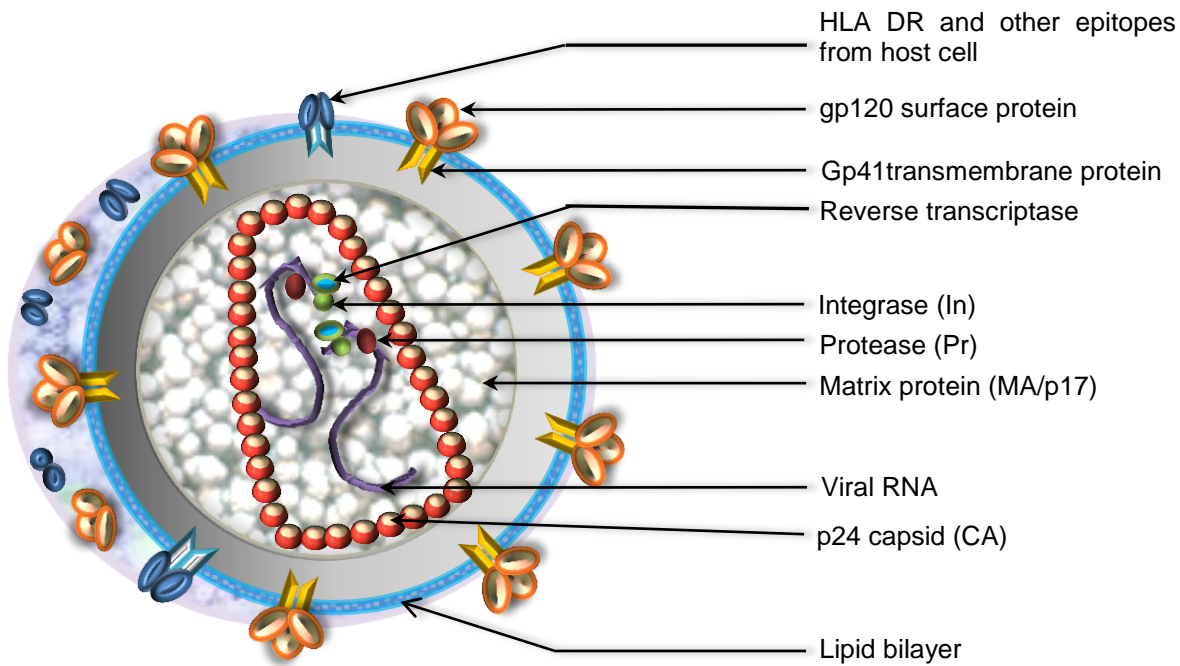
Figure 2.3: Structure of a mature HIV virion showing the conical viral core containing two strands of RNA viral genome and associated viral enzymes.

2.12 Mechanism of infection and HIV life cycle

The glycoproteins adsorb to specific receptors on target cells and is followed by fusion of the viral envelope with the cell membrane and entry of the p24 encapsulated core into the host cell, where viral enzymes convert and integrate the viral genome with that of the host (Nielsen, Pedersen & Kjems, 2005). The entire process is initiated when the gp120 attaches to a CD4 receptor on a host cell. This causes a conformational change in gp120 which then interacts with CCR5 or CXCR4 co-receptors. This interaction causes further conformational change in gp41. Coiling of gp41 pulls the viral particle closer to the host cell, simultaneously creating an opening and fusion of the host membrane with that of the virus (Ganser-Pornillos, Yeager & Sundquist, 2008).

Another proposed mechanism for HIV viral entry into cells is via the endocytic cellular machinery (Bomsel, 1997; Hocini & Bomsel, 1999; Hocini *et al.*, 2001; Lagaye *et al.*, 2001; Vidricaire, Imbeault & Tremblay, 2004). It is proposed that HIV in cell free form can enter cells without the classical CD4 receptor and chemokine co-receptor usage. Studies have shown that trophoblast cells behave like macrophages and macropinocytosis may lead to engulfing of virus particles. Apart from the classical endocytic clathrin, dynamin, caveolae and/or clathrin-caveolae pathways, there may also exist alternate pathways at endothelial exchange zones the permit viral entry into cells (Vidricaire & Tremblay, 2007).

Once internalised the viral core capsid is released into the intracellular compartment of the host cell, thereby injecting two viral RNA copies and enzymes (reverse transcriptase, ribonuclease, protease and integrase) into the cell. The viral capsid (p24) does not last long within the cytoplasm and is most likely broken down by cellular restriction factors and proteasomes (Fassati, 2006). However, the capsid protects the viral RNA long enough to initiate reverse transcription but is removed early enough to allow for effective binding of karyophilic viral and cellular nuclear import proteins. The RNA copies are then reverse transcribed by the accompanying reverse transcriptase enzyme and the DNA so formed is shuttled using the cell's intracellular machinery to import the DNA into the nucleus and integrate it into the host genome via the integrase enzyme (Craigie & Bushman, 2012).

The integrated virus remains latent until the host cell is activated and transcription factors promote DNA transcription (Ganser-Pornillos, Yeager & Sundquist, 2008).

Cells become activated during infection and inflammation, and ironically, it is these cells that are recruited to fight HIV that actually produce more HIV (Ganser-Pornillos, Yeager & Sundquist, 2008). The DNA provirus is transcribed into mRNA, which is spliced and exit into the cytoplasm where it is translated into regulatory proteins Tat and Rev. HIV-1 Tat, found to be neurotoxic, is a potent activator of HIV transcription and is essential of HIV replication (Hui *et al.*, 2012). Rev accumulates within the nucleus and attaches to full length mRNA, which is then transported out of the nucleus. Gag and Env is translated from this cytoplasmic viral mRNA.

Gag then attaches to full length RNA destined for viral assembly at the cell surface. All structural proteins of HIV are sourced from Gag (Ganser-Pornillos, Yeager & Sundquist, 2008). The viral protease cleaves Gag into spacer peptides that include the components necessary for the matrix layer, the nucleocapsid layer and the capsid layers. Gag also assembles at the plasma membrane allowing immature virions to coat with lipid membrane as they shed from the host membrane. All viral enzymes are packaged within the nucleocapsid including viral protease (Ganser-Pornillos, Yeager & Sundquist, 2008).

The Env protein (gp160) is transported through the endoplasmic reticulum to the Golgi apparatus where it is cleaved into HIV envelope glycoproteins gp41 and gp120. These assemble on the plasma membrane of the host cell in the vicinity of the assembling Gag proteins on the inside of the membrane. Gag (p55) and Gag-Pol (p160) containing two strands of viral RNA and all viral proteins assemble as the immature virus buds from the cell. When the Gag spacer peptides are cleaved by

viral protease into matrix, capsid and nucleocapsid proteins the virus matures and becomes infective (Ganser-Pornillos, Yeager & Sundquist, 2008).

2.13 HIV and the Placenta

Direct transmission of HIV generally requires the use of the virus encoded gp120 and gp41 envelope glycoproteins which complexes with the CD4+ receptor and co-receptors to form a fusion pore in the host membrane (Kubo *et al.*, 2012). Whilst HIV preferentially targets CD4+ T helper lymphocytes, other immunological cells such as macrophages and dendritic cells are also vulnerable to infection. CCR5, a co-receptor of the CD4+ receptor, is used by macrophage-tropic (R5) HIV-1 for cell entry and is genetically regulated by the CCR5 gene. It has been demonstrated that HIV infection and progression is inhibited by competitive ligands binding with the CCR5 receptor (Berger, Murphy & Farber, 1999). Other co-receptors such as CXCR4 and CCR3, amongst others, similarly also facilitate active HIV infection (Arias, Muñoz & Muñoz-Fernández, 2003). It is therefore plausible that the placenta, if indeed infected by HIV, bears these structures on its surface exposed to infected maternal blood.

There are, however, conflicting reports on the presence of CD4 receptors and co-receptors on trophoblast cells. Investigators have reported the presence of both CD4 receptor and co-receptors on trophoblasts (David *et al.*, 1992), while others have discounted their presence to contaminating Hofbauer and other immune cells present in the samples of enriched trophoblastic tissue (Arias, Muñoz & Muñoz-Fernández, 2003; Vidricaire, Imbeault & Tremblay, 2004). While the presence of

mediators of classical HIV infection (CD4 and relevant co-receptors) may be contentious, there is substantial evidence that HIV is present within the placenta of HIV infected women (Lewis *et al.*, 1990; Mattern *et al.*, 1992; Bhoopat *et al.*, 2005; Vidricaire, Gauthier & Tremblay, 2007; Kumar *et al.*, 2011a). With the paucity of CD4 receptor mediated viral entry into trophoblasts reported, and the presence of HIV within the placenta, it is plausible that there may be non-receptor mediated mechanisms (or at least a CD4 independent mechanism) that is responsible for infection of, or transient passage of HIV through, the placental barrier.

Indeed, several reports suggest that HIV may use an endocytic pathway to infect the placental tissue (Hocini *et al.*, 2001; Vidricaire, Imbeault & Tremblay, 2004; Vidricaire & Tremblay, 2007), and that such infection may eventually lead to *in utero* infection of the fetus. Many models have been described that afford a mechanism of HIV entry into placental tissue and subsequent infection or transcytosis (Menu *et al.*, 1997; Hocini *et al.*, 2001; Lagaye *et al.*, 2001; Vidricaire, Tardif & Tremblay, 2003; Vidricaire, Gauthier & Tremblay, 2007; Vidricaire & Tremblay, 2007). It is reported that cell free virus is inefficient in crossing or infecting the placental barrier (Lagaye *et al.*, 2001). This means that the trophoblastic barrier is resistant to free virus circulating in the maternal stream. However, it was found that cell-to-cell contact with HIV infected cells resulted in budding of HIV from the attached infected cell and subsequent uptake of the virus particles at the apical surface of polarised trophoblast in *in vitro* models. Incidentally, this is a highly efficient and rapid mechanism of HIV infection and transmission across the materno-fetal barrier (Hocini & Bomsel, 1999; Lagaye *et al.*, 2001; Arias, Muñoz & Muñoz-Fernández, 2003).

The placental barrier provides a continuous barrier with tight junctions that prevent any paracellular transport. Even *in vitro* models that mimic the placental barrier have shown that passage of any particle involves a transcellular transport process (Hocini *et al.*, 2001; Lagaye *et al.*, 2001; Vidricaire, Imbeault & Tremblay, 2004; Vidricaire & Tremblay, 2007). Any exchange between mother and fetus involves transcytosis, where the substance being transported is enclosed within a vesicle that does not come into contact with the cell cytoplasm. This is typical of most polarised epithelial membranes. HIV has a repertoire of cellular targets of infection and a receptor or protein independent mechanism of infection is plausible in the epithelial-like trophoblast (Vidricaire, Gauthier & Tremblay, 2007; Kubo *et al.*, 2012). HIV-1 binds to and enters the apical surface of epithelial cells via the sphingolipid, galactosylceramide and/or via heparin sulphate proteoglycans (syndecans) expressed by all trophoblast cells (Bomsel, 1997).

Moreover, cytokines and chemokines are also involved in the recruitment of inflammatory cells and consequently initiators, mediators or suppressors of inflammation may be involved in recruitment of HIV-infected cells to the trophoblast. Such recruitment would therefore induce cell-to-cell contact between the infected cell and trophoblast and thereby facilitate infection or transcytosis within the barrier.

One such adhesion molecule is leucocyte function antigen-1 (LFA-1) or CD11A. It is used by immune cells for recruitment to sites of infection. It binds to ICAM-1 (CD54) or ICAM-2 (CD102) on antigen presenting cells or endothelial cells (de Fougerolles *et al.*, 1991). When antibodies to LFA-1 where used to block binding sites,

investigators reported that no binding of HIV infected cells occurred hence these cells were not infected by HIV (Douglas *et al.*, 1994; Xiao *et al.*, 1997; Arias, Muñoz & Muñoz-Fernández, 2003; Baurakiades *et al.*, 2011). When ICAM-2 receptors were blocked by a monoclonal antibody CBR-IC2/2, it antagonised binding of CD11A thereby restricting LFA-1 mediated binding of immune cells (de Fougerolles *et al.*, 1991). This eludes to the significance of these adhesion molecules in facilitating binding of HIV infected immune cells to endothelial cells and, possibly trophoblast tissue, which shares many functional attributes of endothelium (Benirschke & Kaufmann, 2006).

It is therefore important to assess the level of expression of ICAM-1 or ICAM-2 within the study groups. Such information will provide insight into the mechanism whereby HIV may infect the placenta thereby leading to infection of the fetus.

Additionally, inadequate invasion of the luminal wall of spiral arteries by trophoblasts may involve changes in ICAM expression which may be implicated in intrauterine growth retardation and may be of significance in pre-eclampsia (Burrows, King & Loke, 1994; Xiao *et al.*, 1997; Zygmunt *et al.*, 1997). Therefore, comparison of cytokine expression between placentae from normotensive HIV participants and hypertensive participants is of scientific and clinical value. Even though it has been reported that HIV does not alter the risk of pre-eclampsia development (Conde-Agudelo, Villar & Lindheimer, 2008), pre-eclampsia is said to be a hyperimmune or pro-inflammatory state, and such a state will influence both the expression of chemokines and recruitment of HIV infected cells to affected sites; thereby

increasing adhesion of these cells to trophoblast cells. Of further importance, this study will also compare and contrast the expression of the LFA-1 receptor ICAM-2 amongst the study groups to see if there are any changes between them, and if such changes are of any significance.

CHAPTER 3

METHODS

3.1 Ethical approval

This prospective study was conducted in the Optics and Imaging Centre, Doris Duke Medical Institute, College of Health Sciences, University of KwaZulu-Natal. This study uses retrospectively collected placental tissue for which institutional ethical clearance was obtained from the Biomedical Research Ethics Committee (BREC; BF 155/08). Thereafter, expedited institutional ethical clearance for this new prospective analysis was obtained (BE 352/13; see Appendices).

3.2 Patient recruitment and consent

3.2.1 Patient selection and inclusion criteria

The archived placental tissue used in this study was sourced at delivery from pregnant Black women attending the Obstetrics & Gynaecology Unit, Prince Mshiyeni Memorial Hospital. This is a district hospital servicing a catchment of at least sixteen clinics in the Durban South region of KwaZulu-Natal, South Africa. In the original study, 90 pre-eclamptic and 90 normotensive participants were recruited. These groups were further divided into HIV positive and HIV negative participants, and the HIV positive group was further stratified according to their CD4 counts. Although participants of varying parities were recruited in the original study (n=180), we restricted our selection to first pregnancies only.

This study was limited to primigravidae and hence study numbers were reduced to 20 per group. There were 2 main groups (refer to Figure 2.1):

a) Normotensive pregnant women (n=40) - HIV positive (n=20)

　　　　　　　　　　　　　　　　　　　HIV negative (n=20)

b) Pre-eclamptic pregnant women (n=40)- HIV positive (n=20)

　　　　　　　　　　　　　　　　　　　HIV negative (n=20)

The overall control group was HIV negative normotensive pregnant women (n=20). The HIV positive normotensive group (n=20) served as a control group for the HIV positive pre-eclamptic group (n=20).

3.2.2 Inclusion criteria for pre-eclampsia

Pre-eclampsia was diagnosed as sustained systolic blood pressure of greater than or equal to 140mmHg and a diastolic blood pressure of greater than or equal to 90mmHg taken on two occasions at least 6 hours apart. Furthermore, patients had to present with new onset proteinuria (1+ on a urine dipstick analysis) or >30mg/dl urine protein concentration at two intervals at least 4 hours apart.

Patients were tested for HIV as a standard of care as per Department of Health recommendation. Only primiparous women were selected.

Normotensive groups were matched for maternal and gestational age with the pre-eclamptic groups i.e. group 1 vs group 2 (control); group 3 vs group 4 (control).
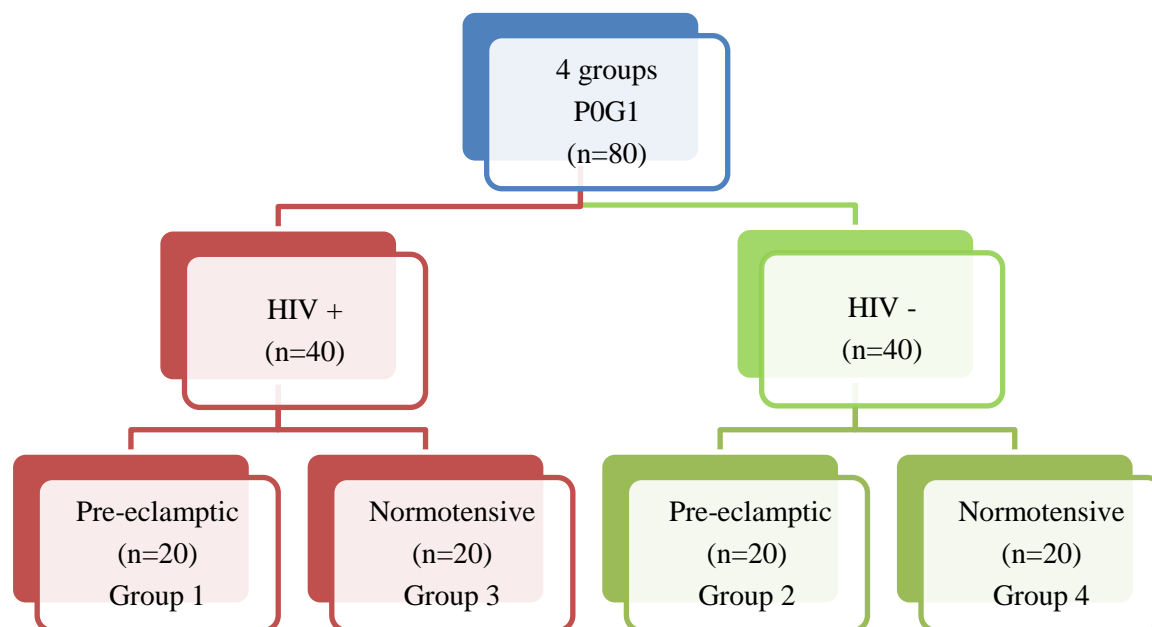
Figure 3.1: Control and study patient groups showing the major division being HIV status and subsequent dichotomy between pre-eclamptic and normotensive participants within each major division.

3.2.3 Exclusion criteria

Women who were previously pregnant were excluded from the study as well as those with a history of smoking and narcotic use. The following disorders also formed part of the exclusion criteria: chorioamnionitis, chronic hypertension, eclampsia, abruptio placentae, intra-uterine death, chronic diabetes mellitus, gestational diabetes, chronic renal disease, connective tissue disease, treatment with aspirin, warfarin, non-steroidal anti-inflammatory drugs, lipid lowering or anti-hypertensive drugs, systemic lupus erythematous, sickle cell disease and anti-phospholipid antibody syndrome; thyroid disease, cardiac disease and active asthma requiring medication during pregnancy and pre-existing seizure disorders.

3.3 Sample collection

This prospective study used archived paraffin wax embedded placental tissue. Originally, the placental tissue was obtained immediately post-delivery with care taken to avoid areas that included major blood vessels, areas of visible necrosis and/or infarction.

3.4 Histological evaluation of the placenta

3.4.1 Sample preparation

Full thickness of placental tissue from the central region of the placenta had been dissected and immersed in 10% buffered formaldehyde (Polychem, KZN, SA). Fixed tissue was subsequently dehydrated, infiltrated with paraffin wax and embedded in cassettes using an automated tissue processor and tissue embedding station respectively (Leica ASP 200S tissue processor; Leica EG 1160 embedding station; Germany). The automated protocol utilized is outlined in Table 3.1.

3.4.2 Microtomy

Serial sections (3-4µm thick) were cut using sterile low profile disposable blades (Leica 819; Germany) on a rotary microtome (Leica Jung RM 2035; Germany). Sections were allowed to spread in a heated water bath (Leica HI210; Germany) and picked up onto coated glass slides (Xtra™ Adhesive, Leica; Germany). All slides were baked overnight at 60°C). Rehydration involved incubation in two xylene baths (5 min each) followed by 3 consecutive washes in a descending series of ethanol (5 min each), with a final washing in tap water for 5 min.

Table 3.1: Brief outline of fixation and embedding of placental tissue

STEP	STAGE	SOLUTION	TIME
1	Fixation	10% phosphate buffered formalin	30min
2	Dehydration	70% Ethanol	30min
		95% Ethanol	30min
		100% Ethanol	30min + 60min X2
3	Infiltration	Isopropanol	45min + 60min X3
4	Impregnation	Paraffin Wax	3 x 60min

3.5 Immunolocalisation of target Antibodies in the placentae

3.5.1 Primary antibodies

Primary antibodies were sourced from different companies (Table 3.2). All antibodies were checked for compatibility with the EnVision™ Flex kit (K8023; DAKO, Denmark) that was used for the immunohistochemistry procedure. Antibody dilutions were optimised and all antibodies were diluted using Envision™ FLEX antibody diluent (K8006; DAKO, Denmark). Table 3.2 summarises the antibodies used in the study.

Table 3.2: Summary of antibodies utilised in study

Antibody	Supplier	Clone	Raised in	Dilution
P24*	DAKO	KAL-1	Mouse	1:10
CD4	DAKO	4B12	Mouse	1:1 Ready to use
CCR5	R&D systems	#45523	Mouse	1:80
ICAM-2	Biolegend	CBR IC2/2	Mouse	1:100

*p24 antibody, required immediate IHC staining and were freshly cut and only baked for an hour before being dewaxed and rehydrated.

3.5.1.1 Mouse monoclonal Anti-Human Immunodeficiency Virus, p24. Clone Kal-1 (DAKO, Denmark)

The antibody was specific for the detection of the p24 viral capsid protein of HIV-1, which is located in the core of the virus (Bhoopat *et al.*, 2005). The antibody labels the HIV capsid within or outside infected cells. Specificity was confirmed using HIV positive lymph node sections (Kaluza *et al.*, 1992). The antibody was used at a 1:10 dilution and confirmed by optimization studies (Table 3.2).

3.5.1.2 Mouse monoclonal anti-human CD4 antibody (4B12, DAKO, Denmark)

This monoclonal mouse anti-human antibody was raised to label thymocytes and T-helper cells ref. It recognizes a 55 kDa glycoprotein with five extracellular domains, a transmembrane and an intracellular domain (CD4). CD4 also serves as a receptor for HIV on T cells and as a co-receptor for MHC class II activation (Davidsson *et al.*,

2013). The antibody was purchased in a pre-diluted "ready to use" format (Table 3.2).

3.5.1.3 Mouse anti-human CCR5 antibody (clone #45523, R&D Systems, USA)

This commercially available mouse anti human CCR5 antibody stains the co-receptor CCR5. CCR5 is a member of the beta chemokine receptor family which is structurally a 7 transmembrane protein. It is expressed by T cells and macrophages and is used by macrophage trophic virus such as HIV as a co-receptor for entry into host cells (Omi *et al.*, 2014). The antibody was diluted 1:80 and confirmed by optimization studies (Table 3.2).

3.5.1.4 Mouse anti-human ICAM-2 antibody (CD102/ CBR-IC2/2, Biolegend, USA)

Intercellular adhesion molecule-2 (ICAM-2) is a 55kD type 1 transmembrane glycoprotein that functions primarily as a ligand for the integrin lymphocyte function-associated antigen-1 (LFA-1). The receptor is expressed on endothelium, platelets, lymphocytes, and monocytes and shares a 35% homology in sequence with ICAM-1, ICAM-3, ICAM-4 and ICAM-5. (Casasnovas, Pieroni & Springer, 1999). The antibody was diluted 1:100 and confirmed by optimization studies (Table 3.2).

3.5.2 Envision™ FLEX Mini Kit, High PH Staining Kit K 8023 (Dako, Denmark)

The Envision™ FLEX Mini Kit is designed for use in immunohistochemistry and contains all the necessary reagents to completely stain formalin fixed paraffin embedded tissue with user provided mouse or rabbit antibodies. The kit comprised undiluted solutions of:

i.   Target retrieval solution (High pH (50x) (DM828)) –a Tris/EDTA buffer, pH 9 used for heat-induced target retrieval (HIER);

ii.   Peroxidase Blocking Reagent (SM801) - a phosphate buffer containing hydrogen peroxide, 15mmol/L NaN3 and detergent;

iii. Wash Buffer (20x) (DM831)- a buffered solution containing Tween 20, pH 7.6;

iv.   HRP detection reagent- a buffered solution containing dextran coupled with peroxidase and goat secondary antibodies against both mouse and rabbit;

v.   3,3'-diaminobenzidine tetrahydrochloride (DAB) + Chromogen (DM827) and Substrate buffer (SM803) -DAB in organic solvent reconstituted 1:20 in substrate buffer;

vi.   Mouse linker (SM804) - a buffered solution of stabilising protein used to enhance visible staining.

3.5.3 Antigen retrieval and blocking

Antigen retrieval was performed using the Envision™ FLEX target retrieval solution (K8004, Dako, Denmark). The sections to be immunostained for p24 were subjected to heat retrieval in a domestic microwave (1200 Watts) for 20min at medium high

power (LG MS2524W). Antigenic sites for the other antibodies were unmasked with the PT link (DAKO, Denmark) for 20 minutes at 95°C.

Once the retrieval solution returned to room temperature, endogenous peroxidase present in the tissue was blocked by incubating slides for 5 minutes in 3% H_2O_2. Additional protein blocking was carried out with 10% bovine serum albumin in Tris buffered saline (A4503, Sigma, USA) solution for 30min.

3.5.4 Immunohistochemical staining procedure for p24, CD4, CCR5 and ICAM-2

Post peroxidase blocking, sections were washed with Tris-buffered saline wash buffer followed by immersion of the slides in wash buffer for 5 minutes on a flatbed rocking station (CAT, Germany). Thereafter, sections were incubated overnight in the primary antibodies listed in Table 3.2 at 4°C in the dark. Immediately after antibody incubation, all sections were washed thoroughly in the wash buffer on the rocking station. ICAM-2 required the LINKER incubation for 15 minutes. Following buffer washes, sections were incubated for 20min in the dark at room temperature with Envision™ FLEX /HRP detection reagent. Slides were then washed thoroughly and immersed in two baths of clean wash buffer for at least 5 minutes each. Slides were then incubated with Envision™ FLEX DAB+ chromogen until there was a visible change in colour of the sections or maximum of 10 minutes elapsed. After thorough washing under running tap water for 5 minutes, slides were immersed in Mayer's haematoxylin for 3-5 minutes. Slides were then dehydrated in a series of alcohols (3x for 5 minutes each), before cleaning in 2 baths of xylene. Dehydrated slides were then mounted in dibutylphthalate xylene (DPX).

During optimization of primary antibodies, negative controls were also included in the run. We used antibody diluent as a substitution of the primary antibody.

Brief summary of the experimental process is depicted in Figure 3.2.
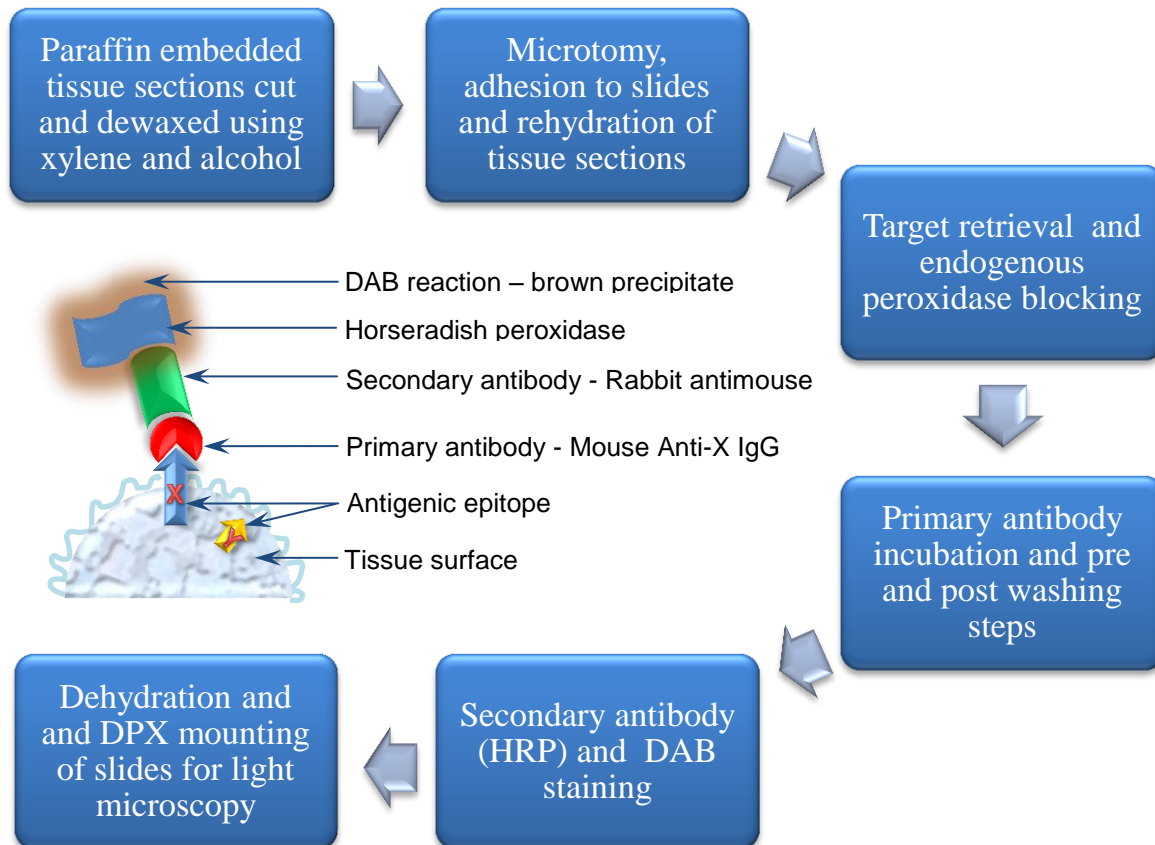
Figure 3.2: A summary of the immunostaining procedure showing the important steps in the process. A detailed summary of the process is provided in the text. Tissue with presence of the epitope of interest is exposed to antibody raised in mouse against that human epitope. A secondary antibody raised in rabbit, for example, against the first antibody is then exposed to the tissue. Only primary antibody/epitope complex present in the tissue will associate and with the secondary antibody and not wash off. Thereafter, horseradish peroxidase (HRP) associates with the secondary antibody and the visual marker diaminobenzidine tetrahydrochloride (DAB) is converted by HRP to form a brown precipitate indicating positive presence of the epitope of interest in the tissue (according to the Envision protocol, DAKO, Denmark).

3.5.5 Morphometric image analysis of antibody expression

All sections were viewed on the Zeiss Axioscope A1 Photomicroscope (Carl Zeiss, Germany). Magnifications of 10x, 20x and 40x were used and some images were captured under oil at 100x magnification. Automated image analysis was utilized in order to obtain quantitative data from images. The automatic measurement protocol included in the AxioVision Image analysis software package version 4.8.3 (Carl Zeiss, Germany) was used to quantify the presence of brown stain on the slides indicating presence of antibody.

Two contiguous images were captured from a centralized area of the tissue section and an attempt was made to avoid capturing areas that contained major stem villi. Areas captured were those that had the greatest exchange villi representation. Images were captured for both CCR5 and ICAM-2 staining. This was not possible with p24 and CD4 due to the paucity of staining within some placental sections as well as complete absence of staining in others. For these antibodies it was only possible to gain a qualitative evaluation of expression within exchange and conducting villi of the placenta.

AxioVision image analysis software (using the auto measurement module) was used to analyse the images for immunoexpression of CCR5 and ICAM-2. Briefly, the randomly selected images were archived at 20x magnification. The frame area was defined as the scaled area of the frame in μm^2. The presence of immunostaining (brown colour) was expressed as a percentage of the frame area and only regions of interest (ROI) were selected by manual segmentation to exclude the maternal

compartment such as maternal red blood cells. It was not possible to completely isolate the exchange villi, therefore the two images captured per sample were evaluated according to area occupied by mainly exchange villi that included intermediate and terminal villi. The data was then tabulated on an SPSS 22 (IBM, USA) spreadsheet for further statistical interrogation.

3.5.6 Statistical analysis

The statistical package IBM®SPSS® Statistics Version 22 (IBM Corp. released 2013, IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) was used for all statistical analyses. The Shapiro-Wilk and normal Q-Q plots were evaluated to indicate whether dependent variables were normally distributed. Parametric data was used directly in analyses while non-parametric data was analysed using the Kruskal-Wallis test and Mann-Whitney U tests. Analyses of Variance (ANOVA) was used to evaluate the effect of HIV on pre-eclamptic and normotensive pregnancies and 2 way ANOVAs were used to compare expression of antibodies amongst groups as follows:

A two way factorial analysis of variance (ANOVA) was used to test the main effect of two factors: HIV status and pre-eclampsia status on variables of interest as well as interactive effects between the factors and each variable using the Fisher's least significant difference (LSD) post hoc analysis as the factors only form a 2x2 matrix (HIV positive and negative; pre-eclamptic and normotensive).

A p-value of less than 0.05 indicated statistical significance unless post hoc tests were used and significance was reduced accordingly. Where data are plotted in boxplots, the horizontal line in the boxes represents the medians for each group. The box represents 50% of the data associated with the median and the vertical lines being at the 10th percentile and end at the 90th percentile.

CHAPTER 4

RESULTS

A total of 80 black African women were recruited into this study and were allocated to 4 groups (Figure 3.1). Fifty percent of the study sample was HIV positive (n=40) and 50% was pre-eclamptic (n=40). Table 4.1 outlines the demographic variables per group as mean ± standard deviation.

Table 4.1: Demographic statistics of study population

	HIV+ pre-eclampsia	HIV- pre-eclampsia	HIV+ Normotensive	HIV- Normotensive
	n=20	n=20	n=20	n=20
Maternal Age	22.2±3.5	21±2.8	23.5±3.5	20.1±2
No. of Weeks of Gestation	37.9±2.1	37.8±1.6	38.7±2	38.6±1.4
Systolic BP on admission	147.1±13.6	149.2±13.8	121.3±8.6	120.3±12.1
Diastolic BP on admission	99.5±9.1	98.4±13.6	71.5±8.3	70.7±10.3
Maternal Body Mass Index	30.2±5.1	31.2±4.9	29.4±4.5	31.4±5.3
CD4 count (cells/mm^3)	269.6±215.6		260.5±168.8	
Baby weight (kg)	2.8±0.5	2.9±0.3	3.2±0.5	3.3±0.4
Placental Weight (grams)	484.3±71.4	455.3±80.6	497.6±133.1	443.5±79.4

Values are presented as means ± standard deviation (SD). CD4 counts were not performed on HIV negative groups.

4.1 Maternal age

The average age ± standard deviation for the study population (n=80) was 21.7 ± 3.2 years old. The mean age per cohort was not normally distributed. According to the independent samples Kruskal Wallis test, age was not equally distributed between the groups. As depicted in Figure 4.1 the mean age of the HIV positive normotensive group (23.5±3.5 yrs) was significantly higher (p=0.01) than the mean age of HIV negative normotensive group (20.1±2.0 yrs).
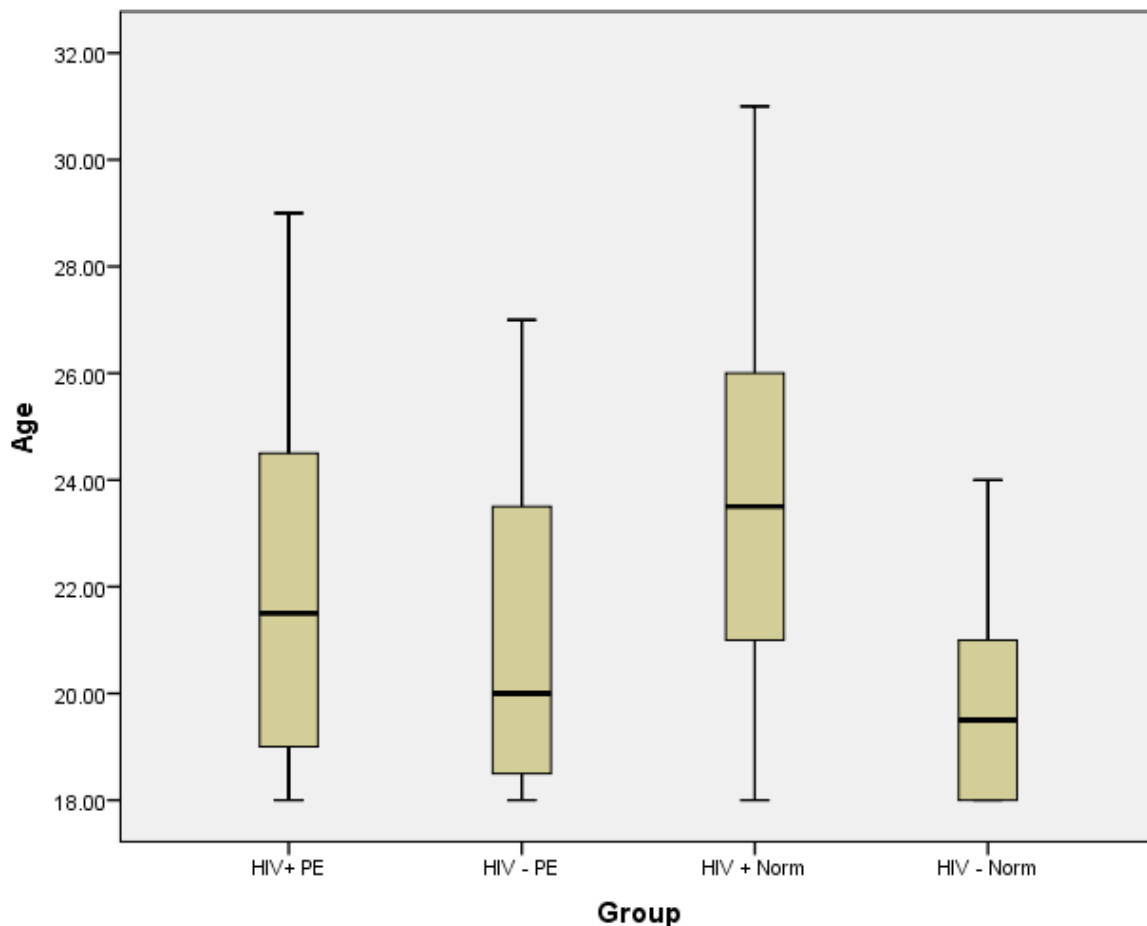
Figure 4.1: Mean maternal age box and whisker plots of the four study groups.

Using a two way factorial analysis of variance (ANOVA), the main effect of HIV status on maternal age showed that maternal age was higher in the HIV positive cohort compared to the HIV negative cohort [F (1, 76) = 11.952, $p<0.001$]. In the HIV-groups, pairwise comparisons showed no difference of maternal age between normotensive and pre-eclamptic groups [F (1, 76) = 0.89, $p= 0.347$]. In the HIV+ groups, there was no difference in maternal age between the normotensive and pre- eclamptic groups [F (1, 76) = 2.01, $p= 0.160$].

There was no main effect of type of pregnancy (normotensive or pre-eclamptic) on maternal age [F (1, 76) =7 .39, $p=0.112$].

4.2 Length of Gestation

A two way ANOVA, showed no main effect of HIV status on gestational age [F (1, 76) = 0.143, p = 0.706]. There was a significant difference between the pre-eclamptic and normotensive groups [F (1, 76) = 4.075, p = 0.47]. The average length of gestation age was 1 week longer in the normotensive cohort compared to the pre-eclamptic cohort regardless of HIV status. Interaction between the pregnancy type and HIV status had no significant effect on gestational age.

4.3 Systolic and Diastolic blood pressure

Maternal blood pressure on admission showed homogeneity within the normotensive and pre-eclamptic groups. Pairwise and univariate analyses of the

main effect of HIV status on systolic blood pressure showed no significant difference between groups [F (1, 76) = 0.041, p =0.8417].

As expected, the systolic blood pressures between the pre-eclamptic and normotensive groups were different due to our inclusion criteria of study groups [F (1, 76) = 100.202, p <0.0001].

The interaction between HIV status and pregnancy type within each pairwise group (HIV+ normotensive *vs* HIV+ pre-eclamptic, HIV- normotensive *vs* HIV- pre-eclamptic) showed a significant main effect on systolic blood pressure [F (1, 76) = 55.941, p <0.0001].

Two way factorial analysis of variance (ANOVA) for diastolic pressure mirrored the main effects of HIV and pregnancy status on systolic pressure with HIV status showing no main effect [F (1, 76) = 0.155, p =0.695] on diastolic pressure . Type of pregnancy did significantly affect diastolic pressure [F (1, 76) = 139.955, p <0.0001], and the interactive effect between the groups were significantly different (p<0.0001).

4.4 Body mass index

The body mass index (BMI) was calculated as $\frac{Weight}{Height^2}$ in kg.m^{-2}. A one way ANOVA of BMI did not show a significant difference between groups [F (3, 76) = 0.713, p=0.547] therefore there was no variance in BMI across the study groups.

4.5 CD4 cell count

CD4 counts (in cells/mm^3) were only available for HIV positive mothers. An independent-samples t-test was conducted to compare CD4 counts in pre-eclamptic mothers and normotensive mothers (Figure 4.2). There was no significant difference between normotensive mothers (M=267.50, SD=167.23) and pre-eclamptic mothers {(M=289.55, SD=207.66);   [t (38) = 3.70, p = 0.714]}.
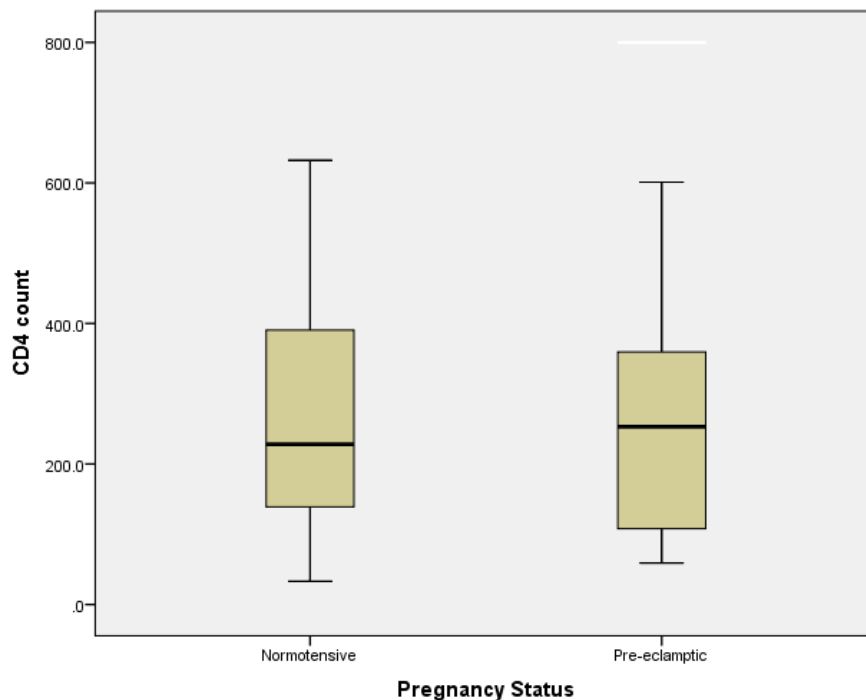
Figure 4.2: Mean CD4 box and whisker plots in the HIV positive groups showing CD4 count in cells/mm^3 in the normotensive and pre-eclamptic groups.

4.6 Baby birth weight

A two factorial ANOVA showed a main effect of pregnancy type on the birth weight of neonates between normotensive and pre-eclamptic groups [F (1, 70) = 14.339, p < 0.0001]. There was no main effect of HIV status on birth weight [F (1, 70) = 1.823, p =0.181]. The interaction between the factors of HIV and pregnancy status showed no effect on birth weight between pre-eclamptic and normotensive pregnancies [F (1, 70) = 0.046, p =0.831] (Figure 4.3).
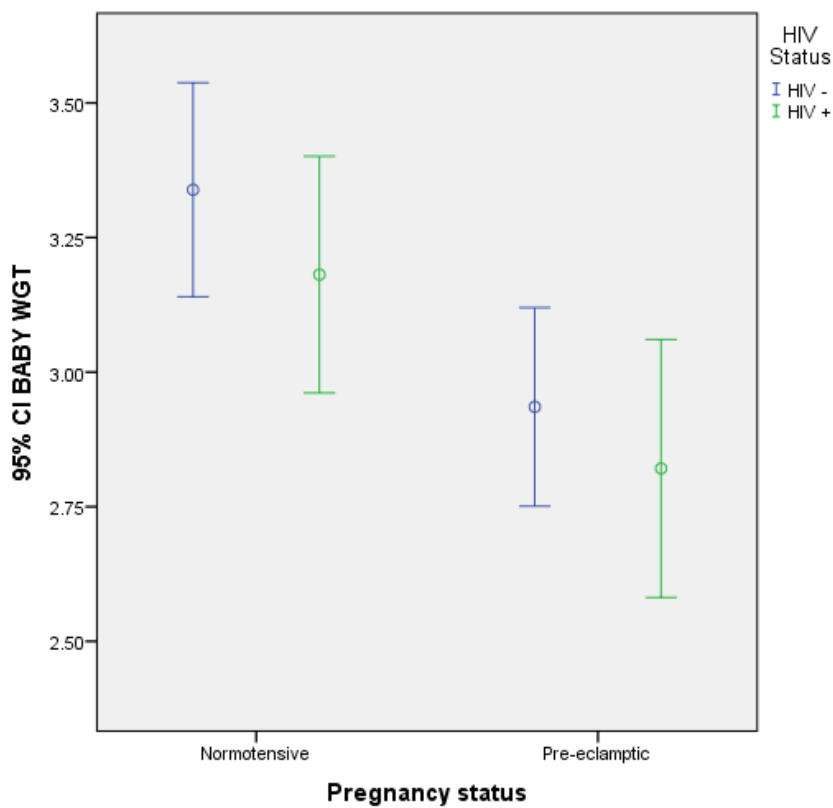
Figure 4.3: Clustered error bar plot showing the effects of HIV status and pregnancy type on birth weight of babies.

4.7 Placental Weight

Placental weight for the study population was 475.5 ± 80g SD and ranged from 300-700g with only 6% under 400g. Placental weight was not normally distributed and therefore a non-parametric analysis of the data was carried out. A Kruskal-Wallis test showed a significant difference across the 4 groups (asymptotic p=0.003). An independent sample Mann-Whitney U test showed no difference in the distribution of placental weight between the pre-eclamptic and normotensive groups (p=0.414). The placental weight in the normotensive HIV positive group was significantly higher than the HIV negative normotensive group (p=0.01). A graphical representation of placental weight data for each of the group is shown in Figure 4.4.
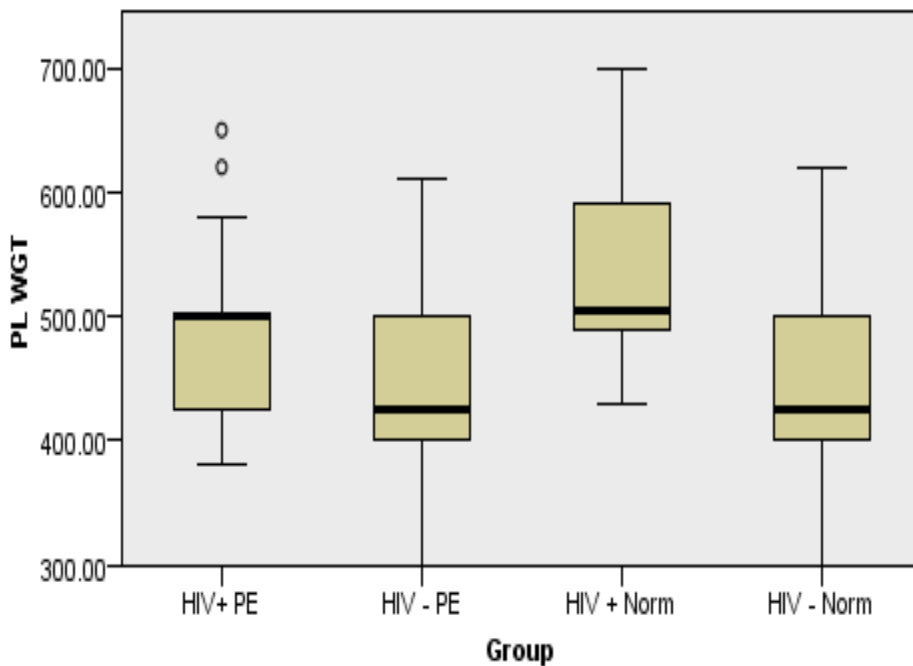
Figure 4.4: Cluster error bar plot illustrating the distribution of placental weight amongst the 4 study groups. Note: HIV positive normotensive group was significantly higher than the HIV negative groups.

4.8 Immunohistological evaluation of the placenta

The histology of haematoxylin and eosin (H&E) stained placental tissue was examined across all study groups.

The amnion was only present in full thickness placental samples. It comprised of an avascular layer of epithelium (either simple columnar or simple cuboidal) and connective tissue that also formed a continuous covering up to and including the umbilical cord. The epithelium faced the amniotic cavity on its apical aspect and was basally attached to a thick basement membrane (Figure 4.5 (a)). The basement membrane was attached to a spongy layer of connective tissue below which lay the fibrous chorion (Figure 4.5 (b)). The internal aspect of the chorionic membrane faced the amnion whilst at its' external aspect was found the villi and the chorionic plate of the placenta.

Three types of villi were observed viz., stem villi (Figure 4.6), intermediate villi (Figure 4.7 (a)) and terminal villi (Figure 4.7 (b)). These villi, all share basically the same structure in that their outer layer was covered by trophoblast cells juxta the maternal blood supply. The trophoblast cell populations were of two types i.e., cyto- and syncytiotrophoblast cells. The syncytiotrophoblast layer consisted of a continuous uninterrupted multinucleated syncytial layer (Figure 4.7). Below the syncytiotrophoblast occasional cytotrophoblast cells (Langhan's cells) that are thought to give rise to the syncytiotrophoblast were observed. This layer separated the maternal blood from the internal compartment of the villi. The trophoblastic basement membrane was found below the syncytiotrophoblast and cytotrophoblast

cells. The villous mesenchymal core consisted of a stroma made up of connective tissue, capillaries, arteries and veins depending on the villus type and maturity.

4.9 Conducting villi - Stem Villi

Large stem villi occurred throughout the placenta and provided a structural as well as a conduit role, evidenced by the larger blood vessels within it as well a reduced complexity within the fibrous stroma (Figure 4.6). Stem villi varied greatly in size as well as position within sections. The blood vessels were composed of endothelium lining the intima of the vessel. This was surrounded by concentric layers of smooth muscle cells (tunica media, (Figure 4.6) and the concentric layers were more pronounced in arteries than veins. The adventitia was continuous with the fibrous stroma. Scattered around the stroma were fibroblasts. Figure 4.6 (a and b) show stem villi of differing maturity and calibre with Figure (a) depicting a larger stem villus with peripheral fibrinoid.

4.10 Exchange Villi

4.10.1 Intermediate villi

Intermediate villi were of two types i.e., mature and immature types. Immature intermediate villi contained blood vessels within a reticular stromal core (Figure 4.7 (a)). Vascularisation was high via thin walled capillaries, arterioles and venules. The immature intermediate villi had large central areas of reticular stromal tissue within a uniform syncytiotrophoblast layer. Mature intermediate villi were elongated and were often associated with terminal villi.

4.10.2 Terminal Villi

The terminal villi are shown in Figure 4.7 (a) as the terminal ends of the villous tree, and are closely associated with intermediate villi. The trophoblastic cells covering terminal villi were thin and highly vascularised with much of the volume of the structure being occupied by dilated capillaries. These sinusoids were very close to the trophoblastic surface. Terminal villi were around 80-100µm in diameter and made up a large portion of the exchange villi exposed to maternal blood.

4.11 Basal Plate

The basal plate was found on the maternal aspect of the intervillous space. The mature term basal plate contained extravillous trophoblast (Figure 4.7 (b)) embedded within a dense fibrinoid layer.
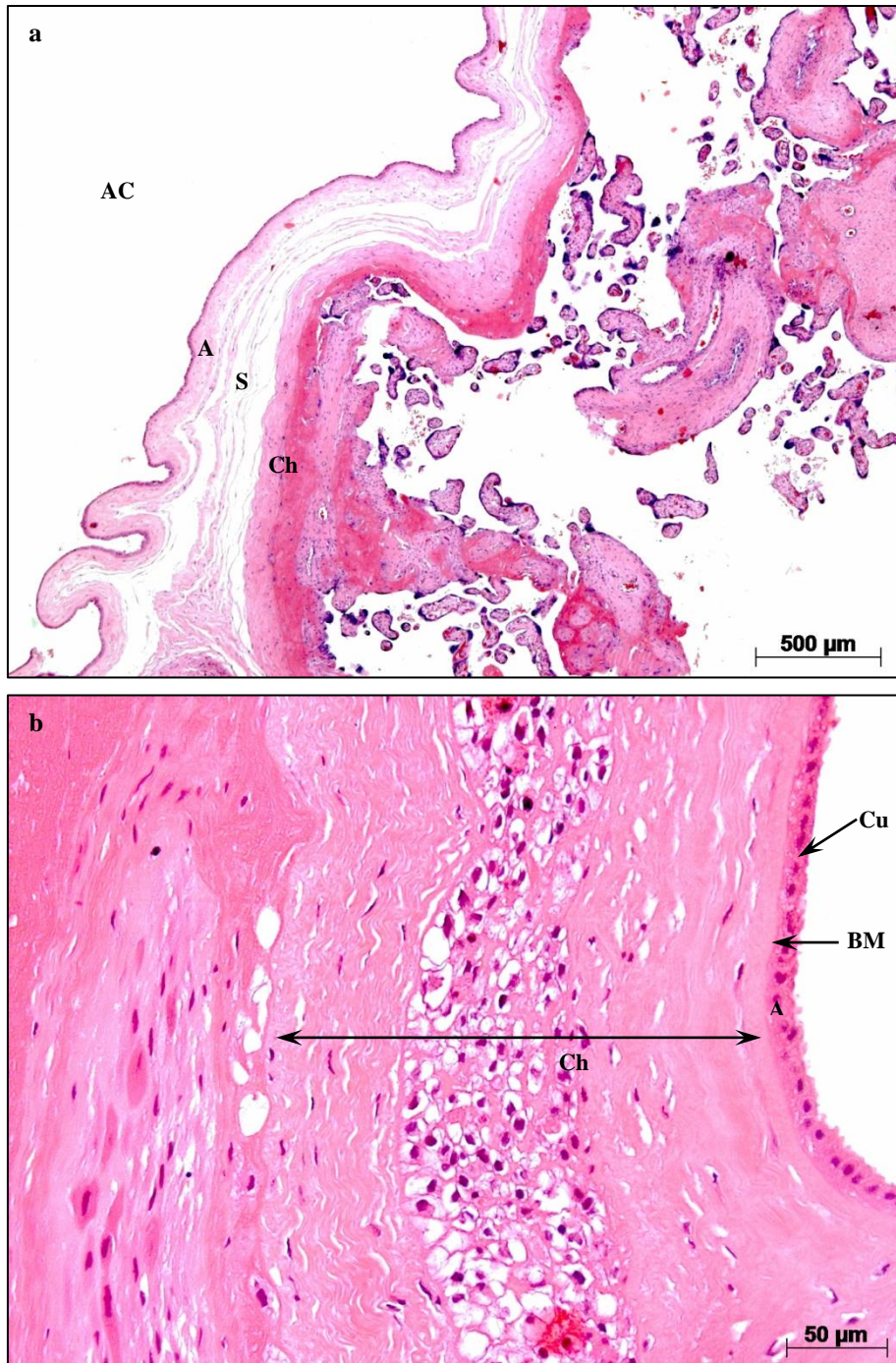
Figure 4.5: Light micrograph of H&E stained placental tissue. (a) Placental section showing the amnion (A) lined by an epithelial layer separating the amniotic cavity from the placental tissue. A spongy layer of connective tissue lies between the amnion and the chorion (Ch). In (b) the cuboidal epithelial layer (Cu) is visible attached to the basement membrane (BM). The amnion (A) extends to the spongy layer (not seen) which is terminated by the fibrous chorion (Ch).
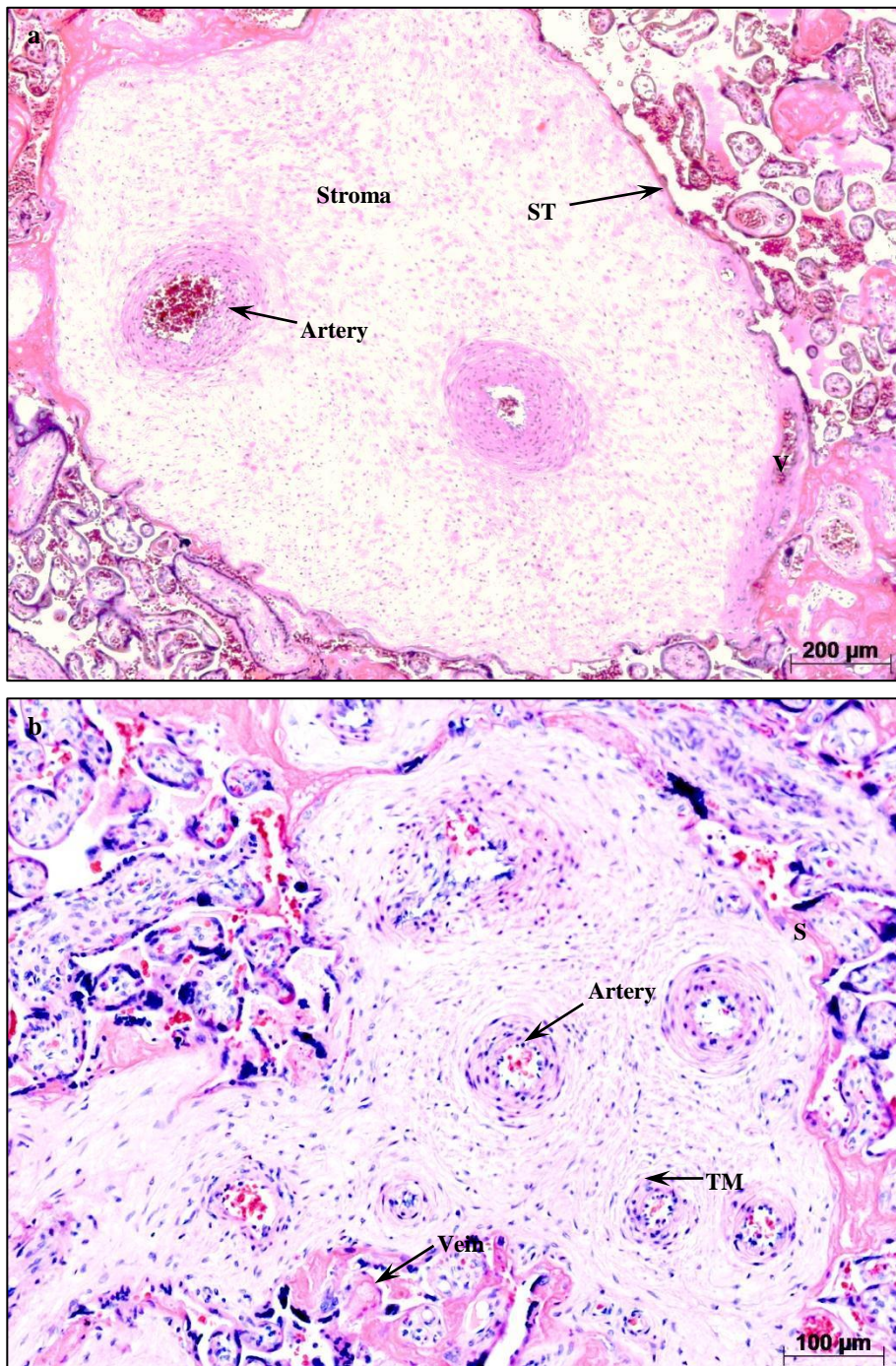
Figure 4.6: Light micrograph of H&E stained placental tissue. (a) Stem villus showing importance and differentiation into a conducting vessel. Central arteries are supported in a stromal matrix. (b) The central structure is a stem villus showing a number of collecting arteries. The arteries are created by a thin layer of endothelium surrounded by layers of tunica media (TM). Stem villus is covered by syncytiotrophoblast (S) and the section shows adjacent exchange villi on the periphery of the image.
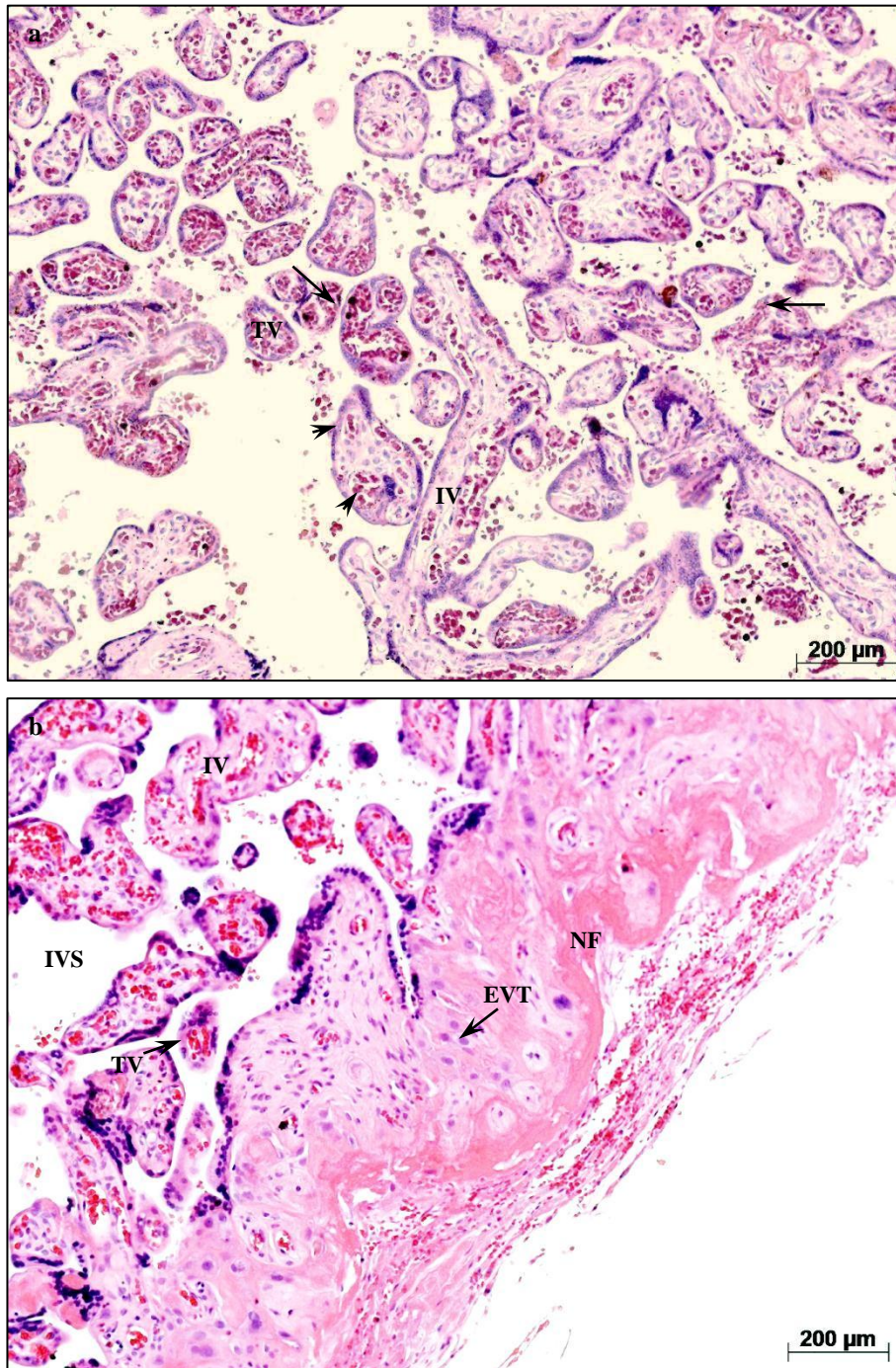
Figure 4.7 (a): Stem, intermediate and terminal villi depicted in section within the IVS. Terminal (TV) and intermediate villi (IV) have capillaries that were close to syncytiotrophoblast (arrows) to facilitate exchange. Arrowheads indicate the capillaries within the terminal villi which was also a common feature in intermediate villi (b) Basal plate showed a mixture of extravillous trophoblast (EVT) cells, Nitabuch's fibrinoid (NF) and decidual cells with the intravillous space shown on the top left and the separation zone of endometrial stroma on the bottom right of the micrograph.

4.12 Pathological Features

A qualitative assessment of histopathological features between the study groups revealed the following:

Perivillous fibrinoid was evident in all four groups but was more prominent in the pre-eclamptic placentae. This fibrinoid region was often found on the basal aspect of the syncytiotrophoblast and cytotrophoblast. It was usually located below the syncytiotrophoblast appearing as an acellular eosinophilic layer (Figure 4.8). Intravillous fibrinoid was observed within exchange villi. Intravillous fibrinoid was also qualitatively associated with HIV affected placentae. The high degree of vascularisation typically seen in normal placental exchange villi was qualitatively reduced in areas of intravillous fibrinoid.

A prominent difference between normotensive and pre-eclamptic pregnancies was an increase in the number of syncytial knotting or Tenney-Parker changes that were present in pre-eclamptic placentae associated with exchange villi. These were nuclear accumulations or peripheral clustering of syncytiotrophoblastic nuclei and were associated with small villi. By visual comparison, HIV status did not seem to affect the density of knots between the positive and negative groups (Figure 4.8).

The density of the exchange villi was also increased in pre-eclamptic placentae. Although the number of exchange villi increased over a given area, the size of the exchange villi decreased (Figure 4.8). Apart from the size, the level of maturity of the intermediate villi differed between the pre-eclamptic and normotensive groups with the pre-eclamptic groups showing a greater concentration of reticular stroma.

Cytotrophoblasts were more easily seen in terminal villi also indicating less mature villi when compared to the normotensive group.
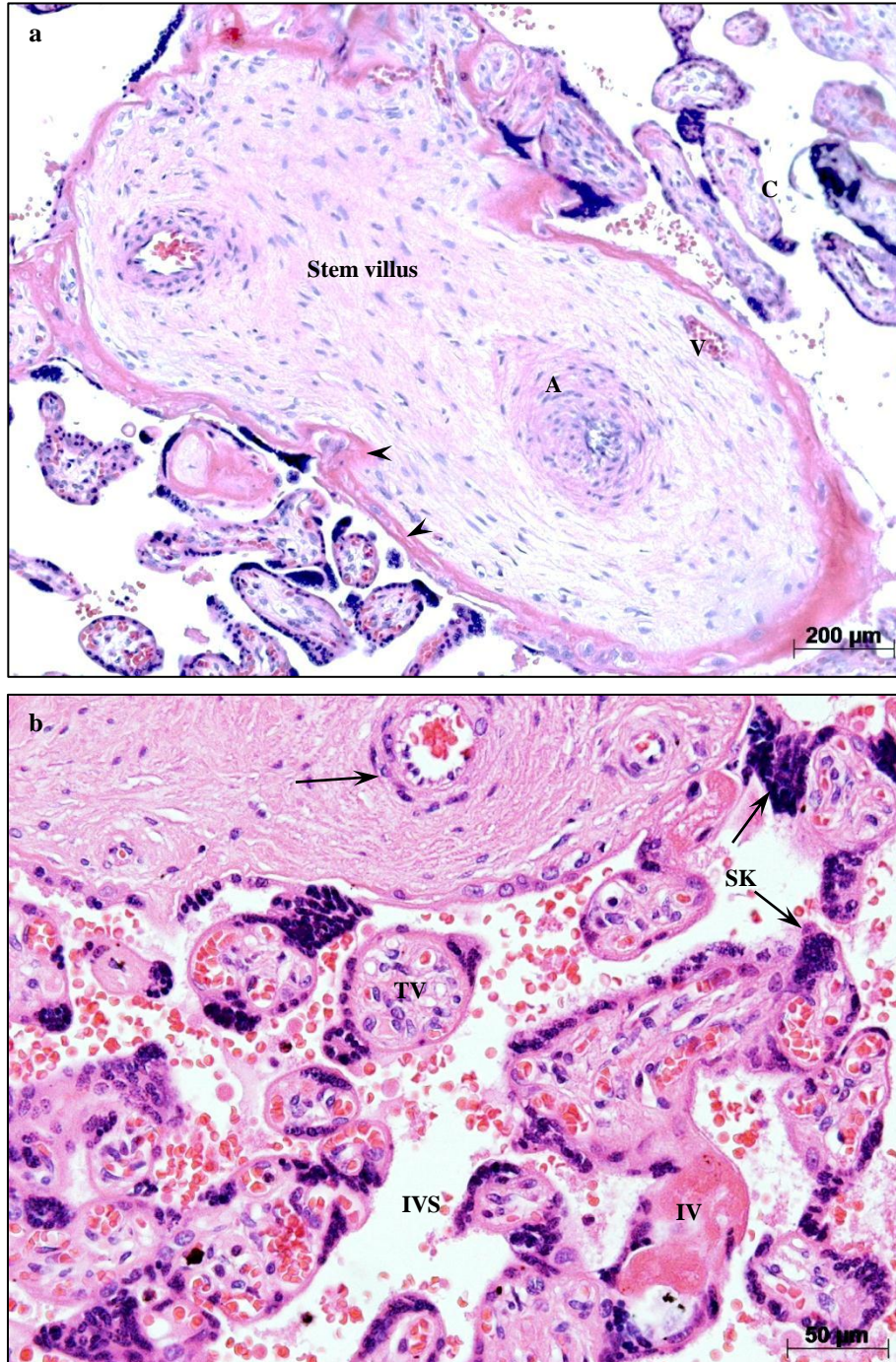
Figure 4.8 Section from pre-eclamptic placenta (a) Perivillous fibrinoid deposition immediately below trophoblast layer in stem villus (arrowheads) Also note terminal villus (Arrow) with increased intravillous fibrinoid and subsequent low degree of vascularisation. (b) Section from HIV positive pre-eclamptic placenta. Syncytial knots (SK) are shown as communication between villi. Terminal (TV) and intermediate villi (IV) show thicker trophoblast exchange membranes.
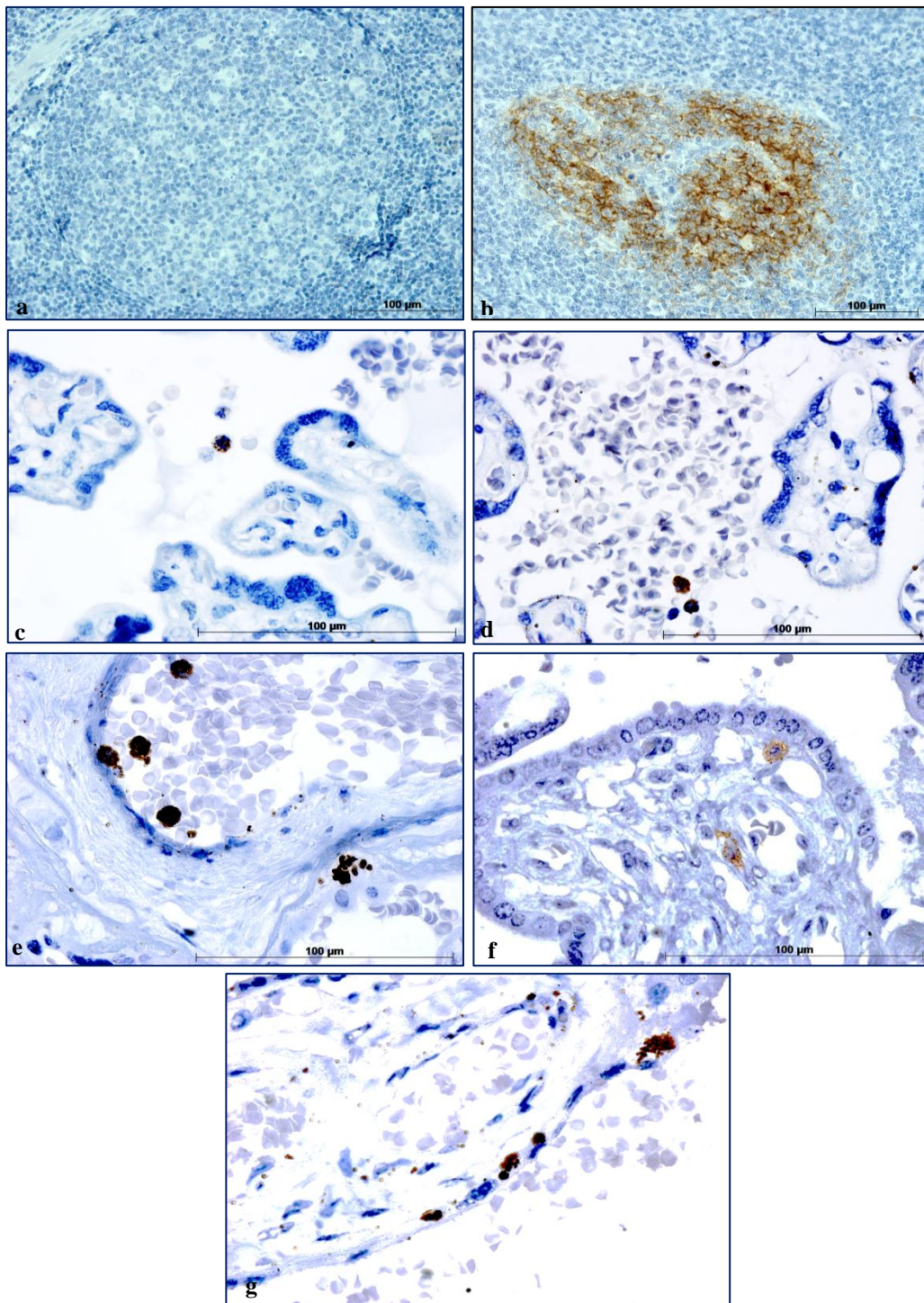
Figure 4.9: Immunostaining for p24 (a) Method control for p24 where primary antibody was omitted in processing of HIV positive lymph node biopsy. (b) Positive control for p24 staining of HIV capsid within HIV positive lymphatic tissue. (c-g) Micrographs show p24 immuno-positive staining in various compartments within the placenta. (c&d) HIV+ leukocytes within the intervillous space of (c) normotensive and (d) pre-eclamptic placenta. In (e) and (f) p24 immunostaining is present within a (e) normotensive fetal vessel and (f) Hofbauer cells within a pre-eclamptic intermediate villus. In (g) a longitudinal section through a stem villus shows positive staining of the syncytiotrophoblast in a pre-eclamptic section (100x).

4.13   p24 Immunostaining

Sections of HIV positive lymph node tissue were used for controls. The negative control substituted diluent buffer for the primary antibody and no staining was observed (Figure 4.9 (a)). The positive control stained positive for p24 (Figure 4.9 (b)).

p24 immunoreaction occurred within HIV positive placentae indicating positive staining of the p24 capsid within and around exchange villi. Circulating maternal leukocytes stained p24 positive (Figure 4.9 (c and d)). Staining did not occur diffusely throughout tissue sections, nor did it occur in all 40 HIV positive samples analysed. Staining was sparse. There was no predilection to any specific regions between samples as there was no obvious pathology noted between frames of view containing staining and those that did not.

p24 staining occurred in conducting as well as exchange villi (Figure 4.9 (c, d and e)). Circulating immune cells within the fetal capillaries stained positive and there was evidence of immunostaining of Hofbauer cells in some samples (Figure 4.9 (f)) Intermediate villi also contained cells within capillaries that stained positive, and there was evidence of staining of syncytiotrophoblast in these structures as well (Fig 4.9 (g)). Both the interface layer as well as the internal compartment of the exchange villi showed immunostaining. This occurred in all positive tissue regardless of pre-eclampsia status.

There was evidence of HIV within exchange villi in both normotensive and pre-eclamptic pregnancies (Figure 4.10 (a and b)). p24 positive cells were found within the fetal circulation (capillaries and arterioles of exchange vessels) in tissue sections of placenta where babies were HIV positive at 6 weeks post-delivery. Figure 4.10 (c and d) show differing magnifications (10x and 40x) of umbilical cord sections of babies born to HIV positive normotensive mothers. Figure 4.10 (e and f) show similar umbilical cord sections of babies born to HIV positive pre-eclamptic mothers. Brown immunoprecipitate in the fetal bloodstream indicates presence of p24 within the fetal blood stream as part of the umbilical cord. Figure 4.10 (c – f) are sections taken from mothers who did not transmit HIV to their babies as the 6 week follow up HIV test was negative.
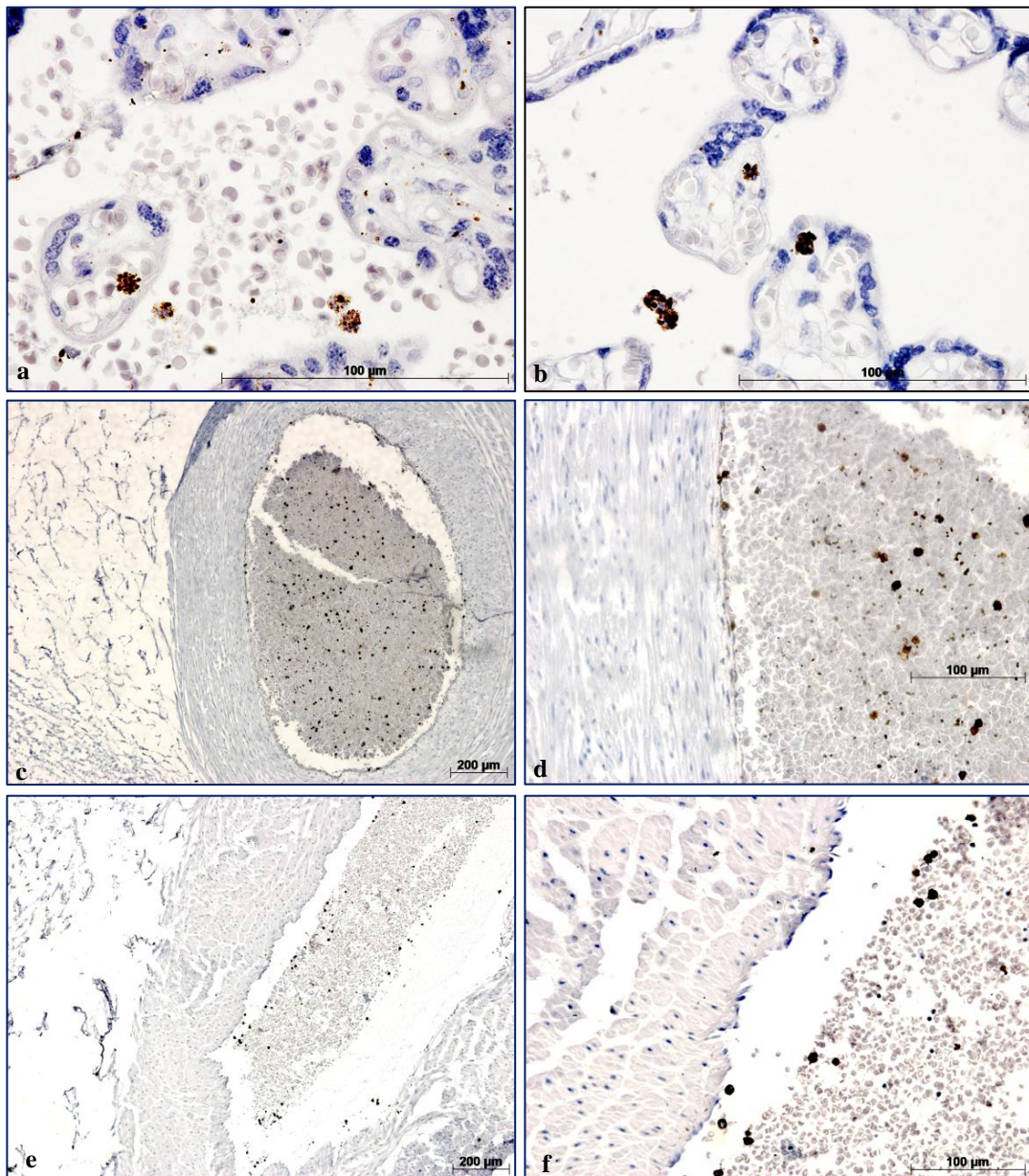
Figure 4.10: Immunostaining for p24 in both maternal and fetal blood circulation.
Micrographs show p24 immuno-positive staining in various compartments within
the placenta. (a-b) HIV+ leukocytes within the intervillous space of (a)
normotensive and (b) pre-eclamptic placenta. (d-f) Low power (left) and high power
(40x) images of umbilical cord vessels showing in (c) and (d) the presence of p24
within the fetal umbilical cord circulation of babies born to HIV positive
normotensive mothers. In (e) and (f), p24 positive staining is seen in umbilical
vessels of babies born to HIV positive pre-eclamptic mothers.

4.14 Immunolocalisation of CD4 in placentae

CD4 immunostaining was present in most samples analysed (Figure 4.11), even in those patients with low CD4 counts. CD4 positive cells were seen in all groups and found in the intervillous space as well as within the fetal bloodstream. CD4 positive cells were distributed variably amongst the groups as well as within the groups. CD4 positive cells were found in the fetal conducting vessels of both normotensive and hypertensive HIV negative placentae (Figure 4.11 (a and b)). HIV positive sections also contained areas and villi that were immunopositive for CD4 antigen (Figure 4.11 (a and b)). These ranged from circulating maternal white blood cells as well as fetal white blood cells within exchange and conducting villi. In all four groups there was evidence of syncytiotrophoblast immunoprecipitation indicating areas of CD4 positivity in the fetomaternal exchange barrier. Figure 4.11 (a and b) show staining of the syncytiotrophoblast in both HIV associated normotensive as well as pre-eclamptic placental tissue, respectively.
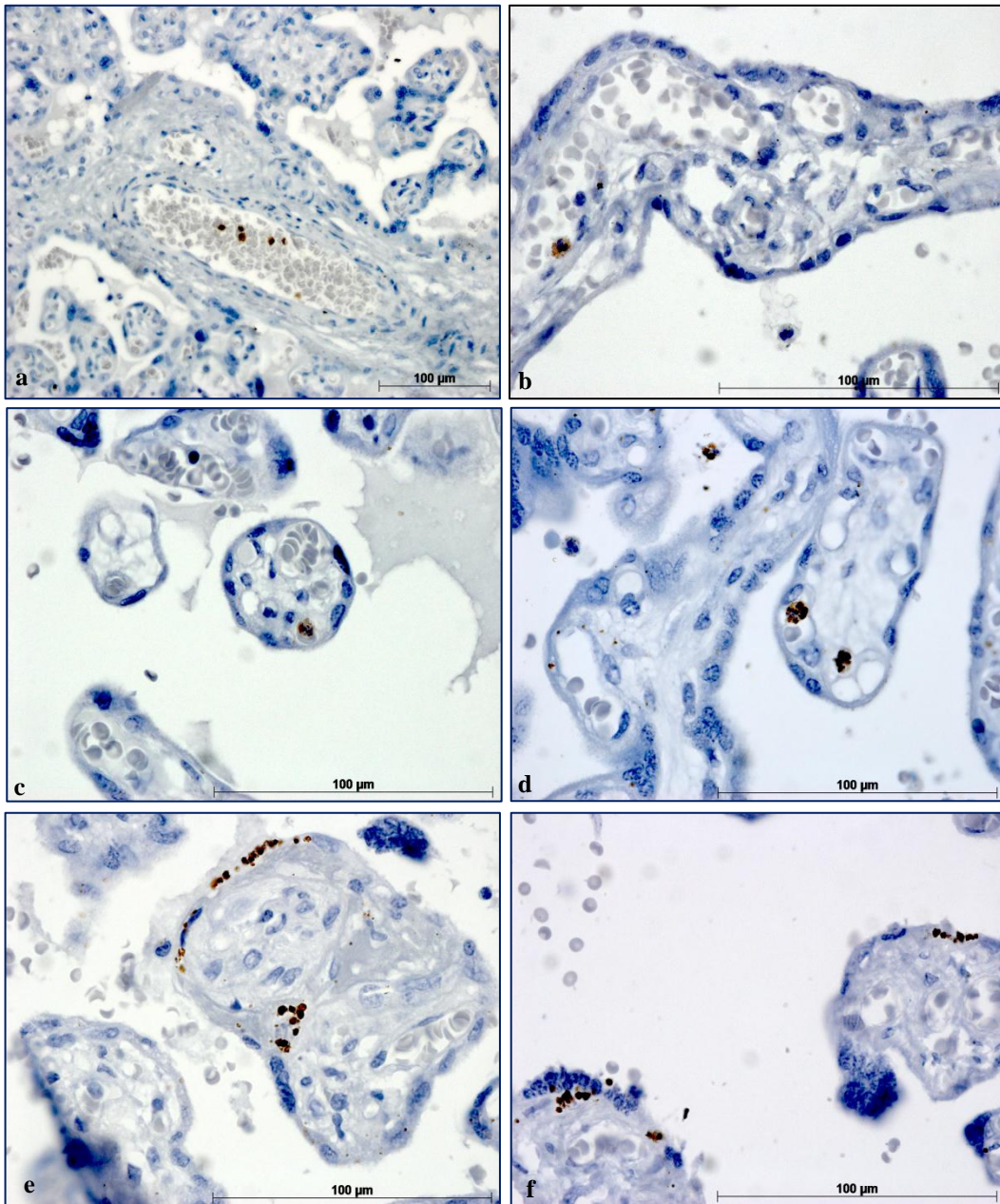
Figure 4.11: Immunostaining for CD4 within the placenta with (a), (c) and (e) showing normotensive sections and (b), (d) and (f) depicting pre-eclamptic sections. Both (a) and (b) show CD4 positive cells within the fetal circulation in stem villi of HIV negative placenta. In (c) and (d) CD4 positively staining cells were present in HIV positive tissue sections. (e) shows CD4 positive staining occurring within the syncytiotrophoblast in HIV positive exchange villi in normotensive placenta as well as pre-eclamptic in pre-eclamptic placentae (f).

4.15 CCR5 Immunostaining

The CCR5 co-receptor used by HIV to enter cells was also immunoprecipitated in placental tissue. The staining of tissue using this antibody was markedly increased compared to both p24 and CD4. As a cytokine CCR5 was distributed widely within the tissue and antibody to CCR5 stained the syncytiotrophoblast of both conduction and terminal villi in all groups (Figure 4.12 (a-e)). The chorionic plate also revealed staining of the amnion (Figure 4.12 (f)). There was a marked visual increase in staining intensity and distribution in the pre-eclamptic group compared to the normotensive groups. Statistical analysis of staining distribution (as a percentage of brown precipitate in a fixed frame area regarded as positive staining) in all 80 samples revealed that the percentage positivity was normally distributed in the four groups. A Levene's test for equality of variances showed that the variances were equal across the groups (F (3,169) =1.515, p = 0.212).

A factorial univariate ANOVA was then performed which showed that HIV and pregnancy status had a significant main effect on expression of CCR5 in placental tissue {[F (1,169) =6.979, p=0.009] and [F (1,169) =8.803, p=0.003]}. There was greater expression in the HIV positive groups compared to the HIV negative groups and the pre-eclamptic compared to the normotensive groups. The interaction between the HIV status and pregnancy status groups indicated a significant interactive effect only between the HIV negative normotensive group and the 3 other groups [F (1,169) =10.377, p=0.02]; while a pairwise comparison between the normotensive and both pre-eclamptic groups showed no significant difference in the means.
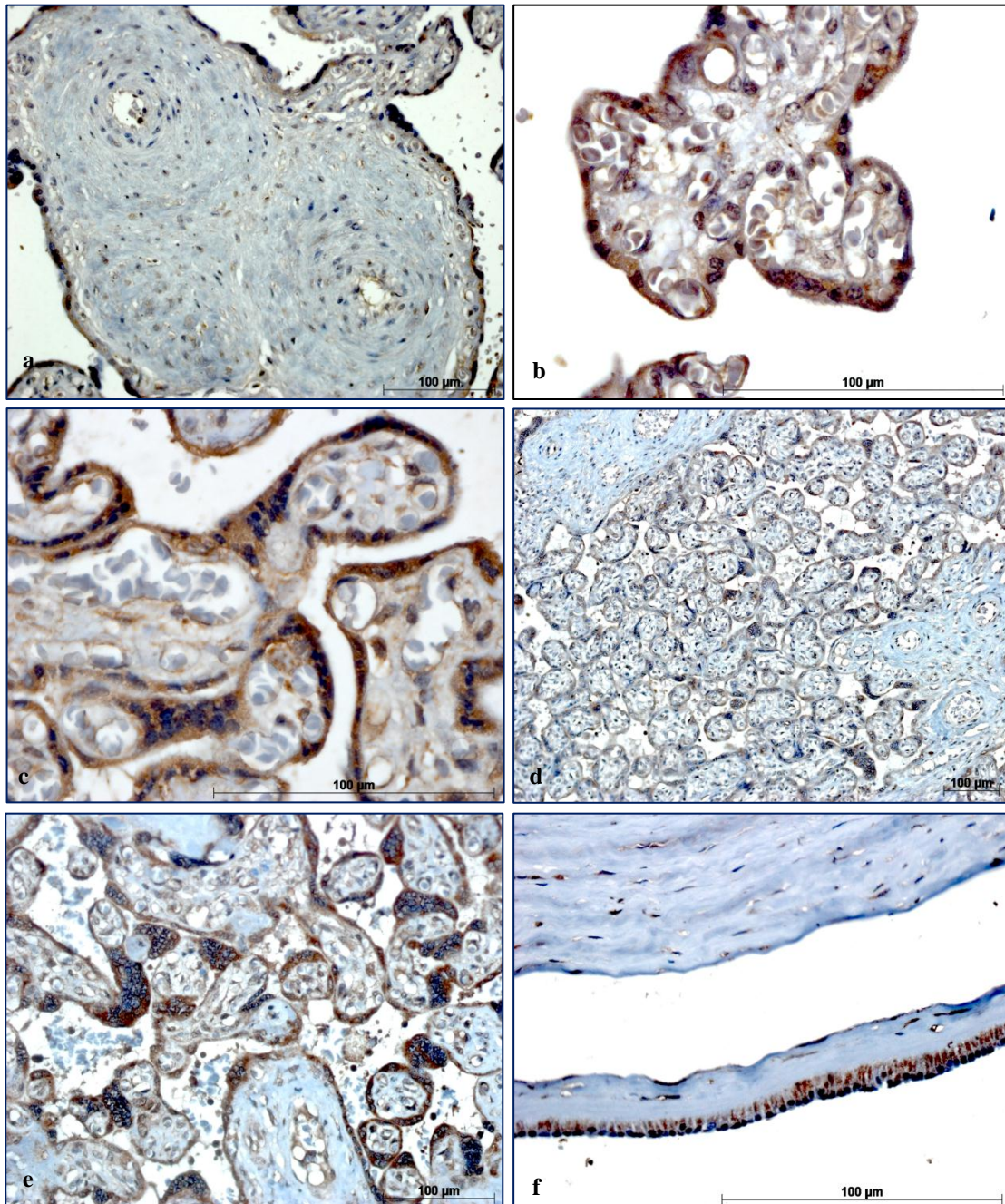
Figure 4.12: Immunostaining for CCR5 of both HIV positive and negative placentae
Both (a) and (b) show CCR5 positive immunoreactivity in (a) normotensive HIV
negative and (b) HIV positive pre-eclamptic placenta. Micrograph (c) depicts staining
of intermediate and terminal villi syncytiotrophoblast in an HIV positive sample.
Syncytial bridges as well as endothelial lining of the fetal vessels showed positive
staining. (d) The density of exchange villi was notably increased in placental tissue of
pre-eclamptic placentae studied. There was also a visual increase in the amount of
syncytial knotting in pre-eclampsia. Micrograph (e) shows the intensity as well as the
distribution of staining in preeclampsia with staining being more concentrated around
the syncytial knots. Micrograph (f) shows CCR5 staining of the amnion in a placenta
from the HIV positive group.

The HIV negative normotensive group's mean expression of CCR5 was significantly lower than the other three groups, with both HIV positivity and pre-eclampsia increasing expression of CCR5. This is illustrated in figure 4.13.
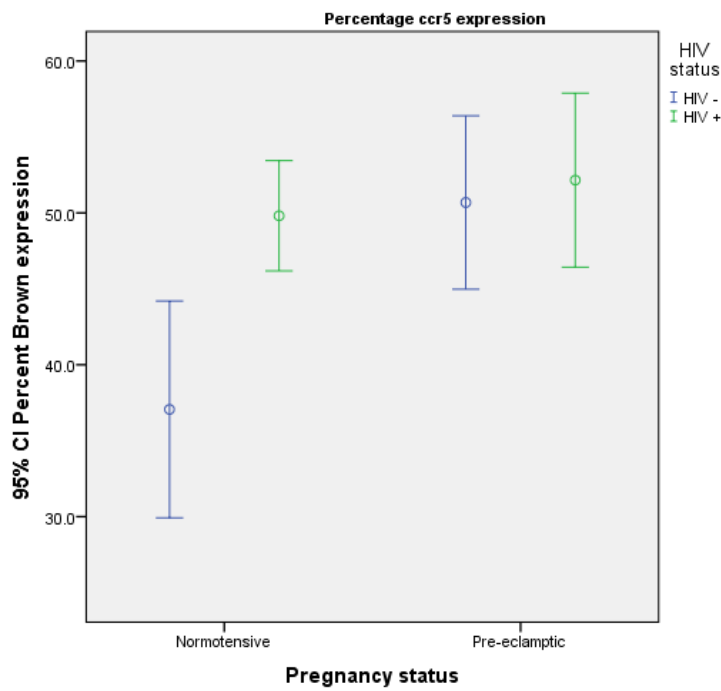
Figure 4.13: Scatter plots of mean percentage CCR5 expression in normotensive vs pre-eclamptic placentae factoring HIV status, thereby reflecting mean expression per study group. CCR5 expression is reported as mean per group after the percent expression of two snapshots of contiguous frames were taken at 20x magnification. Percentages are expressed per frame area.

4.16 ICAM-2 Immunostaining

ICAM-2 was found to be expressed throughout the placental tissue and expressed mainly on the fetomaternal trophoblastic barrier Figure 4.14 (a-d). Expression of ICAM-2 also occurred within the exchange villi wherein circulating fetal blood cells showed immunoreactivity. Where staining occurred in the syncytiotrophoblast, it did not stain all areas with the same morphology throughout the tissue, and was restricted to certain areas of the tissue, or even on the same villus (Figure 4.14 (d)). The percentage positive staining for ICAM-2 was taken as mean percentage brown colour of two contiguous frame areas within the section.
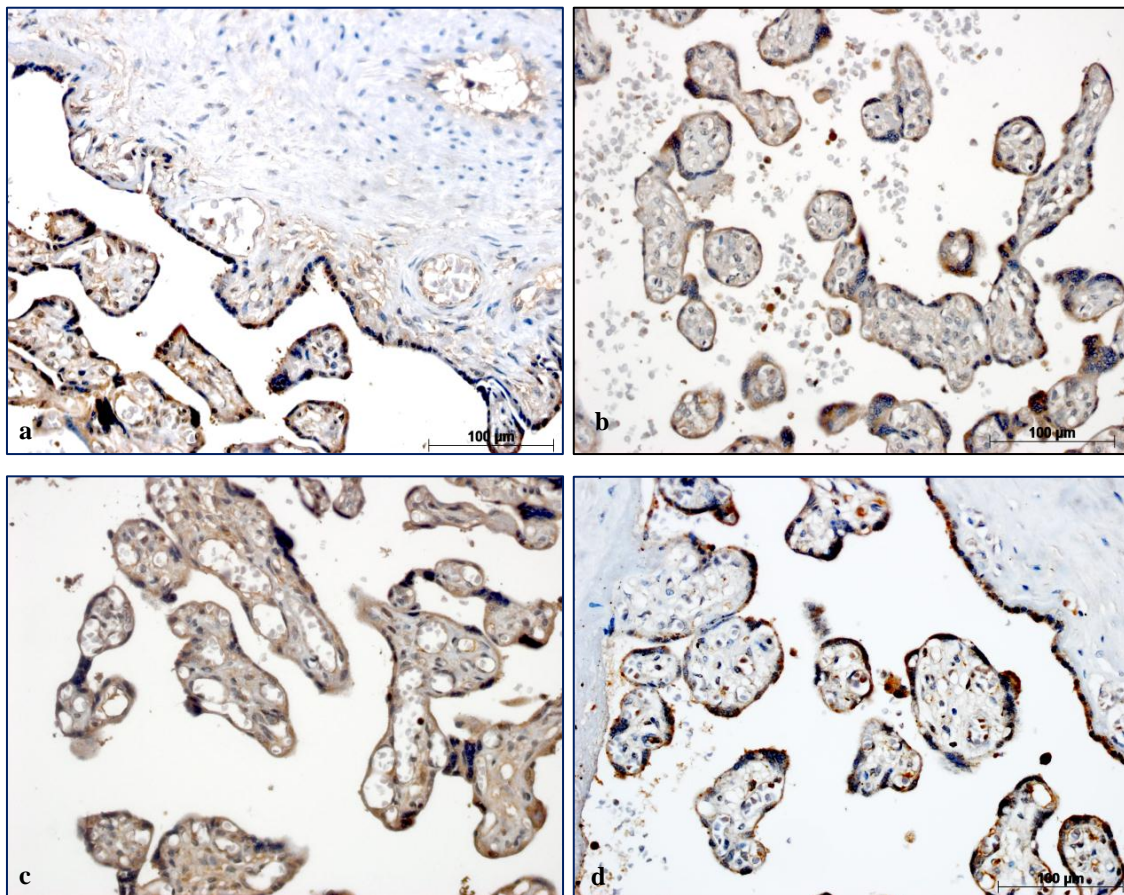
Figure 4.14: Immunostaining for ICAM-2 of HIV positive and negative placentae with (a) and (c) showing normotensive sections and (b), (d) depicting pre-eclamptic sections. Both (a) and (b) show ICAM-2 positive immunoreactivity in normotensive HIV negative (a) and HIV positive placenta (b). Micrograph (c) depicts staining of intermediate and terminal villi syncytiotrophoblast in an HIV positive sample. Micrograph (d) shows distinct staining of the syncytiotrophoblast layer of both stem, intermediate and terminal villi in an HIV positive sample. Cells within the villi were also immunoreactive to the antibody.

ICAM-2 percentage expression was not normally distributed and therefore a non-parametric analysis of the data was carried out. An independent samples Kruskal-Wallis test showed a significant difference between the 4 groups (asymptotic $p < 0.001$). An independent samples Mann-Whitney U test showed a significant difference in the distribution of ICAM-2 expression between the pre-eclamptic and normotensive groups ($p < 0.001$) and the HIV positive groups. The HIV negative pre-eclamptic group showed the greatest expression of ICAM-2 compared to the other groups with a mean rank almost two fold higher than the other groups. A graphical representation of raw score ICAM-2 data for each of the group is plotted as box and whisker plots in Figure 4.15 below to illustrate the difference between groups.


Figure 4.15: Scatter plots of mean percentage ICAM-2 expression in normotensive vs pre-eclamptic placentae factoring HIV status, thereby reflecting mean expression per study group. ICAM-2 expression is reported as mean per group after the percent expression of two snapshots of contiguous frames were taken at 20x magnification. Percentages are expressed per frame area.
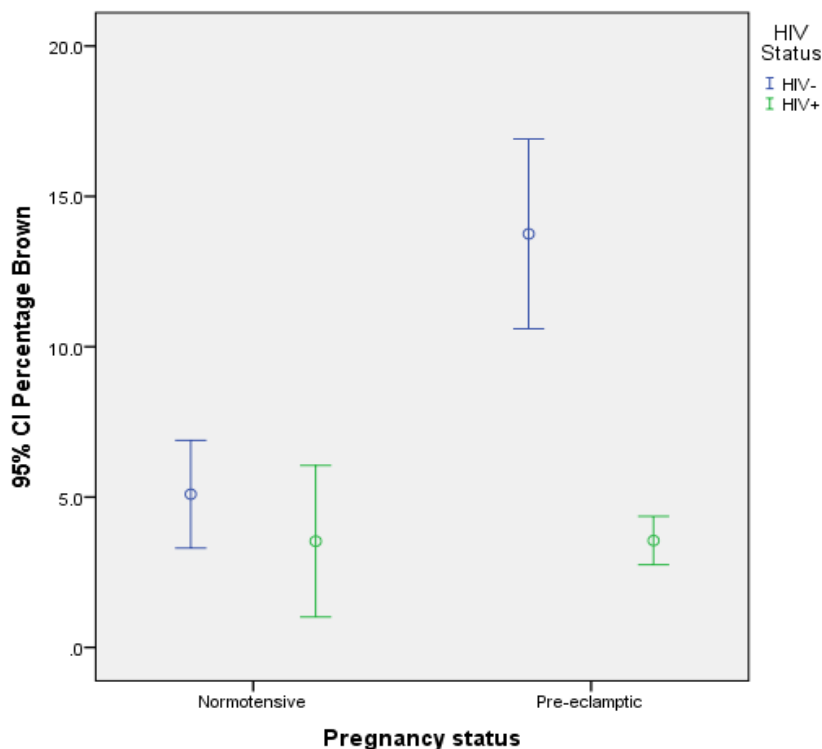
CHAPTER 5

DISCUSSION

5.1 HIV and pre-eclampsia

In this study, we aim to gain better insight into HIV and pre-eclampsia at the forefront of the battle in MTCT: the placenta. 'Insight' is appropriated as this study examines the placenta at a microscopic level with a view to elucidate HIV presence and transmission across placentae in both normotensive and pre-eclamptic pregnancies.

5.2 The placenta

Histomorphological examination of the placentae between the four groups studied characterised typical histology of the placenta, the most important of which for this study was the chorionic villi. It is through these villi that fetomaternal exchange occurs and via which HIV needs to pass in order to enter fetal circulation. Bound by a continuous layer of syncytiotrophoblast, all villi share a similar structure, with an increase in size and complexity as it increases function from purely exchange to conduction of blood.

Placental structure between normal and clinically described pre-eclampsia are identical in histology, and may often be appropriate for gestational age (Roberts & Post, 2008). Changes attributed to pre-eclampsia may reflect gestational age. The

results of our study show that the general age of the placenta is lower in pre-eclamptic pregnancies. Such immaturity may therefore be normal rather than pathological. Diametric considerations are that although histological examination of the placenta may show a normal phenotype, pathology cannot be excluded, and normal term placenta may show changes that are considered abnormal in early gestation (Fox, 1986).

In this study the placenta displayed varying phenotypes ranging from grossly normal to pathological. This variance may be innocuous in normotensive placentae at term or development of pre-eclampsia closer to term, but is clearly more pronounced in pre-eclamptic placentae. A limitation to our study is that we did not differentiate between early and late onset pre-eclampsia as this study utilises retrospectively collected data where that distinction was not originally made. HIV has been reported to affect the villous region of the placenta. Studies have reported villitis and chorioamnionitis (Gichangi, Nyongo & Temmerman, 1993; Ladner *et al.*, 1998; Mwanyumba *et al.*, 2002; Bhoopat *et al.*, 2005) as well as differences in placental weights of HIV infected pregnancies (Gichangi, Nyongo & Temmerman, 1993; Mwanyumba *et al.*, 2002).

The latter studies demonstrate conflicting results on the incidence of placental inflammation and placental weight and/or the development of villitis amongst HIV infected placentae. In our study, chorioamnionitis was an exclusion criterion. Jauniaux *et al.* (1988) described an increase in placental weight in placentae affected by HIV.

Our study demonstrates a significantly higher mean placental weight in the HIV positive normotensive group compared to the HIV negative normotensive group (p=0.01). These results are in contrast to that by Gichangi *et al.* (1993) and Raghavendra *et al.* (2014) who reported a lowered placental weight/baby weight ratio in HIV affected East African and Indian pregnancies respectively. Our observations were limited, however, as we focussed on primigravidae from the original cohort (Haffejee *et al.*, 2013). A limitation of the previous study was that it did not differentiate between early and late onset pre-eclampsia. Early onset pre-eclampsia is correlated with low birth weight and placental weights (Gichangi, Nyongo & Temmerman, 1993), therefore to overcome this limitation we chose only primigravidae so as to maintain a homogeneous population in our study.

The Indian study (Raghavendra, Pai & Vinay, 2014) also compared other regional studies and concurred that the severity of pre-eclampsia was negatively correlated with birth weight. They also reported that primigravidae had more severe forms of pre-eclampsia. Likewise, our study did not show any remarkable differences in placental weight between the normotensive and pre-eclamptic placentae (p=0.414).

Intrauterine growth restriction (asymmetric) is associated with hypertensive disorders of pregnancy and placental pathology (Roberts & Post, 2008), and may be more pronounced in early onset pre-eclampsia. Similar to the Indian study, baby weight in our study was significantly higher in the normotensive compared to the

pre-eclamptic pregnancies (p<0.001). However, in our study HIV did not have any significant effect on birth weight between the groups, in concordance with other studies (Bhoopat *et al.*, 2005; Raghavendra, Pai & Vinay, 2014).

5.3 Pathological features seen in this study

Immature placentas have homogenous trophoblastic thickness with numerous Langhans cells. This is a typical feature of post placental hypoxia, persisting immaturity and gestational diabetes and common in pre-eclamptic placentae (Benirschke & Kaufmann, 2006; Roberts & Post, 2008). These features were observed in the pre-eclamptic placental evaluation of this study.

We noted an increase of villous cytotrophoblast in the pre-eclamptic placentae. Cytotrophoblast proliferation was more frequently observed beneath the syncytiotrophoblast layer sandwiched by the perivillous fibrinoid. These features are consistent with and reflects placental immaturity and correlates with increasing degrees of severity of pre-eclampsia (Fox, 1986; Benirschke & Kaufmann, 2006). Langhans cells are increased in pre-eclampsia and low in placental hypoxia indicating that in pre-eclampsia, where the placenta shows immaturity, such a rise in cytotrophoblasts may be due to a compensatory mechanism to increase syncytiotrophoblast cells to reduce the hypoxia (Ducray, Naicker & Moodley, 2011).

We observed an increased villous density per frame area of placenta. Additionally, we qualitatively observed smaller terminal and intermediate villi in pre-eclamptic

placentae. Roberts (2008) reported an increased density of syncytial sprouting in pre-eclampsia and intra uterine growth retardation. This sprouting was attributed to the hypoxia cause by increased blood flow and high pressure. In our study, HIV infection did not visibly alter the effect of blood pressure between the normotensive and hypertensive groups (p =0.8417).

In our study we observed an increased stromal density in pre-eclampsia indicating an immature phenotype. Coupled with a thickened trophoblast membrane, the efficiency of placental exchange may be reduced, necessitating an increase in the amount of villous sprouting as a compensatory mechanism. This may also reduce the intervillous space thereby increasing the perfusion resistance to blood flow. This increase in syncytiotrophoblast may increase the turnover rate of syncytiotrophoblast, and increase the amount of syncytial knotting and concomitant increase in apoptosis within these aggregates (Naicker *et al.*, 2013); thereby releasing syncytiotrophoblast microvesicles into the maternal circulation, which may cause an increase in pro-inflammatory signalling, coagulation and further oxidative stress (Fox, 1986; Roberts & Post, 2008; Baig *et al.*, 2013).

5.4 p24

In this study we used the p24 Kal-1 antibody. It reacts with the viral inner core capsid protein (p24) as well as the pr55 gag protein during punctate assembly at the plasma membrane of HIV infected cells (Jouvenet *et al.*, 2006; LeBlanc, Perez & Hope, 2008). The antibody can detect virus particles as well as virus like particles (VLPs) developing at the plasma membrane of infected and active virus producing

cells. It could also detect gag or endocytosed regions of engulfed or endocytic trafficked virus particles. Testing p24 serum concentration is standard of practice to assess timing of infection, as the level is raised in serum of newly infected individuals who have not yet developed antibodies.

Immunoreactivity and staining of placental tissue with p24 yields positive staining of HIV present within tissue either in its formed state or in a state of assembly at the plasma membrane (Jouvenet *et al.*, 2006; Sudo *et al.*, 2013). In this study, p24 staining occurred in HIV positive placenta, regardless of ARV treatment or CD4 count. Both maternal and fetal circulating leukocytes were positive for HIV (Figure 4.9 (c-d)). There was focal staining of cells. In these sections leukocytes were clearly stained displaying punctate as well as intracellular staining. There was also evidence of acellular areas that contained globular staining possibly marking the presence of viral particles or VLPs.

Bhoopat *et al.* (2005) found similar staining in preterm placenta of clade E infected mothers. Similar to our observations, they also noted HIV staining in circulating Hofbauer cells. The reticular staining of Hofbauer cells indicates internalisation of HIV and could possibly indicate active production and assembly of pr55 proteins below the cell surface prior to budding or internalisation (engulfment or intracellular trafficking of virus) of whole virus in these cells. In our study similar staining patterns were seen in normotensive and pre-eclamptic placentae.

 Several studies (Lewis *et al.*, 1990; Martin *et al.*, 1992; Bhoopat *et al.*, 2005; Johnson & Chakraborty, 2012) have shown that circulating Hofbauer cells are infected with HIV and suggest that these macrophage-like cells are implicated in the transmission of virus. Johnson and Chakraborty (2012) suggest that although Hofbauer cells were touted as a reservoir for HIV, they offer a protective function by sequestering HIV and releasing Th2 type cytokines to reduce viral replication within these cells. Furthermore, Hofbauer cells do not enter fetal circulation therefore preventing sequestered virus from entering the fetal circulation.

In this study, it was shown that HIV was present in both Hofbauer cells, within the stroma and in the fetal circulation (Figure 4.10). We also demonstrate HIV presence within the umbilical cord vessel of HIV infected babies at 6 weeks post-delivery. Of note and interestingly, we also show circulating HIV in the cord sections of HIV positive mother-infant pairs where the virus was not transmitted to the baby (6 week HIV PCR negative), evidenced by the presence of p24 positive circulating cells in the umbilical artery. Admittedly, further monitoring of the baby is required to detect seroconversion; hence we proposed this for further study. Additionally, monitoring of HIV status immediately post-partum will provide greater insight into timing of infection. Larger sample sizes to accommodate for the low return rate of mothers for the 6 week follow-up visit (Haffejee *et al.*, 2013) may be of value in elucidating associative relationships between HIV presence in the placenta and outcome of HIV infection of infants. Alternatively, educating mothers on the importance of follow-up visits is warranted.

In our study, syncytiotrophoblast also depicted immunostaining (Figure 4.9 (g)), indicative of the infected syncytiotrophoblast showing assembly of virus at either the apical or basal surface. Alternatively it could be HIV trafficking through endocytic vesicles and recycling of virus back to the membrane surface. The incidence of such staining was not common but it occurred in both normotensive and pre-eclamptic pregnancies. Evidence of infection of the syncytiotrophoblast has also been reported by others (Lewis *et al.*, 1990; Martin *et al.*, 1992; Sheikh, Polliotti & Miller, 2000; Bhoopat *et al.*, 2005).

These studies show that villous trophoblasts and fetal tissue contained HIV in early pregnancy (Lewis *et al.*, 1990; Sheikh, Polliotti & Miller, 2000) whilst others show infection of trophoblast and endothelium in term placenta (Martin *et al.*, 1992; Bhoopat *et al.*, 2005). DNA isolation of HIV within trophoblast indicates active infection of the trophoblast and not merely passage of HIV through the barrier. Other studies showed PCR products of HIV DNA within trophoblastic tissue (Sheikh, Polliotti & Miller, 2000; Lagaye *et al.*, 2001). In contrast, one study claimed that there was no infection of the syncytiotrophoblast (Tscherning-Casper *et al.*, 1999), albeit in ARV treated pregnancies, and claim that the trophoblast forms a selective barrier that does not permit HIV passage. In our study, we observed HIV presence within syncytiotrophoblast, irrespective of ARV or HAART administered prior to delivery, in both normotensive and pre-eclamptic pregnancies.

There was a paucity or complete absence of HIV staining in some placental tissue in our study. For example, one non-breastfed baby showed HIV staining in the fetal

umbilical artery, yet did not show presence of HIV in the placental circulation. This observation could reflect the tissue sampling and the chance event of obtaining a 3µm thick 1cm^2 wide section with circulating HIV positive cells. It is plausible to assume that the low HIV staining observed in our study (some with just 1 HIV positive cell) across both pre-eclamptic and normotensive groups is possibly congruent with tissue sampling.

5.5 CD4

There were no significant differences in the CD4 counts between normotensive and pre-eclamptic pregnancies within the HIV positive group. CD4 antibody used in the study is directed towards mature thymocytes and other cells that express the CD4 transmembrane glycoprotein on its surface. A comparison of image analysis of CD4 distribution between groups was not possible due to the sparse staining of circulating maternal and fetal white blood cells. Nevertheless, staining when present was predominantly located on both circulating maternal and fetal leukocytes. Importantly, CD4 staining occurred in the syncytiotrophoblast of terminal villi of HIV positive placenta (Figure 4.11 (e and f) shows immunoexpression of CD4 receptor in both normotensive and pre-eclamptic HIV positive placenta).

This is in contrast to studies which report absence of staining or genetic expression of CD4 in trophoblast cells (Lairmore *et al.*, 1993; Douglas *et al.*, 2001; Parry *et al.*, 2006). Other investigators have found presence of CD4 and cytokines implicated in HIV infection in term (David *et al.*, 1992) and preterm trophoblast tissue (Mognetti *et al.*, 2000). The latter study reflected on both the sensitivity of the test used and the

timing of expression of CD4. These studies demonstrate that the expression of CD4 is not consistent within trophoblast and also differs with gestational age. Our study shows that although rare, there was CD4 staining of syncytiotrophoblast at term.

CD4 is used by HIV to infect cells. Such infection also requires the use of co-receptors. CD4 is a well-known classic receptor used by cell free HIV to infect cells. It is also a popular model to describe infection of circulating CD4 positive T cells and counting CD4 cells are used to assess the disease progression of HIV (Veazey, Marx & Lackner, 2001). However, tissue and tissue associated leukocytes that express CD4 may not mirror the profile in blood (Kumar *et al.*, 2011b), and these authors suggest compartmentalisation of virus may occur based on cytokine expression profile of the tissue. They postulate that the cytokine expression profile of the tissue selects for the virus profile within that tissue. This may be the case with the placenta. Expression of specific cytokines by the placenta may select for those cytokine-trophic virus within that compartment. In the placenta, for example, CCR5 trophic virus may be selected from a quasispecies pool that infects or transmits across the placenta.

5.6 CCR5

The placenta produces many cytokines in order to facilitate fetal growth and development while maintaining a suppressive Th-2 placental milieu in order to prevent rejection of the baby (Behbahani *et al.*, 2000; Douglas *et al.*, 2001; Mulayim *et al.*, 2003; Johnson & Chakraborty, 2012). Abortion and hypertensive disorders of pregnancy have been associated with a pro-inflammatory Th-1 type response.

Placental expression of CCR5 is said to be affected by Th-1 type responses (Al-husaini, 2009). CCR5 is a co-receptor used by HIV for infection. CCR5 trophic (syncytium inducing) virus is the most common virus transmitted to infants, and there exists a selection bottleneck at the placenta where only viral quasispecies of the CCR5 trophic type are transmitted to the infant (Spector, 2001). CCR5 is expressed on activated terminally differentiated T cells engaged in a cell mediated response. This is contrasted to resting T cells which display a CXCR4 (another cytokine) phenotype. CXCR4 expression is normally associated with Th-2 type placentas that are not in an inflammatory state (Behbahani *et al.*, 2000; Veazey, Marx & Lackner, 2001; Al-husaini, 2009).

A placenta expressing CXCR4, therefore, will be more resistant to HIV infection or transmission compared to one in an inflammatory state expressing CCR5. It has been shown that mothers who transmit HIV to their babies demonstrate a Th-1 CCR5 expressing placenta. Most studies have isolated CCR5 in placental lymphocytes and macrophages (Behbahani *et al.*, 2000; Johnson & Chakraborty, 2012), claiming that it is not expressed in trophoblast. In the study by Behbahani (2000), the results do show some immunoreaction of the CCR5 antibody at the syncytiotrophoblast layer although it was not as bright as the leukocyte staining. The expression, (Behbahani *et al.*, 2000) does not indicate expression of only circulating Hofbauer cells. The change in expression of CXCR4 from non-transmitting to transmitting does not correlate with the increase in expression of CCR5 shown if only circulating lymphocyte brown staining was tallied.

In our study, we found diffuse staining of CCR5 throughout the placenta. Trophoblasts did stain positive for CCR5. In normotensive sections, expression of CCR5 was more pronounced near syncytial knots and bridges and macrophage-like fetal cells. Light staining still occurred around syncytiotrophoblast of intermediate and stem villi but this was not regular throughout all sections. Staining in HIV positive and pre-eclamptic placenta was more intense and regular around most of the syncytiotrophoblast as well as within the fetal villi.

As mentioned earlier, CCR5 expression is associated with a pro-inflammatory state We are able to show that trophoblast expression of CCR5 was statistically significantly increased in both pre-eclamptic (p=0.003) as well as HIV positive pregnancies (p=0.009). However, the combination of HIV and pre-eclampsia did not significantly alter the expression of CCR5 compared to the effects of the groups alone (p>0.05).

The expression of CCR5 on the trophoblast membrane could make the placenta more vulnerable to HIV attack therefore explaining the observation of HIV within the placenta in our study. Alternatively the presence of HIV within the placenta may be responsible for increased CCR5 expression, thereby changing the cytokine profile to a pro-inflammatory response. Moreover, such expression may be linked to the initiation of placental defence mechanisms against viral insult. Such a pro-inflammatory response will also lead to expression of other cytokines and signalling molecules involved in leukocyte recruitment in order to neutralise or sequester the invading organism (Saito, Shiozaki, *et al.*, 2007; Saito *et al.*, 2010).

5.7 ICAM-2

Intercellular adhesion molecule 2 (ICAM-2) is a member of the immunoglobulin superfamily, and shares 35% homology with ICAM-1, ICAM-4 and ICAM-5. ICAM-2 is a cell surface protein that functions as a ligand for leukocyte function associated antigen-1 (LFA-1), a function it shares with other ICAMs (de Fougerolles *et al.*, 1991; Casasnovas, Pieroni & Springer, 1999). ICAM-2 is closely related to VCAM-1 and is expressed on lymphocytes, monocytes, platelets and endothelium. It specialises in the binding of integrins and is therefore important for leukocyte recruitment to sites of inflammation, and antigen specific T-cell responses. LFA-1, an important integrin required for cellular adhesion, is responsible for T cell-mediated killing and proliferation as well as T helper and B lymphocyte responses (de Fougerolles *et al.*, 1991).

While ICAM-1 upregulation is caused by stimulated lymphocytes and is therefore implicated as the major ligand for LFA-1 during inflammatory responses, ICAM-2 is expressed in resting endothelium or before expression of ICAM-1. LFA-1 is involved in recirculation of lymphocytes through tissue endothelium and ICAM-2 is responsible for tissue circulation of memory T cells. Memory T cells have greater expression of LFA-1. ICAM-2 is highly expressed in neovascular endothelial cells and mediates neutrophil binding to and migration through vascular endothelium.

ICAM-2 is expressed in stem, intermediate and terminal villi of normal placenta (Lyall *et al.*, 1995). In our study, we found immunoexpression of ICAM-2 at the fetomaternal barrier. Syncytiotrophoblast expression was more pronounced in

normotensive placentae compared to pre-eclamptic placentae (Figure 4.14). Expression in normotensive syncytiotrophoblast was uniform throughout the layer whereas pre-eclamptic villi showed varying immunoreactivity of syncytiotrophoblast. Some immune cells within or attached to the syncytiotrophoblast showed intense staining in the pre-eclamptic group.

Statistical analyses of expression revealed that there was no difference between the normotensive HIV positive and negative, and pre-eclamptic HIV positive group. The mean rank score for HIV negative pre-eclamptic group showed a significantly higher expression of ICAM-2 when compared to the other groups. This is interesting as it is in variance with our expectations of the function of ICAM-2. We anticipated that in a normal 'resting' placenta, we would see a raised expression of ICAM-2. We also expected in the heightened inflammatory states of HIV and pre-eclampsia there would be a drop in expression with a concomitant rise in ICAM-1. Although we did not establish the expression of ICAM-1, we did not see reduced expression of ICAM-2 in the syncytiotrophoblast of HIV positive compared to HIV negative placentae in the normotensive groups. There is either a reduction in the HIV positive normotensive and pre-eclamptic groups or a significant rise in the pre-eclamptic HIV negative group. As this is term or close to term placenta post-delivery, the expression may have changed during delivery of the baby or the placenta. HIV positive mothers are on antiretrovirals and it may be possible that these drugs are maintaining the expression of ICAM-2 in the HIV positive groups at levels comparable to those of the HIV negative normotensive group.

5.8 Summary of findings

In this study we have confirmed the assertions of others that pre-eclampsia changes the morphology of the placenta (Fox, 1986; Naicker *et al.*, 2003; Benirschke & Kaufmann, 2006; Roberts & Post, 2008) and these changes are more pronounced with worsening severity of the syndrome. Most notably, we have seen that the changes correlate with inflammatory and hypoxic conditions of the placenta (Salafia *et al.*, 1995; Bhoopat *et al.*, 2005; Burton & Jauniaux, 2011) leading to the phenotypic changes that we noted. These are typified by histomorphological changes elaborated upon earlier (increased villous density, for example), changes that accommodate for variations in blood pressure, hypoxia, increased apoptosis and nutrient exchange.

Such compensatory mechanisms or runaway catastrophe in pre-eclampsia progression has not been correlated with HIV. However, due to the low sample numbers per group, we cannot provide conclusive evidence, especially since a detailed quantification of pathological presentation may erroneously include normal 'pathology' of pregnancy. Such errors may occur due to the high variability seen in both normotensive and pre-eclamptic placentae and there is no pathognomonic histological feature peculiar to pre-eclampsia. Furthermore, the use of antiretroviral therapy may also mask any significant changes in the placenta, and the study cohort did not include any treatment naïve mothers.

We have shown that HIV was present in Hofbauer cells, within the stroma and in the fetal circulation (Figure 4.10). We also demonstrate HIV presence within the umbilical cord vessel of babies that were infected by HIV 6 weeks post-delivery. Of note and interestingly, we also show circulating HIV in the cord sections of HIV positive mother-infant pairs where the virus was not transmitted to the baby (6 week HIV PCR negative), evidenced by the presence of p24 positive circulating cells in the umbilical artery. Admittedly, further monitoring of the baby is required to detect seroconversion; hence we propose this for further study. Additionally, monitoring of HIV status immediately post-partum will provide greater insight into timing of infection. Larger sample sizes to accommodate for the low return rate of mothers for the 6 week follow-up visit (Haffejee *et al.*, 2013) may be of value in elucidating associative relationships between HIV presence in the placenta and outcome of HIV infection of infants.

CD4 positivity, although rare amongst our cohorts, was found expressed on syncytiotrophoblast in all groups. CD4 is a glycoprotein receptor expressed by lymphocytes, macrophages and monocytes, and once bound is used to activate its host using signal transduction via a tyrosine kinase pathway. This induces expression of mediators of inflammation (cytokines) as well as target proliferation of primed lymphocytes in order to mediate a cellular response to infection (Lairmore *et al.*, 1993: 4; Davidsson *et al.*, 2013: 4). Also, HIV receptor expression varies with gestational age.

CD4, being the main co-receptor used by HIV to facilitate docking and eventual injection of viral capsid into the host cell, would be an important determinant of HIV transmission and any status that increases inflammation, directed towards another invading organism or aberrant release of cytokines would cause cells to increase their expression of CD4 in order to provide directed cellular response to the invader.

However, the expression of CD4 was rare on the syncytiotrophoblast. While others have localised CD4 to circulating leukocytes in both maternal and fetal circulation, we saw presence on syncytiotrophoblast. It was reported that M-trophic viral transmission was reduced drastically following ARV regimens administered to pregnant mothers. Therefore there may be a reduction in the number of CCR5 trophic virus in circulation, thereby reducing the cell mediated infection pathway. However, by virtue of expression of some rare CD4 and other co-receptors on the syncytiotrophoblast, the possibility still exists for a cell free mechanism for HIV to infect the syncytiotrophoblast. And the 2-4% of babies that still become infected *in utero* despite ARV treatment may reflect to some degree a possibility of this type of infection of the placenta.

Alternatively, certain breaks of integrity of the syncytiotrophoblast during gestation or delivery may also account for this persistent transmission (Burton *et al.*, 1996; Naicker *et al.*, 2013).

The presence of the CCR5 co-receptor for macrophage-trophic HIV on the syncytiotrophoblast is important in that coupled with CD4 expression it provides a

mechanism for HIV to infect the syncytiotrophoblast. Some studies have shown that even without CD4, HIV was able to traverse the placental barrier using other cell membrane and endocytic machinery (Megia *et al.*, 2004; Vidricaire & Tremblay, 2007). The widespread expression of CCR5 on the placentae of all groups we studied showed that whatever mechanism HIV used to cross the placenta, if it was CCR5 dependent, the placenta would permit it.

The placenta, by expression of cytokines that suppress maternal immune response or by maintaining a balanced inflammatory response, is able to remain attached and infiltrate the uterine wall. The interface created to facilitate exchange is exposed to a myriad of cytokines, hormones and immune cells within the intervillous milieu (Casasnovas, Pieroni & Springer, 1999; Derrien *et al.*, 2005). In order to defend itself against haematogenous assault, the placenta expresses a gambit of defence mechanisms to ward off attacks. One such mechanism could possibly be expression of ICAM-2 which, in contrast to ICAM-1, is expressed in a docile Th2 type state. ICAM-1 expression on tissue surfaces will recruit and activate circulating cells expressing LFA-1 such as monocytes and lymphocytes, thereby inducing focal damage to that area of tissue and further release of cytokines(Garcia-Lloret, Winkler-Lowen & Guilbert, 2000).

ICAM-2 expression, on the other hand, is expressed in a non-inflammatory state, and is somehow correlated with maintaining an immature inactivated state in the placenta. We have shown that expression of ICAM-2 is up regulated in HIV negative pre-eclamptic pregnancies but is at similar levels in the other groups. The

use of ARVs is known to increase the CD4 count and also to change the immune response from a Th2 to a Th1 type response. As ICAMs are directly associated with an inflammatory state, the effect of ARV is directly associated with expression. It is possible then that such association, by propagating a Th1 type milieu, sustains a pre-eclamptic environment.

5.9 Limitations

Unfortunately, access to hospitals and education about the importance of follow up visits is very poor in South Africa. There needs to be a drive to educate mothers on the importance of follow up visits. This would increase the reliability of the expression data. This would also improve the count of mothers returning for follow-up visits. Numbers of babies who seroconvert could then be properly evaluated. Further follow-up at a later stage will also provide definitive transmission rates, especially if babies at 6 weeks may still be in a window period. Immediate post-partum testing for HIV would also assist in diagnosing HIV infection earlier on, and would therefore resolve any doubt of breastfeeding transmission.

Another platform of HIV testing to confirm HIV within the placenta may also be warranted to corroborate our results. Identification of the circulating immunopositive cells will help understand the role of these cells and whether they perform a selective function or are reservoirs for HIV.

An important exclusion criterion of our study from which our samples were sourced was chorioamnionitis, and it is known that villous inflammation increases the risk of

pre-eclampsia (Ladner *et al.*, 1998; Bhoopat *et al.*, 2005). Placental inflammation and co-infection has also been postulated to increase the rate of mother to child transmission (Ladner *et al.*, 1998; Mwanyumba *et al.*, 2002; Coovadia *et al.*, 2007; Kourtis & Bulterys, 2010). Although the original study selected patients randomly but filtered for the selection criteria, our study refined the selection to primigravidae and included all patients that transmitted HIV to their babies; therefore, our prevalence rates of HIV transmission were artificially higher than the original study (8% *vs* 4% in our study).

It is important to gain a deeper understanding of some of the mechanisms HIV uses to enter the fetal bloodstream eventually leading to the main cause of HIV in children (Nightingale & Dabis, 2006; Joint United Nations Programme on HIV/AIDS, 2011). Although interventions have proven extremely successful, there is still worry about the fairly high rate of MTCT in underdeveloped countries. This study attempted to shed some light on the transmission of HIV through the placenta. Immunolocalisation of HIV within the blood stream of infants, even though they do not seroconvert, raises interesting questions about the selectivity of the placenta to HIV as well as the immunocompetence or naivety of the neonatal immune system. How does the virus pass through the placenta? What is the significance of the virus in the circulation?

5.10 Conclusion

We have found that the virus could use the traditional route to infect the placenta as both co-receptors needed for successful infection were found on the placenta. We have shown that both CD4 and CCR5 are present on the trophoblast. These co-receptors were found in all cohorts studied, and CD4 expression on syncytiotrophoblast was sparse, whereas there was evidence of circulating CD4 positive cells in both fetal tissue and maternal blood stream. CCR5 was more diffuse in expression in all groups and was higher in the HIV and pre-eclamptic groups (p=0.003). When there are confounding pregnancy related conditions such as pre-eclampsia, the expression of these co-receptors are altered which we propose may lead to greater susceptibility to infection, however due to the poor response rate of returning mothers, we could not establish this. Further study outlining these parameters (more frequent HIV testing and follow-up; other HIV associated co-receptors) may provide a more complete picture of orthodox transmission methods across the placenta.

The placental barrier in an activated state may attract HIV infected cells and such intimate contact may increase the propensity of infection. In mothers that are pre-eclamptic and not on HIV treatment, the expression of these attachment signals is increased. We studied the expression of ICAM-2 and found that expression was increased in the pre-eclamptic HIV negative group compared the other groups (p<0.001). Such expression is indicative of the balancing of pro-inflammatory and anti-inflammatory responses exhibited by the maternal and trophoblast barrier in

order to achieve successful pregnancy. ICAM-2 is expressed in a non-inflammatory state or is expressed to create such a state, and increased expression may be a mechanism to effect stability. In HIV positive pregnancies, such increase expression is not evident in either the normotensive or pre-eclamptic trophoblast barrier. This may be due to HIV infection or treatment.

5.11 Future studies

Further study is warranted and greater recruitment and less attrition of study participants should be strived for. A greater complement of cellular adhesion molecules implicated in inflammation need to assessed at the trophoblast barrier to gain a better understanding of the role of the placenta in evading the maternal immune system. Identifying the cell types that are HIV infected in the maternal bloodstream and correlating these with circulating fetal cells will also prove of tremendous value and this is our suggestion for further interrogation, all geared for the protection of children from HIV.

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18 December 2013

Mr V Dorsamy
607 Belmont
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Durban, 4001
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Dear Mr Dorsamy

**PROTOCOL: The Role of Trophoblast Cells in HIV-1 Passage across the Placenta:
REF BE352/13**

EXPEDITED APPLICATION

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 03 October 2013.

The study was provisionally approved pending appropriate responses to queries raised. Your responses received on 17 December 2013 to queries raised on 17 December 2013 have been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given full ethics approval and may begin as from 18 December 2013.

This approval is valid for one year from **18 December 2013**. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

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

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

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

The sub-committee's decision will be **RATIFIED** by a full Committee at its next meeting taking place on **11 February 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

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