

THE ROLE OF LEPTIN IN HIV ASSOCIATED PRE-ECLAMPSIA

By

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DEDICATION

To my son, Taahir

For encouraging me to initiate and pursue the research towards this degree.

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ABSTRACT

HIV and hypertensive disorders in pregnancy, in particular pre-eclampsia, are the main causes of maternal mortality in South Africa. In HIV associated pre-eclampsia, it is biologically plausible that the immune activation associated with pre-eclampsia may be neutralised by the immune suppression of HIV infection. The precise aetiology of pre-eclampsia is unknown, however leptin has been implicated in its development. Leptin is an adipocyte hormone, also produced by the placenta. It has a role in the development of inflammation. Adipose tissue is reduced in HIV infected individuals, resulting in lower leptin levels with consequent impaired immune function. This study aimed to compare serum and placental leptin levels in HIV infected and uninfected normotensive and pre-eclamptic pregnancies. Since insulin levels may affect the secretion of leptin, the study also compared insulin levels in these pregnancies.

Following ethical clearance and hospital permission, 180 participants were recruited during their antenatal period. The groups were HIV- normotensive (n = 30), HIV+ normotensive (n = 60), HIV- pre-eclamptic (n = 30) and HIV+ pre-eclamptic (n = 60). Blood samples were collected ante-natally and placental samples post delivery. Serum leptin and insulin levels were determined by ELISA. Placental leptin levels were determined by ELISA and immunohistochemistry with morphometric image analysis. The placental production of leptin was determined by RT PCR.

There was a non-significant increase in serum leptin levels in HIV- pre-eclampsia compared to HIV- normotensive pregnancies ($p = 0.42$). However leptin was decreased significantly in HIV+ pre-eclampsia compared to HIV- normotensive ($p = 0.03$). Based on HIV status leptin levels were decreased in HIV+ groups compared to HIV- groups in both pre-eclamptic ($p < 0.01$) and normotensive pregnancies ($p < 0.01$). Insulin levels of the HIV positive groups were lower than those of the HIV negative groups ($p < 0.001$). Insulin levels were also decreased in pre-eclampsia compared to normotensive pregnancies, irrespective of HIV status ($p = 0.02$).

Immunohistochemistry demonstrated an increase in immuno-reactivity of leptin in the exchange villi of pre-eclamptic compared to normotensive placentae, irrespective of HIV status ($p < 0.001$). Supporting this finding, ELISA also demonstrated elevated leptin levels in the placenta of pre-eclamptic compared to normotensive pregnancies ($p < 0.001$). Placental leptin levels were similar in both HIV positive and negative pregnancies ($p = 0.36$). However, the placental leptin mRNA expression was up-regulated in HIV negative pre-eclampsia ($p = 0.04$) but not in HIV positive pre-eclampsia ($p = 1.00$).

In conclusion, the elevated placental leptin in pre-eclampsia, irrespective of HIV status, is consistent with hypoxia. These elevated levels are not reflected in the maternal serum which raises the possibility of decreased leptin expression by adipose tissue especially in HIV infection where serum leptin levels are decreased. This would negate the increased placental leptin expression in pre-eclampsia. Furthermore, the elevated placental leptin levels are suggestive of an autocrine role of leptin in the placenta.

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LIST OF ABBREVIATIONS

ACTH	adreno-corticotropin releasing hormone
AgRP	agouti related protein
AIDS	acquired immune deficiency syndrome
ANOVA	analysis of variance
APGAR	Appearance, Pulse, Grimace, Activity, and Respiration
ARV	antiretroviral
AZT	Azidothymidine / zidovudine
BMI	body mass index
BP	blood pressure
BSA	bovine serum albumin
CD4	cluster of differentiation 4
cDNA	copy deoxyribonucleic acid
CI	confidence interval
COX2	cyclo-oxygenase 2
C-section	caesarean section
CSF	cerebrospinal fluid
Ct	threshold cycle
DAB	diamino-benzidine
<i>db</i>	diabetes gene
DEPC	diethylpyrocarbonate
DPX	dibutylphthalate xylene
DNA	deoxyribonucleic acid

DNase	deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid;
ELISA	enzyme linked immuno-sorbant assay
EVT	extravillous trophoblast
F	degrees of freedom
FSH	follicle stimulating hormone
g	gram
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GITC	guanidine-isothiocyanate
GnRH	gonadotropin releasing hormone
gp120	glycoprotein 120
H&E	haematoxylin and eosin
HAART	highly active antiretroviral therapy
HDL	high density lipoprotein
HELLP	haemolysis, elevated liver enzymes and low platelets
HIF-1 α	hypoxia-inducible factor-1 α
HIV	human immunodeficiency virus
HLA-G	human leukocyte antigen-G
H-P-A	hypothalamic-pituitary- adrenal
IgG	immunoglobulin
IL-2	interleukin 2
IL-6	interleukin 6
IUGR	intra-uterine growth restriction
JAK	janus tyrosine kinases
KDa	kilodalton

Kg	kilogram
KZN	KwaZulu-Natal
l	litre
LDL	low density lipoprotein
LH	luteinising hormone
m	metre
mg	milligram
min	minute
ml	millilitre
mM	millimole
mm/Hg	millimetres of mercury
MMPs	matrix metalloproteinases
mol	mole
mRNA	messenger ribonucleic acid
MSH	melanocyte stimulating hormone
MTCT	maternal to child transfer of HIV
mU	milli units
n	number
NHIV ⁻	normotensive HIV negative
NHIV ⁺	normotensive HIV positive
NK cells	natural killer cells
nm	nanometres
NO	nitric oxide
NPY	neuropeptide Y
NVP	nevirapine

<i>Ob-R</i>	leptin receptor
OD	optical density
°C	degrees celcius
<i>p</i>	level of significance
PBMC	peripheral blood mono-nucleocyte
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEHIV ⁻	pre-eclamptic HIV negative
PEHIV ⁺	pre-eclamptic HIV positive
pg	picogram
PMTCT	prevention of maternal to child transfer
POMC	propiomelanocortin
<i>r</i>	correlation co-efficient
RNA	ribonucleic acid
RNase	ribonuclease
ROI	region of interest
rpm	revolutions per minute
RT-PCR	real time polymerase chain reaction
s	second
SD	standard deviation
SEM	standard error of mean
SOCS3	suppressor-of-cytokine-signalling-3
STAT	signal transducers and activators of transcription
TBE	tris borate EDTA
TDF	Tenofovir disoproxil fumarate / Tenofovir

TGF- β	transforming growth factor beta
Th 1	T helper 1 lymphocytes
Th 2	T helper 2 lymphocyte
T _m	melting temperature
TMB	tetramethylbenzidine
TNF- α	tumour necrosis factor-alpha
TRH	thyrotropin releasing hormone
TSH	thyroid stimulating hormone
viz	namely
vs	versus
w	watts
X	times
β	beta
μg	microgram
μl	microlitre

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CHAPTER 1

INTRODUCTION

1.1 PRE-ECLAMPSIA

1.1.1 Definition

Pre-eclampsia is a hypertensive disorder in pregnancy, defined as a systolic blood pressure of at least 140 mm Hg and/or a diastolic pressure of at least 90 mmHg (Alderman *et al.*, 1986; Cnossen *et al.*, 2006; Magnussen *et al.*, 2007). In pre-eclampsia, the hypertension is accompanied by proteinuria of at least 300 mg protein in a 24 hour urine sample or $\geq 1+$ on a urine dipstick measurement (Sibai *et al.*, 2005). Hypertension and proteinuria should be apparent on two different occasions at least 4 – 6 hours apart (Magnussen *et al.*, 2007). Pre-eclampsia usually presents after 20 weeks of gestation (Alderman *et al.*, 1986; Magee *et al.*, 1999; Cnossen *et al.*, 2006). In patients with pre-existing hypertension, pre-eclampsia is diagnosed when proteinuria occurs for the first time in the second half of pregnancy (Evers *et al.*, 2004).

Pre-eclampsia is a multisystem disorder, affecting maternal renal, cerebral, hepatic and clotting functions (Brown, 1995). Women with severe pre-eclampsia (BP $\geq 160/110$ mmHg and/or proteinuria $\geq 2-5$ g in a 24 hour urine sample) may have signs such as renal insufficiency (reduced urinary volume, raised serum creatinine), liver disease (upper abdominal pain, elevated liver enzymes), neurological disturbances (headache, visual disturbances, exaggerated tendon reflexes, convulsions) and haematological abnormalities (thrombocytopenia, disseminated intravascular coagulation, haemolysis, Sibai *et al.*, 2005). Once the placenta is delivered, the symptoms of pre-eclampsia are resolved (Cnossen *et al.*, 2006).

1.1.2 Epidemiology

The incidence of pre-eclampsia is higher in developing countries compared to developed countries (Sahin and Gulmezoglu, 2003). The worldwide prevalence of the condition is 3 – 5% (Magnussen *et al.*, 2007). However in South Africa, a low to middle income socio-economic country, it occurs in 11.5% of pregnancies (Immink *et al.*, 2008). Furthermore, in South Africa hypertensive disorders of pregnancy account for a large proportion (14.1%) of deaths and pre-eclampsia is the most common direct cause of maternal deaths (Saving mother's Report: 2008-2010, 2012).

1.1.3 Risk Factors

There are five categories of risk factors for pre-eclampsia, namely, genetic, immunological, physiological, environmental and pregnancy-related factors (Lorquet *et al.*, 2010).

1.1.3.1 Genetic Factors

First degree relatives of a woman with pre-eclampsia are more likely to develop pre-eclampsia (Esplin *et al.*, 2001). The daughters of women who had a pre-eclamptic pregnancy have more than twice the risk of developing the disorder themselves (Skjaerven *et al.*, 2008). Daughters born from such pregnancies may suffer from long term cardiovascular effects later in life, which could increase the risk for pre-eclampsia (Dekker and Sibai, 1999). A family history of chronic hypertension is a risk indicator of pre-eclampsia development (Emonts *et al.*, 2008).

In addition, men born from a pre-eclamptic pregnancy also have an increased risk of fathering a pre-eclamptic pregnancy, the risk being 1.5 times greater than in men who were

born from a normal pregnancy (Skjaerven *et al.*, 2008). Furthermore, susceptibility for developing pre-eclampsia in female monozygous twins differs, indicating that both maternal and paternal genes are involved in pre-eclampsia development (Dekker and Sibai, 1999).

1.1.3.2 Physiological Factors

Cardiovascular risk factors that are present before pregnancy are associated with an increased risk of developing pre-eclampsia (Magnussen *et al.*, 2007). Pre-eclampsia occurs more frequently in women with a higher body mass index (BMI; Yu *et al.*, 2006; Bhattacharya *et al.*, 2007), there being a three-fold increased risk in obese (30 – 39.9 kg/m²) and a seven-fold increase in morbidly obese women with a BMI greater than 40 kg/m² (Bhattacharya *et al.*, 2007). Magnussen *et al.* (2007) also found an increased risk for pre-eclampsia in women with an increased waist circumference and in women with increased levels of serum triglycerides, total cholesterol and low density lipoproteins. Other studies have linked pre-eclampsia with a higher risk of future cardiovascular disease (Smith *et al.*, 2001). These findings suggest that unfavourable cardiovascular and metabolic profiles may represent primary causes of pre-eclampsia (Magnussen *et al.*, 2007).

Women with diabetes or chronic hypertension before pregnancy have a higher risk of developing pre-eclampsia (Magnussen *et al.*, 2007). Evers *et al.*, (2004) report that the risk of developing pre-eclampsia increases 3-12 fold in diabetic women. Insulin resistance in early pregnancy is associated with lower levels of sex binding globulin which may be implicated in the development of pre-eclampsia. This association however, is only observed in non obese women (Carpenter, 2007). In gestational diabetes mellitus, there is

an over expression of the innate immune response, which in turn is associated with vascular dysfunction which may then result in the development of pre-eclampsia (Carpenter, 2007).

Other factors that increase the risk of developing this pregnancy disorder include the occurrence of migraines both before and during pregnancy, since endothelial dysfunction, platelet activation and inflammation are common to both disorders (Sanchez *et al.*, 2008).

Renal abnormalities and the formation of intra-uterine haematoma at around 13 – 14 weeks of pregnancy increase the risk of pre-eclampsia (Ihle *et al.*, 1987). Another factor may involve high circulating levels of very low density lipoproteins, which have been reported to adversely affect the endometrium (Dekker and Sibai, 1998).

1.1.3.3 Pregnancy Related Factors

The risk of pre-eclampsia is higher in first pregnancies (Chesley, 1984; Campbell *et al.*, 1985; Seidman *et al.*, 1989; Eskenazi *et al.*, 1991; Roberts and Cooper, 2001), with 75% of the disorder occurring in nulliparous pregnancies (Chesley, 1984). However, pre-eclampsia disposition in subsequent pregnancies is only reduced if the first pregnancy was normotensive, had a gestational age greater than 37 weeks and if the birth weight was greater than 2.5 kg (Campbell *et al.*, 1985). A first pregnancy that is miscarried also reduces the risk of pre-eclampsia in the second pregnancy (Campbell *et al.*, 1985, Seidman *et al.*, 1989).

A new pregnancy with a change in partner increases the risk of pre-eclampsia to that of a nulliparous woman (Robillard *et al.*, 1993). Sexual cohabitation under six months also increases the risk, the length of co-habitation being inversely proportional to the risk of

pre-eclampsia (Robillard *et al.*, 1993). Previous exposure to paternal antigens in seminal plasma is thus protective (Roberts and Cooper, 2001). Furthermore, decreased exposure to semen causes an increase in the production of interleukin 2 (IL-2), high levels of which are implicated in the development of pre-eclampsia (Postovit *et al.*, 2001).

In addition, consanguineous marriages are associated with a lower incidence (Stevenson *et al.*, 1971) whilst a dissimilar race of mother and father is associated with a 1.5 fold increase in the risk of the disorder (Alderman *et al.*, 1986).

The onset of pre-eclampsia prior to 37 weeks of gestation has been reported to be more severe than that occurring after 37 weeks (Ihle *et al.*, 1987). In addition, placental dysfunction with lower oestriol secretion, low birth weight and perinatal death are more common in early onset pre-eclampsia (Ihle *et al.*, 1987). The disorder appears to be more severe when the diastolic pressure is higher. Furthermore, an earlier onset increases the severity of pre-eclampsia (Symonds, 1980). However, it is the appearance of proteinuria that increases the risk to both mother and fetus (Symonds, 1980).

1.1.3.4 Environmental Factors

Smokers (current or former) have a lower risk for pre-eclampsia than non-smokers. (Magnussen *et al.*, 2007).

1.1.4 Aetiology of Pre-eclampsia

The precise aetiology of pre-eclampsia is still unknown and appears to be multifactorial (Cnossen *et al.*, 2006). However, it has been described as a two-stage disorder (Figure 1.1) where shallow placentation occurs in the first stage followed by placental oxidative stress and inflammation in the second stage (Redman and Sargent, 2009; Lorquet *et al.*, 2010).

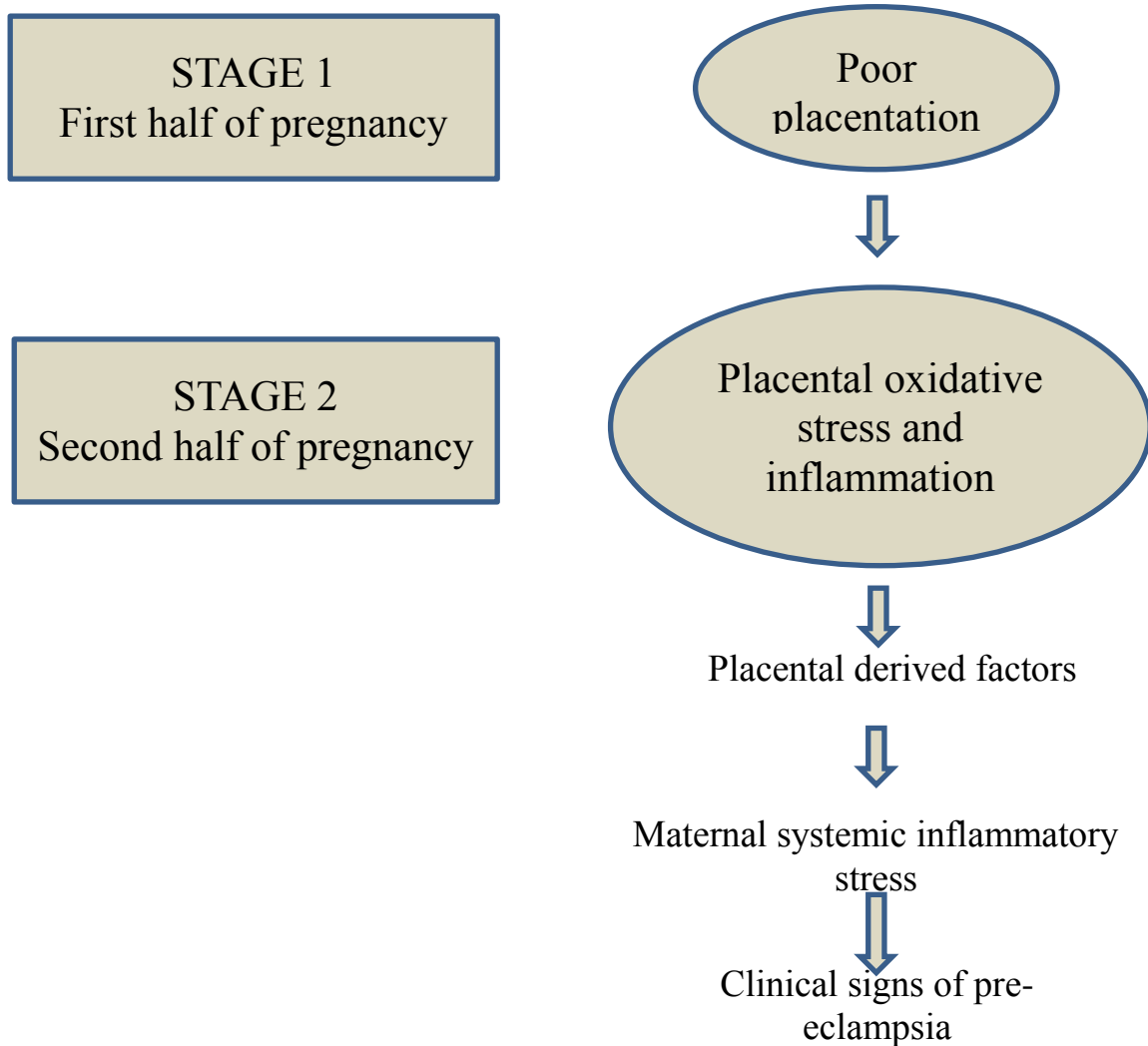


Figure 1.1 The two-stage model of pre-eclampsia. Poor placentation results in oxidative stress and inflammation which eventually cause the maternal symptoms of hypertension and proteinuria (Adapted from Redman and Sargent, 2009)

In a normal pregnancy, trophoblast cells migrate into the lumen of the spiral arteries and then replace the endothelium of these arteries (Brosens *et al.*, 1967; Lorquet *et al.*, 2010). The muscular wall of the spiral arteries is replaced by a fibrinoid-like material (Naicker *et al.*, 2003), which leads to large, low resistance blood vessels thus allowing an adequate blood supply to the placenta and the growing fetus (Dekker and Sibai, 1999; Lorquet *et al.*, 2010).

On the other hand, in pre-eclampsia there is insufficient trophoblast invasion of the spiral arteries in the myometrium and this limits early structural changes in these arteries (Brown, 1995; Naicker *et al.*, 2003; Lorquet *et al.*, 2010). A possible mechanism for the decreased trophoblast invasion of the spiral arteries is an increased placental production of nitric oxide (NO) which induces trophoblast cell death (Postovit *et al.*, 2001). The spiral arteries then remain small in diameter and in turn become high resistance blood vessels that are unable to meet the increasing demand for blood supply to the placenta (Khong *et al.*, 1986; Redman and Sargent, 2009). In addition, the arteries may also be obstructed due to the formation of atherosclerotic plaques, which further decrease the blood flow (Redman and Sargent, 2003). The reduced blood flow to the placenta can cause hypoxia, oxidative stress and syncytiotrophoblast shedding into the maternal circulation (de Jager *et al.*, 2003; Redman and Sargent, 2009).

A response to low oxygen levels is an increase in the production of haemoglobin, which is produced in excess by the pre-eclamptic placenta. As a result free haemoglobin is released into the placental blood vessels and the breakdown of the excess amounts of this molecule results in the release of heme which damages cell membranes and oxidises lipids, in turn damaging the endothelium (Centlow *et al.*, 2008). A further source of oxygen free radicals are activated leukocytes, which are also increased in pre-eclampsia (Dekker and Sibai, 1999). In normal pregnancy, lipid peroxides and oxygen free radicals do increase but there is a concomitant increase in anti-oxidant molecules which counteract the effect of the peroxides, but this is lacking in pre-eclampsia (Dekker and Sibai, 1999). The lipid peroxides increase the production of thromboxane A₂ and cell adhesion molecules in the utero-placental vasculature (Wang and Walsh, 2001), which results in vasoconstriction and platelet adhesion (Jauniaux *et al.*, 2006).

Reactive oxygen species can propagate pro-inflammatory signals (Redman and Sargent, 2003). The inflammatory response is increased in normal pregnancy, particularly with the activation of monocytes and neutrophils but this inflammatory response is exaggerated in pre-eclampsia (Dekker and Sibai, 1999; Redman and Sargent, 2009). Pro-inflammatory cytokines like tumour necrosis factor-alpha (TNF- α) and complement protein are increased in this condition (Dekker and Sibai, 1999; Redman and Sargent, 2003). Furthermore there is an increase in activated platelets and intravascular coagulation especially when complicated with the HELLP (haemolysis, elevated liver enzymes and low platelets) syndrome (Redman and Sargent, 2003). The cytokine, transforming growth factor beta (TGF- β), is also elevated and may damage the endothelium (Tremellen *et al.*, 1998; Naicker *et al.*, 2002). TGF- β may also lead to an increased production of interleukin-2 (IL-2) which could induce trophoblast cell death (Postovit *et al.*, 2001). Furthermore, IL-2 is a macrophage chemo-attractant (Bellardelli, 1995). An increase in the number of macrophages in cross sections of vessels from pre-eclamptic placental tissue has been observed (Reister *et al.*, 1999). Thus increased production of IL-2 at the fetal-maternal interface during the first trimester of a pre-eclamptic pregnancy attracts and activates macrophages, which may then result in an increased production of NO which in turn induces trophoblast cell death thereby preventing adequate remodelling of the spiral arteries (Postovit *et al.*, 2001).

Human leukocyte antigen-G (HLA-G) may provide immune tolerance between maternal and fetal tissue but levels of this antigen in pre-eclampsia appear to be contradictory. Some studies have reported that levels of this antigen are decreased in pre-eclampsia, resulting in low trophoblast invasion and vascular abnormalities (O'Brien *et al.*, 2001; Le Bouteiller

and Tabiasco, 2006). However, other studies report normal levels of HLA-G in pre-eclampsia (Humphrey *et al.*, 1995; Aldrich *et al.*, 2000; Bermingham *et al.*, 2000). Thus other possible antigens may also play a role.

Other evidence that links immunological factors to the development of pre-eclampsia is indicated by the lower incidence of the condition in nulliparous women who have previously received blood transfusions (Feeney *et al.*, 1977). Chronic maternal infections like periodontal disease and cytomegalovirus related villitis are associated with a higher risk of developing pre-eclampsia (Moore *et al.*, 2004).

In pre-eclamptic patients, there appears to be increased fibrin deposition and reduced fibrinolytic activity which may contribute to the narrowing of blood vessels and elevation of blood pressure (Ducray *et al.*, 2011). Furthermore, arteriolar vasospasm, especially of the uterus and the kidney directly elevates the blood pressure (Symonds, 1980).

In this condition there is an increased sensitivity to angiotensin II, which may possibly be a consequence of low levels of vasodilators (Abdalla *et al.*, 2001; Symonds, 1980). These substances include the vasodilators prostaglandin E and 6-keto prostaglandin F, which may play a role in elevating the blood pressure, as these are present in low concentrations in pre-eclampsia (Moodley *et al.*, 1984). Low levels of these prostaglandins result in vasoconstriction and thus an elevation of the blood pressure (Symonds, 1980).

In contrast, increased levels of vasoconstrictors such as endothelin, have also been postulated to play a role in the pathophysiology of pre-eclampsia. Endothelin may contribute to the vasospasm that is characteristic of the condition (Khedun *et al.*, 2002).

Naiker *et al.* (2001) reported increased endothelin production by the fetal membrane of women with pre-eclampsia. This increased production could compromise blood flow to the developing fetus resulting in growth restriction that is characteristic of infants born to mothers with pre-eclampsia (Naiker *et al.*, 2001).

1.2 STRUCTURE OF THE PLACENTA

The placenta is composed of both fetal and maternal tissue. The fetal component is derived from the chorion and is referred to as the chorionic plate, whilst the maternal component is derived from the endometrium (Gude *et al.*, 2004). In the mature placenta this maternal aspect is referred to as the basal plate (Gude *et al.*, 2004). In the early placenta, extravillous trophoblast cells form a cytotrophoblastic shell which later disintegrates and becomes partially invaded by the underlying decidual cells. This structure later becomes the basal plate which is thus derived from a combination of both maternal and fetal tissue (Boyd and Hamilton, 1970).

In between the chorionic and basal plates lies the intervillous space, which contains finger-like projections of the chorion, called chorionic villi, which are the areas of maternal-fetal exchange (Figure 1.2). The villi are connected by cell columns to the basal plate (Benirschke *et al.*, 2006). Circulating maternal blood enters the inter-villous space through the spiral arteries, bathes the villi and drains back into the maternal circulation via the endometrial veins (Gude *et al.*, 2004). The villi can be subdivided into different types according to their structure and position in the villous tree. The following types of villi are present in the human placenta: stem, immature intermediate, mature intermediate and terminal villi (Figure 1.2). The stem villi have a fibrous stroma with arteries and veins.

Collagen fibres, fibroblasts and macrophages may be present in the stroma. Large stem villi connect the villous tree to the chorionic plate. Branches of these large stem villi extend into the periphery of the villous tree. The stem villi provide mechanical support to the villous tree (Benirschke *et al.*, 2006).

Stem villi branch to form immature intermediate villi, which branch further to form the mature intermediate villi. The immature intermediate villi are the growth points of the villous tree and mature intermediate villi sprout off these areas. These villi are the sites of exchange in the first two trimesters of pregnancy (Challier *et al.*, 2001). The terminal villi are the final branches of the mature intermediate villi. Capillaries and sinusoids which are the main areas of feto-maternal exchange are present in the terminal villi. In the normal third trimester placenta, the terminal villi occupy over half the surface area of the villous tree (Challier *et al.*, 2001).

The outer portion of all villi is made up of a multinucleated layer called the syncytiotrophoblast (Figure 1.2; Wang and Schneider, 1987). At term the presence of microvilli on the syncytiotrophoblastic surface multiplies the total villous surface area of about 12 square metres by a factor of 7.67, thus ensuring an enormous materno-fetal interface (Teasdale and Jean-Jacques, 1986). Beneath the syncytiotrophoblast are mononucleate cytotrophoblast cells, which are present in about 20% of villous surfaces. In third trimester placentae, the trophoblast can be degenerated and replaced by perivillous fibrinoid (Benirschke *et al.*, 2006).

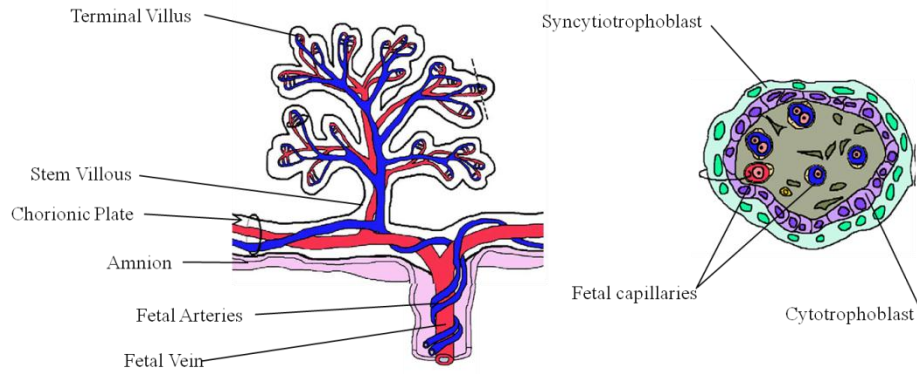


Figure 1.2 Schematic representation of the chorionic villi of the placenta (Adapted from Gude *et al.*, 2004)

1.3 LEPTIN

Leptin is a protein hormone produced primarily by adipocytes and is thought to play a role in the aetiology of pre-eclampsia (Zhang *et al.*, 1997; Laivuori *et al.*, 2006).

1.3.1 Structure Of Leptin

Leptin is a 167 amino acid, 16kDa protein, made up of four polypeptide chains which are connected by two long cross-over links and one short loop as shown in Figure 1.3 which represents the structure of human leptin (Zhang *et al.*, 1997).



Figure 1.3 The structure of human leptin. Four polypeptide chains (A, B, C, and D) make up the protein leptin. These polypeptide chains are connected by two long crossover links (AB and CD) and one short loop (BC; adapted from Zhang *et al.*, 1997).

Leptin is released into the blood as a 146 amino acid molecule (Zhang *et al.*, 1994). The amino terminal is referred to as the secretory signal terminus and is essential for the receptor binding activity of leptin (Imagawa *et al.*, 1998). In circulation, the molecule has a single disulphide bond. This gives it a C-terminal region with a loop structure (Figure 1.4). This loop is important for stability and solubility of the molecule as it enhances the function of the N-terminal region (Imagawa *et al.*, 1998). One should take cognisance that these data are derived from experiments on mice and may therefore not be directly extrapolated to the human.



Figure 1.4 Schematic illustration of the C-terminal loop structure of mouse leptin (Adapted from Imagawa *et al.*, 1998).

1.3.2 Leptin Gene

In humans the leptin gene is located on chromosome 7 (Margetic *et al.*, 2002). Its nucleotide sequence, 5'-TACGTACCCATCCAAAAAGTCCAA-3', produces the wild type leptin (Imagawa *et al.*, 1998).

1.3.3 Source Of Leptin

The main source of leptin is white adipose tissue, with subcutaneous adipose tissue representing a larger source than visceral adipose tissue (Margetic *et al.*, 2002). The size of the adipocyte also affects the amount of leptin that is produced, with bigger cells producing more leptin (Margetic *et al.*, 2002). Circulating leptin levels are thus equivalent to the amount of body fat (Margetic *et al.*, 2002). Other sources of leptin are brown adipose tissue, the placenta, mammary glands, testes, ovaries, endometrium, stomach and

hypothalamus (Haynes *et al.*, 1997). Food intake, corticosteroids and insulin levels directly increase leptin secretion, whilst cold temperature and catecholamines decrease its secretion (Haynes *et al.*, 1997; Trayhurn *et al.*, 1998).

The secretion of leptin into the blood stream is pulsatile at intervals of 30 minutes, with a nocturnal rise (Mantzoros *et al.*, 2001; Balthasar *et al.*, 2004). Leptin circulates in the free form but may also be bound to leptin binding proteins (Margetic *et al.*, 2002; Bluher and Mantzoros, 2007). The free form of leptin is rapidly removed from the plasma, its half life equal to 3.4 minutes whilst the bound form is retained for a longer time, with a half life of 71 minutes. The plasma concentration of leptin in normal adults averages at 10 ng/ml (Margetic *et al.*, 2002).

1.3.4 Leptin Receptor

The leptin receptor is coded for by the diabetes gene (*db*) (Tartaglia, 1997). Six isoforms of the receptor have been identified and these have been termed *Ob-Ra*, *Ob-Rb*, *Ob-Rc*, *Ob-Rd*, *Ob-Re* and *Ob-Rf* respectively (Lee *et al.*, 1996). All the leptin receptor isoforms, except *Ob-Re*, are cell membrane spanning receptors with a 34 amino acid trans-membrane region. *Ob-Re* is the soluble form of the receptor and it is the leptin binding site in plasma (Otero *et al.*, 2005). The other isoforms have an identical extra-cellular N-terminal of over 800 amino acids. The intracellular C-terminals of these isoforms differ. *Ob-Ra*, *Ob-Rc*, *Ob-Rd* and *Ob-Rf* are short isoforms with an intracellular component of 30-40 amino acids. *Ob-Rb* is the long isoform with an intracellular component of 302 amino acids (Otero *et al.*, 2005; Harvey, 2007).

The long form of the leptin receptor (*Ob-Rb*) is present in the hypothalamus, endothelial cells, beta cells of the pancreas, ovary, haematopoietic bone marrow precursors, monocytes/macrophages, T and B lymphocytes and the placenta. *Ob-Rb* is the functional form of the leptin receptor (Friedman and Halaas, 1998; Sanchez-Margalet and Martin-Romero, 2001; Margetic *et al.*, 2002; La Cava and Matarese, 2004; Otero *et al.*, 2005; Harvey, 2007). It is not yet fully understood why so many short forms of the receptor exist. In humans, two short functional leptin receptor isoforms (*Ob-Ra* and *Ob-Rc*) play a role in the translocation of leptin into tissues. They may also be important in the degradation and clearance of leptin in the liver and kidneys (Margetic *et al.*, 2002; La Cava and Matarese, 2004; Otero *et al.*, 2005; Bernotiene *et al.*, 2006). The short form of the leptin receptor is responsible for the modulation of cell proliferation and cell differentiation in adipose tissue, pancreas, stomach, liver, kidneys, arteries, and immune cells (Djiane and Attig, 2008).

1.3.4.1 Leptin Receptor Signalling

The leptin receptor is a class 1 cytokine receptor, which activates target cells via cytoplasmic kinases which cause phosphorylation of a cascade of proteins, eventually leading to transcription of genes within the target cell (Margetic *et al.*, 2002). When leptin binds to the extracellular portion of the receptor, the intracellular portion undergoes a conformational change. This activates janus tyrosine kinases (JAK) particularly JAK2, which leads to the phosphorylation of tyrosine residues located within the intracellular portion of the receptor. Signal transducers and activators of transcription (STAT) are then activated, dissociated from the receptor and translocated to the nucleus (Figure 1.5). STATs bind to DNA to activate transcription of target genes resulting in the functional effect of leptin (Friedman and Halaas, 1998; Sanchez-Margalet and Martin-Romero, 2001;

Balthasar *et al.*, 2004; Matarese *et al.*, 2005; Harvey, 2007). The above appears to be the main signalling pathway and it occurs in the hypothalamus, which is a target organ for leptin. Cascade pathways other than STATs may also be involved in leptin receptor signaling (Sanchez-Margalet and Martin-Romero, 2001).

In addition to causing the transcription of certain functional genes, other genes that function as part of the negative feedback loop are also transcribed. Transcription of the suppressor-of-cytokine-signalling-3 (SOCS3) gene and cytokine-inducible sequence inhibit the JAK-STAT pathway, thereby switching off cytokine signal transduction (Bjørnbæk *et al.*, 1998; Friedman and Halaas, 1998; Margetic *et al.*, 2002(a); Matarese *et al.*, 2005; Bernotiene *et al.*, 2006; Figure 1.5).

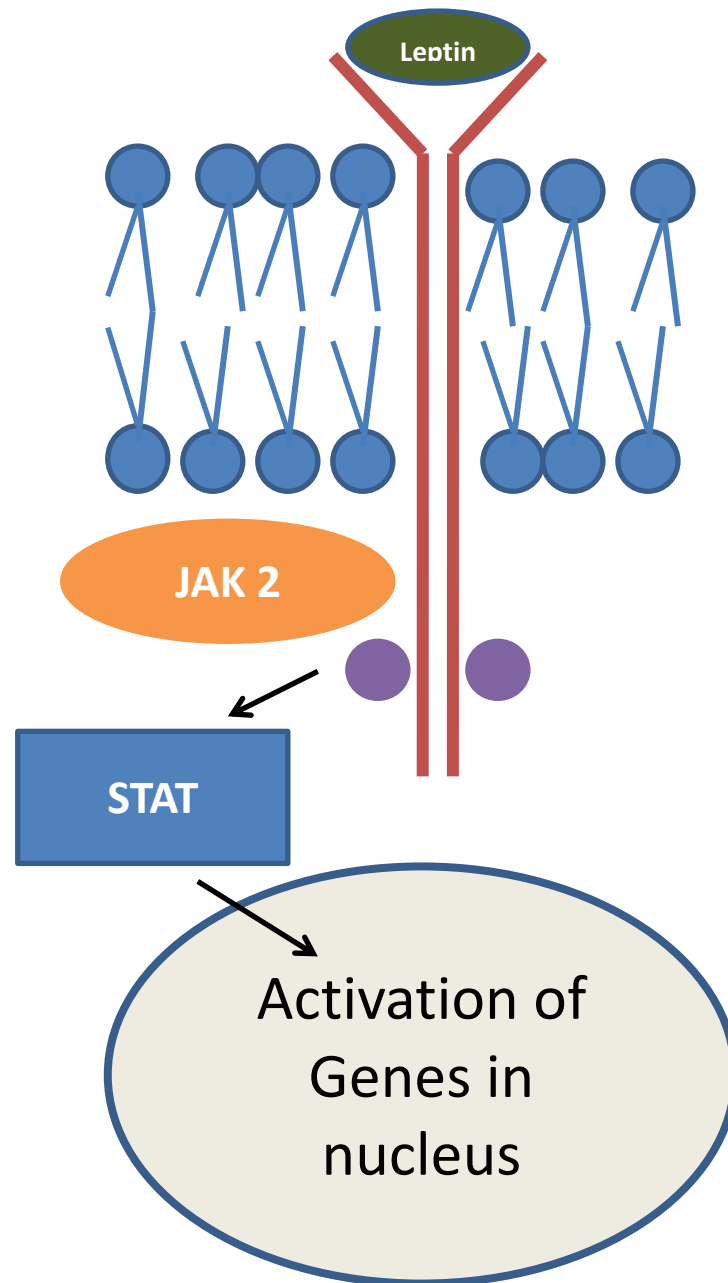


Figure 1.5 Pathway illustrating leptin binding to its receptor and subsequent activation of the JAK-STAT pathway. STATs bind to DNA to activate transcription of target genes resulting in the functional effect of leptin.

JAK2 = janus tyrosine kinases, STAT3 = signal transducer & -activation of transcription, SOCS3 = suppressor of cytokine signaling (Adapted from Balthasar *et al.*, 2004).

1.3.5 Functions Of Leptin

Leptin is involved in the control of body weight and also affects reproductive, endocrine and immune functions.

1.3.5.1 Control Of Body Weight

Leptin is involved in the regulation of body weight. Since it is primarily produced by adipose tissue, its levels are directly proportional to the amount of body fat and hence body mass index (BMI; Margetic *et al.*, 2002; Bluher and Mantzoros, 2007). Body weight may be controlled by a feedback loop which controls food intake and energy expenditure (Friedman and Halaas, 1998; Farooqi *et al.*, 2002). Leptin reduces plasma low density lipoprotein (LDL) cholesterol and triglycerides and increases plasma high density lipoprotein (HDL) cholesterol.

Leptin exerts its effect by acting on neurons in the arcuate nucleus of the hypothalamus (Figure 1.6). The arcuate nucleus contains two sets of neurons that express leptin receptors. Both sets of neurons have opposing effects on food intake and energy metabolism (Schwartz *et al.*, 2000). High leptin levels stimulate neurons that secrete proopiomelanocortin (POMC) which then stimulates the synthesis of melanocyte stimulating hormone (MSH), which is a satiety signalling protein (Niswender and Schwartz, 2003). MSH suppresses the appetite and increases energy expenditure by heat production (Guzik *et al.*, 2007). It thus mobilises fuels and inhibits energy storage (Guzik *et al.*, 2007). On the other hand, low leptin levels stimulate neurons that express neuropeptide Y (NPY) and agouti related protein (AgRP) which in turn stimulates food intake (Niswender and Schwartz, 2003). The NPY release stimulates energy storage, while AgRP inhibits the production of MSH and therefore stimulates an increased intake of food (Niswender and Schwartz, 2003; Guzik *et al.*, 2007). In addition, increasing leptin levels stimulate the sympathetic nervous system to send signals to increase energy expenditure (Margetic *et al.*, 2002).

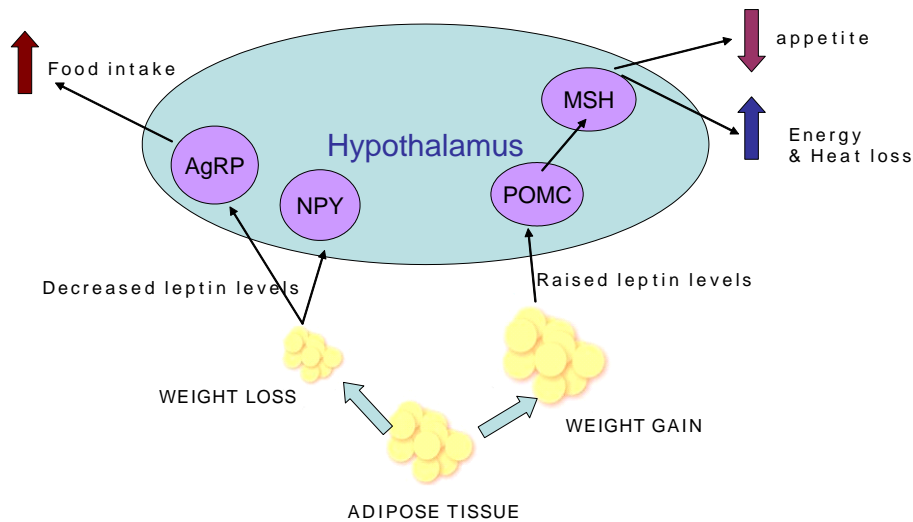


Figure 1.6 Leptin exerts its effects on feeding via neurons in the hypothalamus. Raised leptin levels stimulate the release of POMC and in turn MSH which decreases appetite and increases energy loss whilst decreased leptin levels stimulate the release of NPY and AgRP which increase food intake.

Exposure to cold temperatures and fasting inhibits leptin production. This is mediated by sympathetic nerves which transmit increasing nerve impulses via β_3 adrenergic receptors to adipose tissue in order to decrease leptin production (Trayhurn *et al.*, 1998; Margetic *et al.*, 2002). The body's response to low temperature prevents the mobilisation of fats for use by muscle and promotes its use for temperature regulation (Margetic *et al.*, 2002).

Obesity is associated with high levels of circulating leptin, without a suppressive effect on food intake or an increase in energy expenditure (Haynes *et al.*, 1997). There appears to be resistance to leptin in the hypothalamic receptors (Brabant *et al.*, 2005; Bluher and Mantzoros, 2007; Djiane and Attig, 2008) and therefore high leptin levels fail to decrease food intake and energy expenditure (Margetic *et al.*, 2002). Leptin is normally transported into the brain via the cerebrospinal fluid (CSF), where the short form of the receptor *Ob-Ra* mediates its transport. Defects in *Ob-Ra* result in less leptin entering the brain, resulting in obesity (Harvey, 2007).

Leptin also plays a role in thyrotropin releasing hormone (TRH) synthesis in the hypothalamus and therefore affects the synthesis of thyroid stimulating hormone (TSH) and thyroid hormones. Leptin deficiency has been associated with decreased TSH secretion (Mantzoros *et al.*, 2001). This is beneficial as it leads to a drop in the basal metabolic rate so that less energy is used in times of starvation (Flier, 1998).

1.3.5.2 Inter-Relationship Between Leptin And Insulin

The function of insulin is to decrease blood glucose concentration. It stimulates glucose, free fatty acid and amino acid uptake into body tissues. It then promotes the build up of glycogen, fats and protein in the tissues. Insulin release occurs from the pancreas when blood glucose levels are high (Margetic *et al.*, 2002). Although insulin levels vary depending on food intake, fasting and 24 hour integrated insulin levels are indicative of the body's fat stores (Niswender and Schwartz, 2003). Leptin is released when body fat stores are high. Since both hormones are involved in energy homeostasis, there is a link between the function of the two (Margetic *et al.*, 2002). Both insulin and leptin exert their effects via the same neurons of the hypothalamus (Niswender and Schwartz, 2003). Leptin is therefore able to modify the action of insulin. In the liver and muscle, leptin enhances glucose uptake and glycogen synthesis. It may also decrease insulin secretion in pancreatic β cells (Niswender and Schwartz, 2003).

Hyperinsulinaemia increases plasma leptin concentrations. Whilst normal concentrations of leptin do not influence the release of insulin by the pancreas, high concentrations of leptin act on insulin receptors in the β cells of the pancreas, causing an increase in the secretion of insulin (Margetic *et al.*, 2002).

High levels of leptin are associated with insulin resistance by acting on the insulin receptors to impair insulin mediated glucose uptake, glycogen synthesis, lipogenesis and protein synthesis. Glycogen synthesis in muscle is also inhibited by high leptin levels. In muscle tissue, increased leptin levels stimulate fatty acid oxidation. Hence, in obese people who have a greater proportion of fat mass, leptin inhibits glucose transport into adipose tissue whilst allowing fatty acid oxidation in muscle. Although insulin increases fatty acid uptake by muscle, it inhibits their oxidation. This action of insulin is suppressed by leptin. Thus while insulin is anabolic and tends to increase fat storage, leptin appears to mobilise fatty acids for use (Margetic *et al.*, 2002).

Leptin may be responsible for insulin resistance in type 2 diabetes (Gulturk *et al.*, 2008). Leptin has also been implicated in the development of type 1 diabetes which is an autoimmune disease that results from inflammatory destruction of insulin producing pancreatic β cells (Matarese *et al.*, 2002b).

Reduced serum leptin levels activate the hypothalamic-pituitary-adrenal (HPA) axis. The activation of this axis results in the production of the glucocorticoid, corticosterone which in turn promotes the release of glucose from the liver and muscles into the blood. This would keep the blood glucose concentration within normal limits in times of starvation (Flier, 1998).

1.3.5.3 Leptin In Immunity

Leptin enhances immunity by modulating the function of many of the body's immune cells, such as macrophages, neutrophils, natural killer cells and lymphocytes (La Cava and Matarese, 2004).

Phagocytosis of macrophages is enhanced by leptin. It stimulates the release of cyclooxygenase 2 (COX2), leukotriene B4, nitric oxide and the pro-inflammatory cytokines, tumour necrosis factor alpha (TNF- α), interleukin 1(IL-1) and interleukin 6 (IL-6) from monocytes and macrophages (La Cava and Matarese, 2004; Matarese *et al.*, 2005; Otero *et al.*, 2005). These all enhance inflammation by inducing fever, causing chemotaxis of neutrophils and the release of oxygen free radicals such as hydrogen peroxide and superoxide anion which then destroy the pathogen. In addition, the cytokines can cross the blood brain barrier to enter the hypothalamus and anterior pituitary gland to regulate the synthesis of adreno-corticotropin releasing hormone (ACTH; La Cava and Matarese, 2004).

ACTH causes the adrenal gland to release the glucocorticoid, corticosterone, which provides a feedback mechanism that limits the activity of the immune system by allowing just sufficient immune cell activity required to destroy the pathogen without causing autoimmunity (Turnbull and Rivier, 1999).

Leptin also increases the inflammatory response by enhancing the development and activation of natural killer (NK) cells and T helper 1 lymphocytes (Th1). The proliferation of naive T cells is enhanced with the Th1 response being stimulated but the T helper 2 lymphocyte (Th2) response which leads to antibody formation is inhibited (Figure 1.7). The release of pro-inflammatory cytokines and adhesion molecules by the Th1 cells is promoted. This leads to increased activation, migration and clustering of macrophages at the site of inflammation (La Cava and Matarese, 2004; Matarese *et al.*, 2005; Otero *et al.*, 2005; Lago *et al.*, 2007). Leptin can also protect T lymphocytes from corticosteroid induced apoptosis. Furthermore, activated T lymphocytes secrete leptin which sustains

their proliferation in an autocrine positive feedback mechanism (Bernotiene *et al.*, 2006). Leptin stimulates the production of growth hormone by blood monocytes and lymphocytes. Growth hormone maintains the survival and proliferation of these cells and also increases T lymphocyte adhesion (Dixit *et al.*, 2003; La Cava and Matarese, 2004).

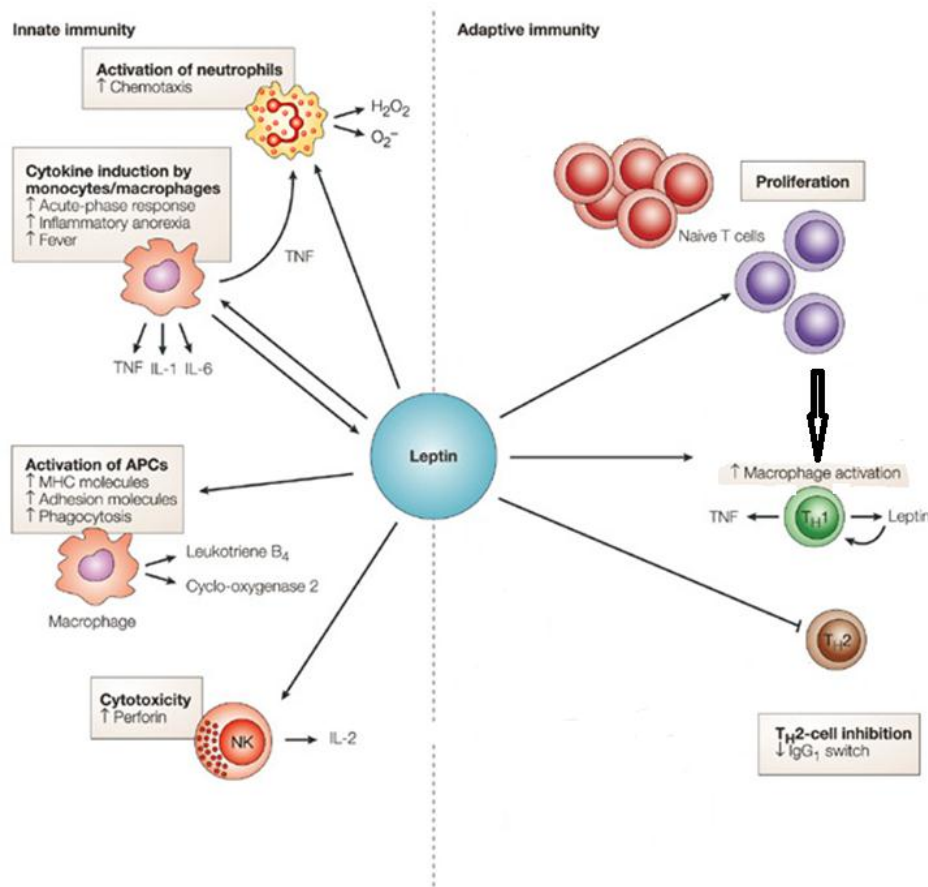


Figure 1.7 The effects of leptin in immunity. Leptin stimulates the innate immune response by enhancing the function of macrophages, neutrophils and natural killer cells. It also stimulates the development of Th1 cells but inhibits the Th2 response, decreasing antibody production. (Adapted from La Cava and Matarese, 2004)

By promoting the Th1 pro-inflammatory response, leptin may be involved in the development of inflammatory conditions like type 1 diabetes mellitus. In this condition there is an inflammatory destruction of the β cells of the pancreas and raised levels of

leptin have been observed prior to the onset of the disease (Matarese *et al.*, 2002b). Leptin may also be responsible for the development of inflammatory conditions that are prevalent in obese individuals (Otero *et al.*, 2005).

Raised leptin levels are also found in other inflammatory conditions, with the exception of newborn sepsis and tuberculosis. Other factors that lower leptin levels must therefore be involved in these conditions (Bernotiene *et al.*, 2006).

A fall in leptin concentration, as occurs in starvation, causes immuno-suppression by decreasing pro-inflammatory responses (Figure 1.8; Lord, 2002). Indeed congenital leptin deficiency has resulted in childhood infections and mortalities (Matarese *et al.*, 2005) as well as thymic atrophy (La Cava and Matarese, 2004). On the other hand, very high leptin levels, as in obesity, may lead to leptin resistance with consequent immune dysregulation (Figure 1.8; Matarese *et al.*, 2005)

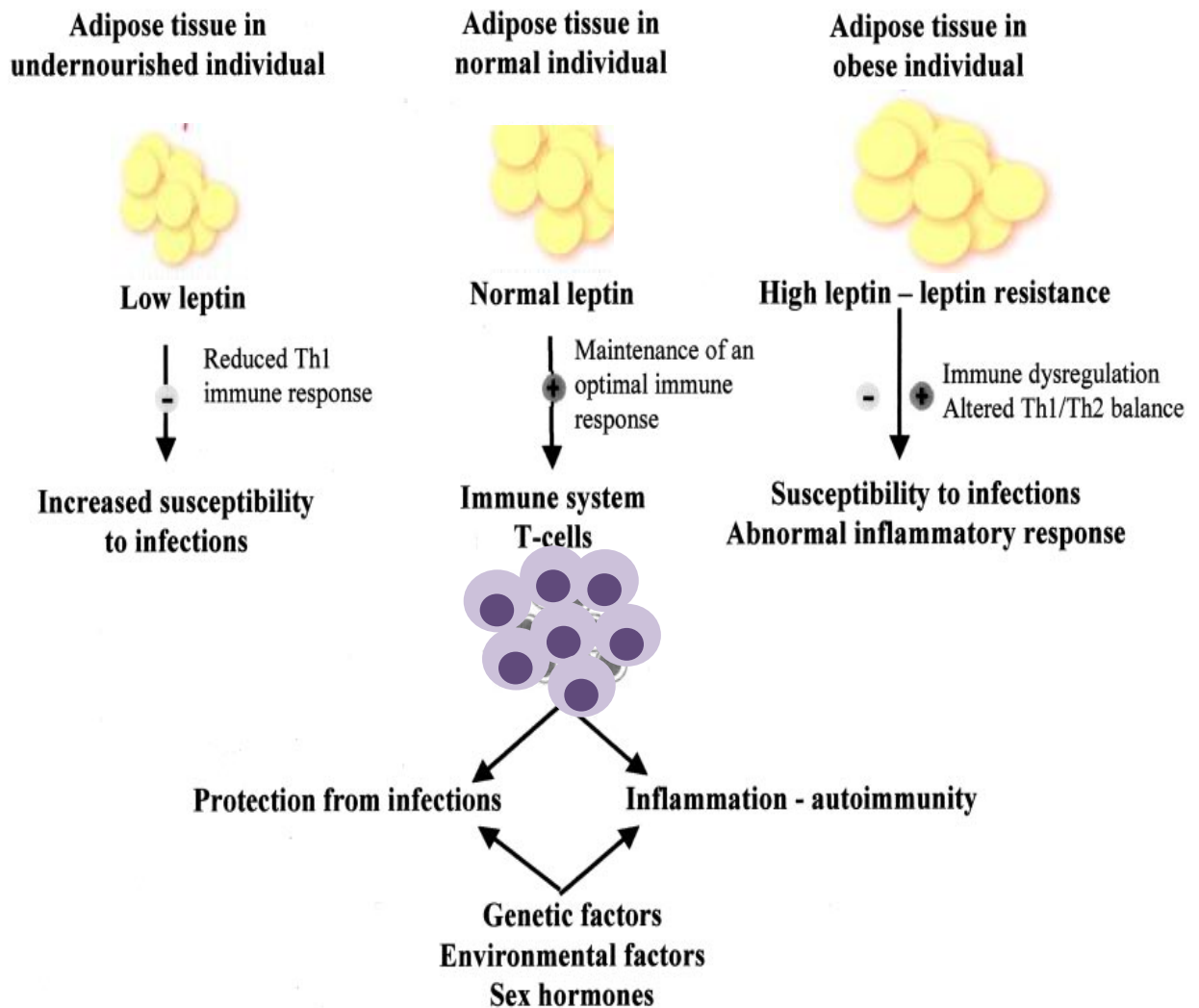


Figure 1.8 The effect of body weight on leptin in immunity. In normal individuals (middle), the presence of leptin sustains an optimal immune response. Loss of weight (left) causes a reduction in serum leptin and consequent impairment of the pro-inflammatory response. Obesity leads to leptin resistance and immune dysregulation (Adapted from Matarese *et al.*, 2005).

1.3.5.4 Effects Of Leptin On The Cardiovascular System

In the circulatory system, leptin is involved in stimulation of endothelial cell growth and angiogenesis. This improves the lipid release from fat stores, improves heat dissipation from adipose tissue and assists in wound repair (Sierra-Honigmann *et al.*, 1998; Margetic *et al.*, 2002). Leptin also stimulates blood cell formation (Umemoto *et al.*, 1997; Gainsford and Alexander, 1999) by proliferation and differentiation of haemopoietic precursors (Margetic *et al.*, 2002).

It appears to stimulate sympathetic nerve activity to the kidney, resulting in renal excretion of sodium, which in turn could lead to the lowering of blood pressure (Haynes *et al.*, 1997). Leptin also causes vasorelaxation of large blood vessels thus lowering the blood pressure (Guzik *et al.*, 2007). Leptin resistance may therefore contribute to renal sodium retention and constriction of blood vessels in turn contributing to hypertension in obesity (Haynes *et al.*, 1997).

1.3.5.5 Leptin In Reproduction

Leptin plays a role in reproduction and high levels stimulate the release of gonadotropin releasing hormone (GnRH) from the hypothalamus and the release of luteinising hormone (LH) and follicle stimulating hormone (FSH) from the pituitary gland (Farooqi *et al.*, 2002; Bluher and Mantzoros, 2007). By stimulating the release of these hormones, leptin affects the development of puberty. As a result, menarche occurs at an earlier age in girls with a greater body weight and hence higher leptin levels compared to girls with a lower body weight. Decreased leptin in the plasma suppresses the hypothalamic-gonadal axis in order to prevent pregnancy as insufficient energy stores would not be suitable for fetal development and may also compromise the health of the mother. Low leptin levels in anorexia nervosa therefore result in hypothalamic amenorrhea (Bluher and Mantzoros, 2007).

In addition to stimulating the hypothalamic–gonadal axis, leptin also stimulates the enlargement of the uterus and ovaries with associated follicular development. Furthermore, leptin induced puberty is accompanied by the growth spurt as well as behavioural changes that occur at puberty (Farooqi *et al.*, 2002).

On the other hand, obesity results in disruption of the hypothalamic-gonadal axis. Increasing obesity is accompanied by a high number of anovulatory cycles due to ineffective follicular development. This is due to hyperleptinemia in the obese being accompanied by leptin resistance. Obesity may also be associated with hypogonadism (Bluher and Mantzoros, 2007).

Leptin levels are higher in females than in males, both during childhood and adolescence. In males, leptin levels rise prior to puberty but the increasing testosterone levels post puberty causes a decline in leptin levels so that in adult males there is an inverse relationship between testosterone and leptin levels (Bluher and Mantzoros, 2007). However, reduced plasma leptin results in the suppression of this axis in males, but the reason for this is as yet unknown (Flier, 1998).

1.3.6 Leptin In Pregnancy

The primary function of the placenta is nutrient and gas exchange between mother and baby. It also has an endocrine function and secretes hormones that have a functional role in the maintenance of pregnancy and in fetal development (Malik *et al.*, 2005). One of these hormones is leptin (Mise *et al.*, 1998; Malik *et al.*, 2005; Haugen *et al.*, 2006). Placental leptin is identical to adipose tissue leptin (Ashworth *et al.*, 2000).

Placental leptin is expressed by the syncytiotrophoblast that has contact with maternal blood and by the vascular endothelial cells that have direct contact with fetal blood. Leptin may therefore be released into both the maternal and fetal circulation (Schubring *et al.*, 1997; Lea *et al.*, 2000). Release of placental leptin into the maternal circulation increases serum leptin by 2-4 times the level before pregnancy (Masuzaki *et al.*, 1997), with levels

peaking in the second trimester (Kafulafula and Moodley, 2001) and returning to pre-pregnancy levels within the first few days post partum (Laivuori *et al.*, 2000). It has been suggested that leptin may cause fat mobilisation (Hoggard *et al.*, 2001). However, the increase in leptin during late pregnancy when maternal nutrient requirements are increased also suggests maternal leptin resistance (Hoggard *et al.*, 2001). The binding of leptin to its soluble receptor may protect leptin from degradation and excretion giving rise to a peak in maternal leptin levels. This also prevents its binding to the signalling form of the receptor, thereby giving rise to leptin resistance (Hoggard *et al.*, 2001). Early studies indicated that maternal weight at delivery does not correlate with maternal serum leptin (Schubring *et al.*, 1997). More recent studies however indicate that maternal weight and BMI do correlate with maternal leptin levels (Kafulafula and Moodley, 2001; Ozkan *et al.*, 2005; Woelfer *et al.*, 2005). Furthermore Kafulafula and Moodley (2001) show that maternal leptin levels also correlate with waist and mid arm circumference measurements during pregnancy.

The leptin receptor is present at the maternal side of the placenta, indicating a possible autocrine role for leptin (Lea *et al.*, 2000). It promotes the invasion of cytotrophoblasts, stimulates placental growth and angiogenesis and regulates immune function at the maternal-fetal interface (Hoggard *et al.*, 2001; Mise *et al.*, 2007).

Leptin is also found in amniotic fluid and umbilical cord blood. It can enter the amniotic fluid from the placenta or the fetus. There is an inverse relationship of amniotic fluid leptin levels with the size of both the fetus and the placenta (Woelfer *et al.*, 2005). However, leptin levels in umbilical cord blood correlate with birth weight (Schubring *et al.*, 1997). In the umbilical cord, leptin in the arterial blood is present in higher levels than that of venous blood, suggesting a larger amount of leptin synthesis by the fetus as compared to the

placenta (Schubring *et al.*, 1997). Leptin is produced by the fetal heart, bone, cartilage and hair follicles (Trayhurn *et al.*, 1998) and it promotes growth during fetal development (Hoggard *et al.*, 2001; Souren *et al.*, 2008).

Leptin stimulates the release of growth hormone and also directly stimulates cell proliferation. Its growth promoting effects are thus both central as well as peripheral (Djiane and Attig, 2008). Placental leptin production is decreased in pregnancies with small for gestational age neonates and a growth retarded twin has lower placental and umbilical cord blood leptin than its normal size twin (Lea *et al.*, 2000; Xiaoming *et al.*, 2001). Placental leptin production is increased in pregnancies with large for gestational age neonates (Xiaoming *et al.*, 2001). Higher placental leptin levels are also seen in diabetic pregnancies. Since poorly controlled maternal diabetes is associated with macrosomia, the high leptin levels may lead to the increased neonatal size (Lepercq *et al.*, 1998 ; Lea *et al.*, 2000).

A smaller dysfunctional placenta is present in intra-uterine growth restriction (IUGR; Shekhawat *et al.*, 1998). In pregnancies complicated with IUGR, leptin levels are increased in the maternal and umbilical cord blood (Mise *et al.*, 2007; Kyriakakou *et al.*, 2008). Similarly in other obstetric complications associated with IUGR, such as multiple births and pre-eclampsia, placental and maternal leptin levels have been shown to be raised (Hytinanti *et al.*, 2000; Laivuori *et al.*, 2006). It has been postulated that maternal insulin resistance may be a possible cause for the raised maternal leptin levels in these conditions (Shekhawat *et al.*, 1998).

1.3.7 Leptin In Pre-Eclampsia

In pre-eclampsia, maternal serum leptin levels are higher than those of normal pregnancies with levels rising further with increasing severity of the condition (Mise *et al.*, 1998; Ouyang and Chen, 2007). In pre-eclampsia, leptin levels decrease post delivery but this decrease is less significant than after a normotensive pregnancy (Laivuori *et al.*, 2000).

Plausible interrelationships between leptin and pre-eclampsia include:

- A major clinical manifestation of pre-eclampsia is hypertension with arteriolar vasoconstriction. This causes a reduction in uteroplacental blood flow, leading to placental hypoxia, as well as fetal growth retardation. Leptin secretion is increased in cell cultures with low oxygen levels. Increased placental leptin in pre-eclamptic pregnant women thus possibly reflect placental hypoperfusion and hypoxia (Mise *et al.*, 1998; Mise *et al.*, 2007).
- Increased leptin levels signal high energy stores and inhibit the secretion of the hypothalamic protein, neuropeptide Y (NPY) (Guzik *et al.*, 2007). In addition to stimulating food intake, NPY is also involved in vasorelaxation. Raised placental leptin thus indirectly causes blood vessel constriction (Li *et al.*, 2007).
- Leptin increases noradrenalin output to brown adipose tissue. This increased sympathetic outflow also increases maternal blood pressure (Ozkan *et al.*, 2005).
- Leptin promotes arteriosclerosis, which could lead to the development of pre-eclampsia (Ouyang and Chen, 2007).
- High placental leptin levels are associated with abnormal trophoblast proliferation which is a hallmark of pre-eclampsia (Masuzaki *et al.*, 1997).
- Leptin promotes the release of cytokines such as TNF- α and IL-6 (La Cava and Matarese, 2004). Elevated levels of these cytokines cause endothelial dysfunction

(Dekker and Sibai, 1998; Baker and Kingdom, 2004) and could thus promote the development of pre-eclampsia.

On the other hand, it has been postulated that reduced renal clearance rather than increased leptin production may account for raised leptin levels (Ozkan *et al.*, 2005). There is a positive correlation between serum leptin and proteinuria in pre-eclampsia. Impaired renal function, a pathophysiological consequence of pre-eclampsia and subsequent reduced renal clearance may be the reason for high leptin levels in women with this condition (Ozkan *et al.*, 2005).

1.3.8 Leptin In HIV Patients

Human immunodeficiency virus (HIV) infected individuals have less body fat than healthy individuals because of a reduction in subcutaneous adipose tissue. Weight and BMI are thus lower in these individuals (Matarese *et al.*, 2002a; Kosmiski, 2008). Low leptin levels are features of reduced body mass and are associated with impaired immune function. This may exacerbate the immunodeficiency in AIDS patients (Matarese *et al.*, 2002a).

1.4 THE HUMAN IMMUNODEFICIENCY VIRUS (HIV) AND ACQUIRED IMMUNE DEFICIENCY SYNDROME (AIDS)

1.4.1 HIV Biology

The human immunodeficiency virus (HIV) is a retrovirus with a genome encoded by ribonucleic acid (RNA; Roth, 1989; Turner and Summers, 1999). It has an outer lipid bilayer envelope, with a 120 kDa glycoprotein, referred to as glycoprotein 120 (gp120),

which is essential for the interaction of the virus with a receptor of the host cell (Turner and Summers, 1999). The virus attaches to the host cell membrane when gp120 binds with the CD4 receptor on the membrane of a host cell, which is usually the T helper lymphocyte (Figure 1.9; Jameson *et al.*, 1988). HIV then enters the host cells by endocytosis (Roth, 1989; Turner and Summers, 1999).

Following membrane fusion, the viral coat is removed and the viral RNA is reverse-transcribed to viral DNA in the cytoplasm of the host cell, by the viral reverse transcriptase enzyme. Once synthesized, the viral DNA is transported to the nucleus of the infected cell and becomes covalently integrated into the host genome. Messenger RNA is transcribed and transported out of the nucleus for translation of viral proteins in the host cytoplasm. Thousands of copies of the viral proteins are made and assembled into provirus particles inside the cell. The virus then leaves the cell by exocytosis taking along virus glycoprotein molecules that were embedded in the cell membrane of the host cell which now becomes the outer coat of the virus (Figure 1.9; Turner and Summers, 1999).

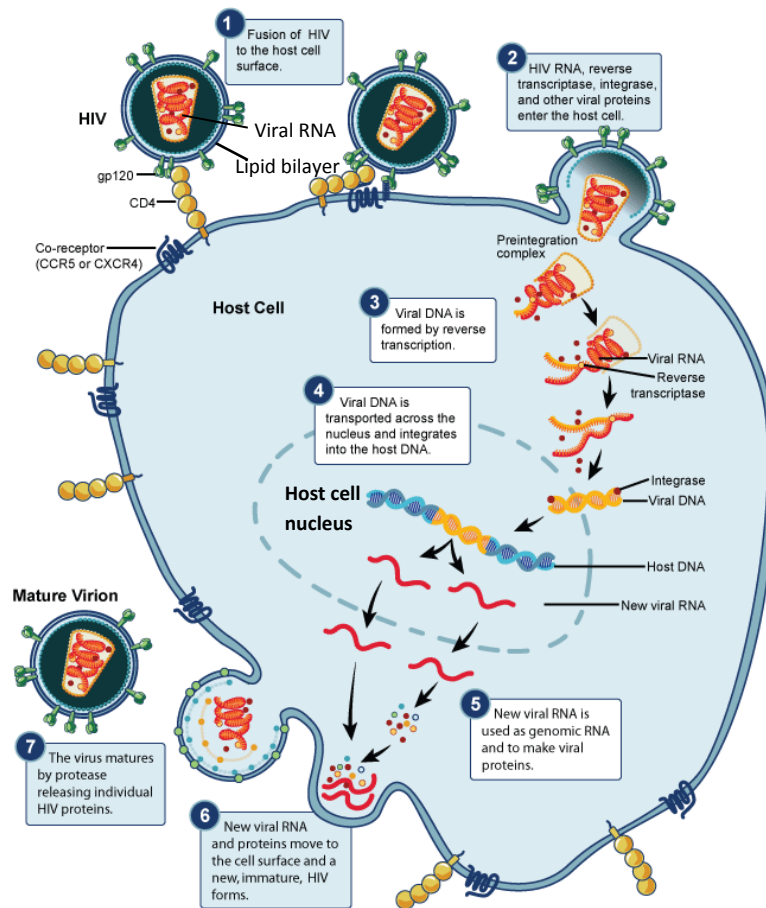


Figure 1.9 HIV interaction with host cell. The virus molecule is surrounded by a lipid bilayer with gp120, which interacts with the CD4 receptor of the host cell which it then enters. Viral RNA integrates with host DNA, eventually forming more viral proteins. These are exocytosed out of the host cell to form new HIV molecules (Adapted from NIAID (2010)).

Based on genetic similarities, the numerous virus strains are classified into subtypes, HIV-1 and HIV-2. Worldwide, the predominant virus is HIV-1. HIV-2 is endemic in West Africa. Outside this region, HIV-2 infection is sporadic and has been reported in Portugal, France and India. In areas where HIV-2 is found, mixed infections of both types of the virus may occur (Esteves *et al.*, 2000). HIV mutates very readily resulting in many different strains of the virus.

Viral proteins bind to host cell surface CD4 receptors and form a coat around them. This results in endocytosis of the CD4 receptor which is subsequently degraded by the cell's endosomes. The decrease of CD4 molecules on the surface of infected cells serves as a

means of avoiding an immune response. Rapid growth of the virus in CD4 T lymphocytes/CD4 T cells eventually kills these cells. This destruction occurs only in those CD4 T lymphocytes that are actively involved in immune reactions (Turner and Summers, 1999). HIV infected CD4 lymphocytes can fuse with each other forming giant multinucleated cells. These cells produce a large amount of virus and then die after a few years (Roth, 1989). The falling CD4 cell count is an indicator of a failing immune system and disease progression (Ibrahim *et al.*, 2004) and people with CD4 cell counts below 200 cells/ μ l are at highest risk for severe HIV related morbidity (Miller *et al.*, 1999). In addition, the progression to AIDS occurs when the CD4 T lymphocytes are below 200 cells/ μ l of blood (Miller *et al.*, 1999).

1.4.2 Weight Loss in HIV/AIDS

Weight loss of 10% of body weight is one of the first signs of acquired immune deficiency syndrome (AIDS; Wilson *et al.*, 2002) and patients with a greater weight loss tend to have more co-morbidities (Siddiqui *et al.*, 2009).

Lipodystrophy syndrome, which is characterised by a selective loss and redistribution of body fat, is common in HIV infection. HIV lipodystrophy may be grouped as follows:

- a. Lipoatrophy - patients with generalised fat depletion
- b. Lipohypertrophy – patients with predominant fat accumulation
- c. Mixed lipodystrophy patients with fat distribution around the abdomen and breasts and the development of a buffalo hump. The increased fat distribution in these areas is accompanied by a loss of fat in the limbs (Martinez and Gatell, 1999; Martinez *et al.*, 2001).

Patients with lipoatrophy have the lowest leptin levels and this is due to due to fat loss, with decreased synthesis and release of leptin from adipocytes (Nagy *et al.*, 2003). Patients with lipohypertrophy have the highest leptin levels due to either an over production of leptin or leptin resistance. This is also accompanied by insulin resistance (Nagy *et al.*, 2003). Fat loss in the extremities of HIV-1 patients is associated with low leptin levels, increased visceral fat and metabolic abnormalities (Estrada *et al.*, 2002).

1.4.3 HIV in Pregnancy

During a normal pregnancy, immunological adaptations occur in order to maintain the pregnancy (Burns *et al.*, 1996). There is an alteration of local immune response at the fetoplacental level with a decline in cell mediated immunity and an increase in antibody-mediated immunity. There is a decrease in CD4 T cells during normal pregnancy (Wegmann *et al.*, 1993). These changes may be of significance in HIV infected pregnant women because they are already immuno-compromised (de Groot *et al.*, 2003). In an HIV infection, depletion of the CD4 T cells result in immune dysfunction, in which both cellular and humoral immune components are decreased (Ibrahim *et al.*, 2004).

Since an HIV infection is an immunosuppressive condition, it increases morbidity and mortality during pregnancy as well as adverse perinatal outcomes which include spontaneous abortion, stillbirth, neonatal and infant mortality (Brocklehurst and French, 1998; de Groot *et al.*, 2003). Furthermore, there is also an increased risk of low birth weight (< 2.5 kg), preterm delivery, preterm rupture of membranes, antepartum and post partum haemorrhage (Braddick *et al.*, 1990; Sukwa *et al.*, 1996; Brocklehurst and French, 1998; Leroy *et al.*, 1998; Stratton *et al.*, 1999; de Groot *et al.*, 2003; Bodkin *et al.*, 2006; Gray and McIntyre, 2008).

In HIV pregnancies, factors associated with low birth weight are CD4 T cell counts < 200 and a higher incidence of prematurity which may be due to chorio-amnionitis, syphilis, anaemia, a maternal BMI less than 20 kg/m² (Leroy *et al.*, 1998; Stratton *et al.*, 1999; Traisathit *et al.*, 2009).

Adequate nutrition and the provision of highly active antiretroviral therapy during pregnancy for the prevention of perinatal transmission for immunocompromised women may reduce the risk of premature delivery (Traisathit *et al.*, 2009).

Infants born to HIV positive mothers are more commonly affected by diarrhoea and pneumonia which contribute to a three-fold increase in the infant mortality rate (Taha *et al.*, 1995). Maternal sexually transmitted diseases are also associated with higher infant mortalities, possibly through damage of the feto-placental unit with consequent prematurity (Stratton *et al.*, 1999). Infant mortality is also due to transmission of HIV to the neonate. Furthermore, there is a 2.2 fold increase of TB in neonates and infants born of HIV positive mothers (Kruger and Bhagwanjee, 2003).

Adverse effects of HIV, on the mother herself include an increased risk of post partum haemorrhage (Leroy *et al.*, 1998). Haemoglobin levels are lower in HIV positive women (Sukwa *et al.*, 1996). Anaemia in pregnancy results in a reduced ability to handle post partum haemorrhage and could contribute to the increasing number of deaths due to post partum haemorrhage. Increased anaemia is also found in pregnancy induced hypertension in HIV positive women (Bodkin *et al.*, 2006). In addition, there is more abnormal vaginal discharge in HIV positive women (Bodkin *et al.*, 2006).

1.4.4 Maternal Mortality

Maternal death is defined as the death of a woman during pregnancy or within 42 days of termination of pregnancy, from any cause related to or aggravated by the pregnancy (Webb *et al.*, 2006). It is estimated that the maternal mortality ratio for South Africa is about 150/100 000 live births (Saving mother's Report: 2008-2010, 2012). The major cause of the maternal deaths is AIDS, which accounts for 27.9% of these deaths. Complications of hypertension are the third leading cause of maternal deaths in South Africa and account for 14% of maternal deaths (Saving mother's Report: 2008-2010, 2012). Of these complications, pre-eclampsia is the most common (Moodley, 2007). Other factors that contribute to the maternal mortality are nutritional status, socio-economic factors, poor rural health care services and poor access to secondary health care (Kruger and Bhagwanjee, 2003).

In South Africa, the maternal mortality rates are lower than in most other African countries. However the rate has been rising in recent years (Kruger and Bhagwanjee, 2003) and this is mainly due to the high incidence of HIV/AIDS (McIntyre, 2003). Obstetric causes of maternal morbidity and mortality are more severe in women infected with HIV as they are more susceptible to infections and post surgical complications (McIntyre, 2003).

Post partum haemorrhage is more common in HIV infected women and may be more serious if associated with pre-existing anaemia. The most common post partum morbidity events are fever without infection, haemorrhage or severe anaemia, endometritis, urinary tract infections, and caesarean wound complications. Post caesarean section complications

are higher in the severely immunosuppressed. The stillbirth rate has also doubled and HIV infection is known to be associated with stillbirth (Kruger and Bhagwanjee, 2003).

1.4.5 Antiretroviral Treatment

There has been a decrease in AIDS related mortality in resource rich settings and this has been attributed to the availability of antiretroviral treatment (McIntyre, 2003). The management of HIV in pregnancy involves triple therapy with antiretroviral (ARV) or highly active antiretroviral therapy (HAART) (Wilson *et al.*, 2002). HAART is a combination therapy that includes nucleoside reverse transcriptase inhibitors and protease inhibitors (Martinez *et al.*, 2001; Spollett, 2006; Li *et al.*, 2009). This therapy decreases the viral load, slows HIV replication, increases the CD4 T lymphocyte/cell count and also reduces the incidence of opportunistic infections (Spollett, 2006). The combination therapy that is used in public sector hospitals in South Africa includes the drugs Stavudine, Lamivudine and Nevirapine. In addition, Nevirapine is administered at the onset of labour for the prevention of maternal to child transfer (PMTCT) of HIV (Bodkin *et al.*, 2006).

Short term therapy with protease inhibitors is associated with an increase in body weight and improvement in nutritional status of HIV infected patients (Martinez and Gatell, 1999). Whilst the long term use of HAART has resulted in the suppression of HIV replication, it is also associated with the development of metabolic syndrome characterised by insulin resistance, hyperlipidemia and body fat redistribution or lipodystrophy (Carr *et al.*, 1999; Tsiodras and Mantzoros, 2006). Lipodystrophy, which is characterised by central obesity together with subcutaneous lipoatrophy, has resulted in an increase in circulating triglycerides and cholesterol (Martinez *et al.*, 2001). This increases the risk of

cardiovascular disease, increases blood lactic acid levels and decreases bone mineral density (Carr *et al.*, 1999; Tsiodras and Mantzoros, 2006; Samaras *et al.*, 2007).

Despite the availability of effective antiretroviral therapy, weight loss remains a problem among patients with HIV, 10% of who have HIV associated weight loss (Siddiqui *et al.*, 2009). Patients with HIV associated weight loss are generally older (Siddiqui *et al.*, 2009).

1.4.6 HIV and Pre-eclampsia

The data on the incidence of pre-eclampsia in HIV infected women is conflicting.

A normal pregnancy is a pro-inflammatory state and pre-eclampsia may be an exaggeration of this immune response. On the other hand, there is immune suppression in an HIV infection which is characterised by loss of CD4 lymphocyte activity which could result in a low incidence of pre-eclampsia in untreated HIV infections (Wimalasundera *et al.*, 2002). In contrast, another study found no reduction in risk of developing preeclampsia amongst untreated HIV positive women (Frank *et al.*, 2004).

The incidence of pre-eclampsia in HIV infected pregnant women on HAART is higher than in women who were untreated or on mono or dual therapy and is also higher than in women who are not infected with the HIV virus (Wimalasundera *et al.*, 2002; de Groot *et al.*, 2003; McIntyre, 2003; Coll *et al.*, 2004; Suy *et al.*, 2006; Conde-Agudelo *et al.*, 2008). HAART results in immune reconstitution and could thus re-instate the pathological processes that result in pre-eclampsia (Wimalasundera *et al.*, 2002). Suy *et al.* (2006) propose that the increased incidence of pre-eclampsia in HIV infected women on HAART

is due to endothelial inflammation as well as insulin resistance since insulin levels are raised in pre-eclampsia.

On the other hand, another study detected lower incidence of pre-eclampsia in HIV women on antiretroviral treatment (Mattar *et al.*, 2004). These authors suggest that anti-retroviral therapy restores different immunological parameters to those that play a relevant role in the development of pre-eclampsia.

1.4.7 Maternal To Child Transmission of HIV

Each year about 700 000 infants become HIV positive from their mothers (Ilf *et al.*, 2005). Vertical transmission of HIV from mother to child can occur across the placenta or via breast milk (John and Kreiss, 1996). HIV-1 has been detected in vaginal, cervical, amniotic fluid and breast milk samples (John and Kreiss, 1996). The risk of transmission is related to maternal health, obstetric factors and infant prematurity. A more severe infection, indicated by higher viral load and lower CD4 lymphocyte counts, is associated with an increased risk of viral transmission (Lyll *et al.*, 2001).

In non breastfeeding infants most vertical transmission occurs late in pregnancy or during parturition with an increased risk if the delivery is prior to 34 weeks of gestation (John and Kreiss, 1996). Vertical transmission is 50% lower when the mother undergoes a Caesarian section before the onset of labour or rupture of membranes (Lyll *et al.*, 2001). Infant exposure to the virus is increased with placentitis, ascending genital tract infections during the peripartum period, use of instruments at delivery and vaginal delivery (John and Kreiss, 1996).

Transfusion of the mother's blood to the fetus during labour contractions, infection after rupture of membranes and direct contact of the fetus with infected secretions or blood from the maternal genital tract may result in the transfer of the virus (John and Kreiss, 1996). Chorio-amnionitis, prolonged rupture of membranes and premature birth are all associated with mother to child transmission of HIV since infants born prematurely have an immature immune system, and prematurity is associated with increased transmission of HIV-1 (John and Kreiss, 1996).

Vitamin deficiency is associated with an increase in vertical transmission from a mother to child as vitamin A is important in the maintenance of mucosal integrity, it could affect either maternal mucosal viral shedding or infant mucosal susceptibility to infection and thus influence maternal transmission (John and Kreiss, 1996). Antiretroviral therapy decreases vertical transmission by decreasing the maternal viral load and prophylactic protection of the infant post natally (John and Kreiss, 1996).

About 40% of the mother to child transmission is through breast milk (Iliff *et al.*, 2005). Transmission via breastfeeding is higher in the early stages of breastfeeding due an immature immune system, increased gut permeability or high antigen levels in colostrum (Dunn *et al.*, 1994).

In developed countries like Britain, where safe infant feeding alternatives are available, HIV positive women are advised to refrain from breastfeeding (Lyall *et al.*, 2001). However, the avoidance of breastfeeding is not realistic in developing countries and a large proportion of HIV positive women breastfeed their infants due to social pressure (Coutsoudis *et al.*, 2001; Becquet *et al.*, 2005).

The rate of maternal to child transfer of HIV is higher in babies that receive both breast milk and formula compared to those that are exclusively breastfed (Iliff *et al.*, 2005). Exclusive compared to mixed breast feeding is also associated with a lower incidence in diarrhoea, respiratory illness and allergy (Coutsoudis *et al.*, 2001). Mixed feeding may be associated with less maternal antibodies from breast milk as a result of consumption of less breast milk. Additionally, contaminated fluids and food in mixed breastfed babies may damage the bowel, impair mucosal integrity and facilitate the entry of the HIV virus into the infant tissues from the breast milk (Coutsoudis *et al.*, 2001).

1.5 JUSTIFICATION FOR THE STUDY

Sub-Saharan Africa has 24.7 million people that are HIV infected. South Africa is a low to middle income country with a population of 50 million people of which 9-10 million reside in the province of KwaZulu-Natal. This province is regarded as the epicentre of the HIV pandemic with a prevalence of 39.5%. In South Africans, this HIV prevalence is highest between the ages of 25 and 29, with more than 50% of infections occurring female (UNAIDS, 2012).

HIV and hypertensive disorders in pregnancy in particular pre-eclampsia are common conditions in South Africa, where 14.0 % of maternal deaths are due to hypertensive disorders, of which pre-eclampsia is the most common (Saving mother's Report: 2008-2010, 2012). There is conflicting evidence that infection with HIV may influence the incidence of pre-eclampsia negatively or positively (Wimalasundera *et al.*, 2002; Frank *et al.*, 2004; Mattar *et al.*, 2004). Pre-eclampsia is a pro-inflammatory condition whilst an

HIV infection is anti-inflammatory as a result of depressed immunity. Since leptin is pro-inflammatory, the levels of this hormone may be increased in HIV negative pre-eclampsia. However it is unknown how the interaction between HIV infection and pre-eclampsia will affect the levels of this hormone. It is also unknown how leptin would affect the pathophysiology of pre-eclampsia that is accompanied by an HIV infection.

1.6 AIM OF STUDY

1.6.1 Primary Aims and Objectives

- Compare serum and placental leptin levels in normotensive and pre-eclamptic pregnant women.
- Compare serum and placental leptin levels in HIV positive and negative pregnant women.
- Compare serum and placental leptin in the subgroups of HIV positive patients.

1.6.2 Secondary Aims and Objectives

- Compare serum insulin levels in normotensive and pre-eclamptic pregnant women
- Compare serum insulin levels in HIV positive and negative pregnant women.
- Compare serum insulin in the subgroups of HIV positive patients.

1.6.3 Tertiary Aims and Objectives

- Compare birth weights and APGAR scores of neonates from normotensive and pre-eclamptic pregnancies.

- Compare birth weights and APGAR scores of neonates born to HIV positive and negative pregnant women.
- Compare birth outcomes like neonatal weights, APGAR scores, gestational age, neonatal mortalities and morbidities in the subgroups of HIV positive patients.
- Compare the transmission of the Human Immunodeficiency Virus between normotensive and pre-eclamptic HIV positive pregnant women by examination of CD4 T cell counts of mother and baby at birth.

CHAPTER 2

MATERIALS AND METHODS

2.1 STUDY SITE, ETHICS APPROVAL AND DISCLOSURE

This prospective study was conducted at the Optics & Imaging Centre, Doris Duke Medical Research Institute, University of KwaZulu-Natal (PG 005/08; Appendix I) and Prince Mshiyeni Memorial Hospital, Umlazi, Durban, South Africa. Ethical clearance for the study was obtained from the Biomedical Research Ethics Committee, University of KwaZulu-Natal (BF 155/08; Appendix II) and the South African Department of Health (HRKM076/09; Appendix III). Permission to collect samples at the Obstetric Unit, Prince Mshiyeni Memorial Hospital was obtained from the hospital manager (Appendix IV). Written informed consent was obtained from all participants (Appendices V and VI).

2.2 STUDY POPULATION

Participants were recruited in their antenatal period by a qualified research nurse, employed for the purposes of this study, at the Obstetric Unit of the Prince Mshiyeni Memorial Hospital between the period September 2009 and December 2010. All participants were managed by standard Department of Health protocol. Participants (n = 180) that were recruited were divided into 2 study groups viz., pre-eclamptic (n = 90) or normotensive (n = 90) based on specific inclusion and exclusion criteria.

2.2.1 Inclusion Criteria

Pre-eclampsia was defined as a systolic blood pressure of at least 140 mmHg and a diastolic pressure of at least 90 mmHg with proteinuria \geq 1+ on a urine dipstick (Cnossen *et al.*, 2006; Magnussen *et al.*, 2007). Participants were categorized as pre-eclamptic (n =

90) or normotensive (n = 90) with a further categorization according to HIV status and CD4 T cell count where participants with a CD4 T cell count greater than 200 formed one group and those with CD4 T cell counts less than 200 formed another group. The study groups are graphically depicted in Figure 2.1.

The normotensive pregnant HIV negative group served as the overall control group and the HIV positive normotensive pregnant groups (CD4 > 200 and CD4 < 200) served as controls for the HIV positive pre-eclamptic groups (CD4 > 200 and CD4 < 200). All participants were over the age of 18 years.

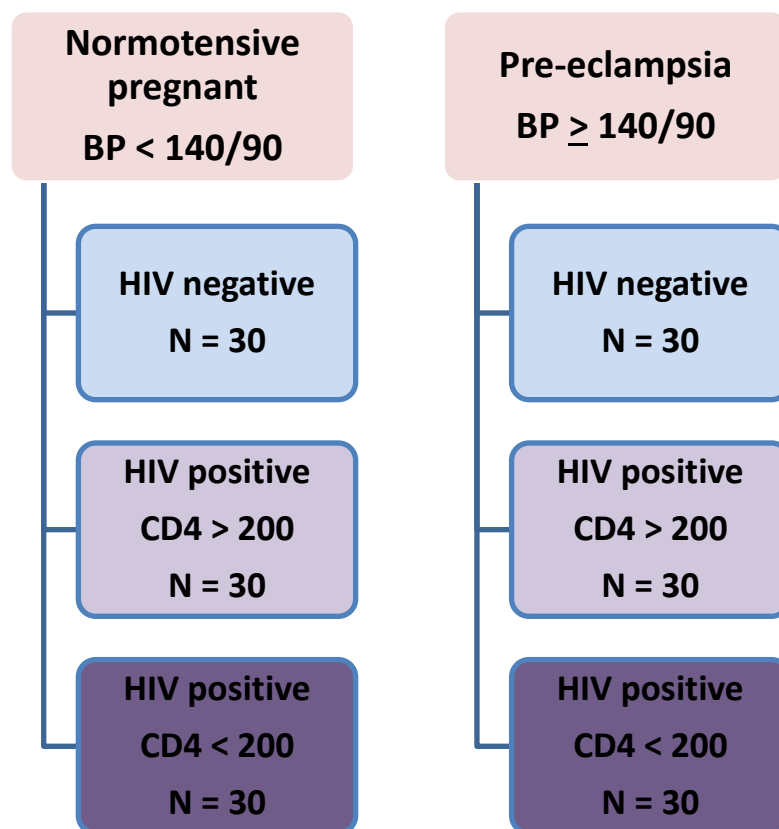


Figure 2.1 Schematic Representation of Study Groups

2.2.2 Exclusion Criteria

Women with the following disorders were excluded from the study: chronic hypertension, diabetes mellitus, gestational diabetes, epilepsy, chronic renal disease, connective tissue disease, heart failure, abruptio-placentae, intra-uterine death, chorio-amnionitis, systemic lupus erythematosus, sickle cell disease, anti-phospholipid antibody syndrome, thyroid disease, active asthma requiring medication during pregnancy and treatment with aspirin, warfarin, non-steroidal anti-inflammatory drugs, antibiotics, lipid lowering or anti-hypertensive drugs. None of the participants were smokers.

2.3 SAMPLE COLLECTION

2.3.1 Participant Demographics

The following participant demographics were collated into a pre-designed data sheet (Appendix VII) by the research nurse during the ante-natal period: age, blood pressure, gestational age, maternal weight, skin-fold thickness, mid-arm circumference, maternal height, neonatal weight, APGAR scores and maternal HIV status.

The Body Mass Index (BMI) was calculated by dividing the pregnant body weight in kg by the height in cm squared ie:

$$\text{BMI} = \frac{\text{weight}}{\text{height}^2}$$

Obesity was defined as a BMI of at least 30 kg/m² (Kafulafula and Moodley, 2001).

The mid arm circumference was taken with a measuring tape at the midpoint between the olecranon and the acromion process of the right arm. The triceps skin-fold thickness was measured with the participant standing with arms loosely hanging at her sides. The subcutaneous tissue and skin of the midpoint of the posterior part of the right arm was measured with a skin-fold caliper. The mid-arm muscle circumference was calculated by using the following formula:

Mid-arm muscle circumference = mid-arm circumference – (3.14 X triceps skin fold thickness)
(Huang *et al.*, 2001).

2.3.2 Blood Collection

Systemic venous blood was collected ante-natally in plain tubes, centrifuged at 3 500 rpm for 10 minutes (Heraeus, Megafuge 1.0R, ThermoScientific, Waltham, U.S.A.) and the serum stored at -80°C until required for ELISA. The HIV tests and CD4 T cell counts were conducted as standard of care practice.

2.3.3 Placental Tissue

Placental tissue was obtained post-partum. A segment from the central and peripheral region of each placenta was carefully excised with care taken to avoid macroscopic infarction and major blood vessels. Each sample was divided into two and then immersed into

- a) RNA *later* solution (Catalogue number AM7020; AEC Amersham, Sandton, South Africa), refrigerated at 4°C for 24 hours and then stored at -80°C until required for analysis of gene expression.
- b) 10% formaldehyde solution and stored for histological processing and assessment.

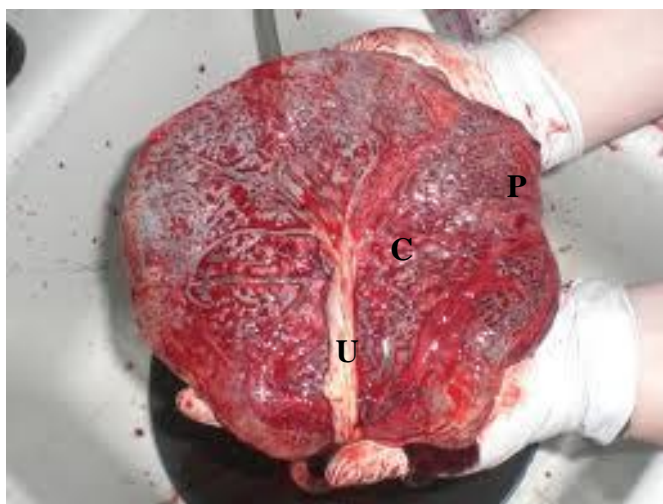


Figure 2.2 Human placenta showing the central (C) and peripheral (P) regions of the placenta as well as the umbilical cord (U)

2.4 DETERMINATION OF SERUM LEPTIN LEVELS BY ELISA

2.4.1 Principle of the Serum Leptin Detection

Serum samples were diluted 100 fold and leptin levels were determined using a commercially available Quantikine Human Leptin enzyme-linked immunosorbent assay (ELISA) kit (catalogue number: DLP00; R&D Systems, Minneapolis, USA). The assay utilized a quantitative sandwich enzyme immunoassay technique in which monoclonal antibody specific for leptin has been precoated onto a microplate. Leptin present in standards and samples are then bound by the immobilised antibody. After washing away unbound substances, an enzyme-linked monoclonal antibody specific for leptin was added to the wells. Following further washes to remove unbound antibody enzyme reagent, a substrate solution was added and colour development occurred in proportion to the amount of leptin bound in the initial step. This procedure is illustrated schematically in Figure 2.3. The technique had a sensitivity limit of 7.8 pg/ml.

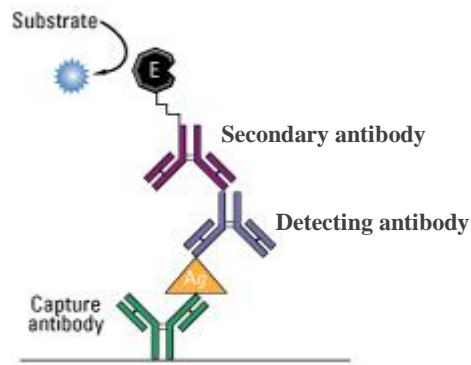







Figure 2.3 A schematic diagram illustrating the principle of a sandwich ELISA (1) Plate is coated with a capture antibody ; (2) sample is added, and any antigen  present binds to capture antibody; (3) detecting antibody  is added, and binds to antigen; (4) enzyme-linked secondary antibody  is added, and binds to detecting antibody; (5) substrate is added, and is converted by enzyme to detectable form  (Adapted from Thermo Scientific, 2012)

2.4.2 Procedure for Leptin ELISA

The immunoassay procedure was in accordance with the manufacturer's instructions. It was carried out in triplicate at room temperature in a 96 well microplate that was pre-coated with a monoclonal mouse leptin antibody.

For the binding of leptin in the sample or standard solution to the capture antibody, 100 μ l assay diluent containing a buffered protein base and 100 μ l sample/standard was added to each well and incubated for two hours. Following aspirations of the solutions, the wells were washed four times with a wash buffer to remove unbound substances. Thereafter a leptin antibody, conjugated to horseradish peroxidase (200 μ l), was added to each well and incubated for one hour, followed by four washes. A substrate solution (200 μ l), containing hydrogen peroxide and tetramethylbenzidine (TMB) as the chromogen, was added to each well and incubated for 30 minutes in the dark. This reaction was stopped by adding 50 μ l stop solution containing 2N sulfuric acid. Thereafter the optical density was read within 30 minutes at 450 nm on a Bio-Rad ELISA plate reader (model 3350, Bio-Rad Laboratories, Berkeley, California, USA). The assay procedure is summarised in Table 2.1.

Table 2.1 Test Procedure for determination of serum leptin levels.

STEP	PROCESS	METHOD	TIME
1	Leptin in sample bound to primary antibody on microplate well	100 µl of assay diluent + 100 µl of standard or sample	2 hours
2	Wash	Aspirate and wash	4X
3	Secondary antibody	200 µl leptin conjugate	1 hour
4	Wash	Aspirate and wash	4X
5	Colour development	200 µl substrate solution	30 min In dark
6	Ends the reaction	50 µl of stop solution	

Standard solutions with concentrations of 15.6, 31.2, 62.5, 125, 250, 500 and 1000 pg/ml were used to draw a standard curve for each ELISA run (Figure 2.4). The linear regression of the standard curve was calculated at 0.996. Sample values were determined from the standard curve and expressed in pg/ml. The intra-assay coefficient of variation was 6.5%, whilst the inter-assay coefficient of variation was 9.3%.

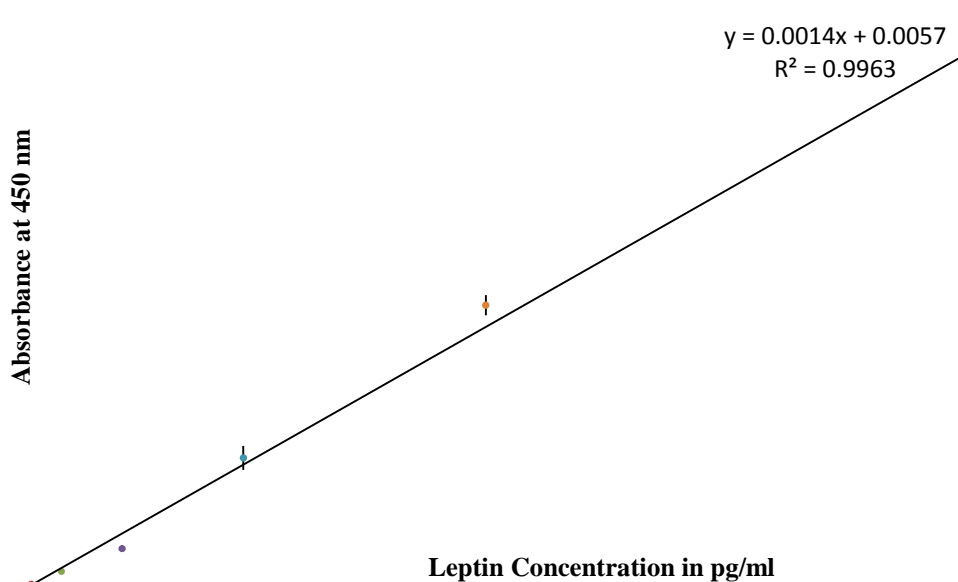


Figure 2.4 A Sample Leptin Standard curve for ELISA

2.5 DETERMINATION OF PLACENTAL LEPTIN CONCENTRATION BY ELISA

A 10 mg piece of placenta was lysed in 0.5 ml cell culture reagent (E153A; Promega, Madison, U.S.A) and subsequently centrifuged at 4 000 g for 40 minutes at 4°C on a Heraeus centrifuge (Megafuge 1.0R, ThermoScientific, Waltham, U.S.A.). The supernatant was collected and diluted 1:1 with distilled water. This diluted supernatant was used for the ELISA procedure, as for serum ELISA determination, outlined in Table 2.1.

2.6 DETERMINATION OF SERUM INSULIN

Serum insulin levels were determined using a commercially available Quantikine Human Insulin enzyme-linked immunosorbent assay (ELISA) kit (catalogue number EIA-2337; DRG International, New Jersey USA). This kit is based on a direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. In this assay, insulin in the sample reacted with anti-insulin antibodies and peroxidase-conjugated anti-insulin antibodies which were bound to the microplate. All tests were carried out in triplicate.

The procedure followed was according to manufacturer's instructions (Table 2.2) and was carried out at room temperature in a 96 well microplate. Sample or standard (25 µl) and 100 µl enzyme conjugate was added to each well on the microplate and incubated for one hour. The solutions were aspirated and washed six times with a wash buffer. Tetramethylbenzidine solution (TMB, 200 µl) was added to each well and incubated for 30 minutes in the dark. This allowed colour to develop according to the amount of insulin present in the sample or standard solution. The reaction was stopped by adding 50 µl

sulphuric acid. The optical density was read at 450 nm on a Bio-Rad ELISA plate reader (model 3350, Bio-Rad Laboratories, Berkeley, California, U.S.A.). Standard solutions provided in the kit were used to draw a standard curve (Figure. 2.5). The concentrations of the standards used were 0.15, 1.0, 3.0, 10.0 and 20.0 mU/l. The calibrator 0 solution was used as a blank.

The linear regression of the standard curve was 1.00. The sample values were determined from the standard curve. The intra-assay coefficient of variation was 0.39% whilst the inter-assay coefficient of variation was 5.0%. The assay had a detection limit of < 0.07 mU/l.

Table 2.2 Test Procedure for determination of serum insulin levels

STEP	PROCESS	METHOD	TIME
1	Insulin binding	25 µl sample or standard + 100 µl enzyme conjugate	1 hour
2	Wash	Aspirate and wash with wash buffer	6X
3	Colour development	200 µl substrate TMB	30 min in dark
4	Ends the reaction	50 µl stop solution	

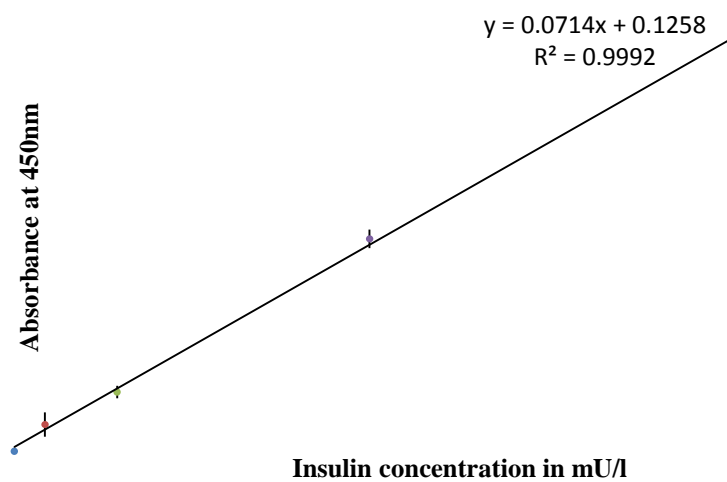


Figure 2.5 A Sample Standard Curve for Insulin ELISA

2.7 DETERMINATION PLACENTAL LEPTIN PRODUCTION

Placental leptin production was determined by real-time polymerase chain reaction (RT-PCR). One mg placental tissue from each of the central and peripheral regions of the placenta was placed in 1.5 ml of RNA-*later* solution (Catalogue number AM7020; AEC Amersham, Sandton, South Africa), refrigerated at 4°C for 24 hours to allow penetration of the RNA-*later* solution into the tissue and then stored at -80°C until required for analysis.

2.7.1 RNA Extraction

The RNeasy Mini Kit (Catalogue number 74106; Qiagen, Santa Clarita, CA, USA) was used to extract RNA.

The RNeasy technology combines the selective binding properties of a silica-gel-based membrane with the speed of microspin technology. A specialized high-salt buffer system allows up to 100 µg of RNA that is longer than 200 bases to bind to the RNeasy silica-gel membrane.

Placental tissue (30 mg) was first lysed and homogenized in the presence of 1.5 ml RLT buffer (1:9 β-Mercaptoethanol: RLT buffer), a highly denaturing guanidine isothiocyanate (GITC)-containing buffer, which immediately inactivates RNases to ensure isolation of intact RNA. Ethanol (70%; 700 µl) was added to an equivalent amount of homogenate to provide appropriate binding conditions, and 700 µl of this solution was then applied to an RNeasy mini column, where the total RNA binds to the membrane and the contaminants are efficiently washed away.

The RNeasy mini column was centrifuged for 1 minute at 13 000 rpm at the end of which the flow-through was discarded (Heraeus, Biofuge; ThermoScientific, Waltham, USA). Thereafter a wash buffer (RW1 - 700 μ l) was loaded onto the column and centrifuged for one minute at 13 000 rpm and its flow through discarded. Buffer RPE (500 μ l) was loaded onto the column and centrifuged for 1 minute at 13 000 rpm. After discarding the flow-through, a further 500 μ l of buffer RPE was added to the column, centrifuged for two minutes at 13 000 rpm and the flow-through discarded. High quality RNA was then eluted in 50 μ l of RNA-free water by centrifuging for one minute at 13 000 rpm. The eluate, which contained the RNA, was used in the subsequent steps for cDNA synthesis.

The RNA quality and quantity was determined on a spectrophotometer, NanoDrop 2000 (Thermo Scientific, Fermentas Canada Inc., Burlington, Ontario) and samples were used only if the optical density at 260 nm (OD₂₆₀/OD₂₈₀) ratio was ≥ 1.90 .

2.7.2 Removal Of Genomic DNA From Prepared RNA

The DNase I, RNase-free kit (catalogue number EN0521; Fermentas, Burlington, Ontario) was used to remove any endogenous DNA and RNase from the extracted RNA. Ten μ l of reaction mixture consisting of 1 μ g of extracted RNA, 1 μ l reaction buffer containing MgCl₂, 1 μ l DNase I and RNase-free DEPC-treated water was made. The latter mixture was incubated at 37°C for 30 minutes on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, California, USA). One μ l of 50 mM EDTA was added and incubated at 65°C for 10 minutes. The total RNA concentration was quantified on a spectrophotometer, NanoDrop 2000 and samples were used only if the optical density ratio at 260 nm / 280 nm was ≥ 1.90 . This prepared RNA was subsequently converted to cDNA.

2.7.3 cDNA Synthesis

The iScript cDNA synthesis kit (catalogue number 170-8891; BIO-RAD, Berkeley, California, USA) was used for the preparation of cDNA from the RNA.

Twenty µl of reaction mixture consisting of 1 µg of RNA, 4 µl of 5x iScript reaction mixture, 1 µl iScript reverse transcriptase in nuclease free water was made. The above mixture was incubated in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, California, USA) at 25°C for 5 minutes, 42°C for 30 minutes, 85°C for 5 minutes and 4°C for 30 minutes. The purity of the DNA was tested by reading the optical density on a spectrophotometer, NanoDrop 2000 and samples were used only if the optical density ratio at 260 nm / 280 nm was in the range of 1.80 and 1.83.

2.7.4 Real Time Polymerase Chain Reaction (RT PCR)

Leptin gene expression was determined by relative quantification which is based on the expression levels of the target gene (leptin) versus a reference gene. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene. Relative quantification determined the change in the levels of the target gene in each sample relative to the level of the reference gene of that sample. The suitability of GAPDH for use as a reference gene was previously determined for PCR efficiency in our laboratory (Singh *et al.*, 2011). The PCR primers for the genes are provided in Table 2.3.

Table 2.3 Primers used for PCR

Gene	Genebank accession number	Primer Sequence
GAPDH	NM_002046	5'-AAGGTCGGAGTCAACGGATT-3' (F) 5'-CTCCTGGAAGATGGTGATGG-3' (R)
Leptin	XM_004625	5'-CCAAAACCCTCATCAAGACAATT-3' (F) 5'-ACCGGTGACTTTCTGTTTGA-3' (R)

(F): forward primer

(R): reverse primer

Ten μ l of reaction mixture consisting of 0.25 μ l of the respective forward primer, 0.25 μ l of the respective reverse primer, 5 μ l Fast Start SYBR green I (Roche), 1 μ l cDNA and 1.5 μ l water was made. Reactions were run on a LightCycler® 480 (Roche Molecular Systems, New Jersey, USA) as indicated in Table 2.4.

Table 2.4 Reaction cycles for PCR

Process	Number of cycles	Temperature	Time
	1	95°C	10 min
denaturation	45	95°C	15s
annealing	45	60°C	15s
extension	45	72°C	15s

To confirm amplification specificity, the PCR products were subjected to a melting-curve analysis on the LightCycler® 480 (Roche Molecular Systems, New Jersey, USA) and agarose gel electrophoresis. Serial dilutions of cDNA from total RNA were performed for the GAPDH and leptin genes. Concentrations of 1, 2, 10, 20, 100, 200 and 1 000 μ l of cDNA was used to generate the standard curves for both genes (Appendix IX a and b). The PCR efficiency of GAPDH was 98.5% whilst that of leptin was 100%. The relative

concentration of the leptin gene for each sample was calculated as leptin concentration / GAPDH concentration.

2.7.5 AGAROSE GEL ELECTROPHORESIS

A 2% gel was prepared by dissolving 4 X 0.5 g agarose tablets (Bioline; catalogue number Bio-41027, London, UK) in 100 ml TBE (Tris borate EDTA buffer; Sigma catalogue number T4415-1L; Sigma, St. Louis, USA). The gel was poured into a casting tray and allowed to set for half an hour. Loading dye (2 µl) was added to 5 µl of sample. Five micro litres of the sample mixture was loaded into each well on the gel plate. The gel was run for two hours at 80 volts.

2.8 HISTOLOGICAL ANALYSIS

2.8.1 Fixation and Tissue Processing

Placental tissue, fixed in 10% buffered formaldehyde solution, was sliced and inserted into embedding cassettes prior to tissue processing.

The tissue was dehydrated via an ascending series of ethanol with isopropanol and xylene serving as the intermediate and final dehydrating mediums, infiltrated with paraffin wax and embedded in cassettes using an automated tissue processor and tissue embedding station (Sakura 5, Torrance, California, USA; Leica EG 1160 embedding station; Germany). This schedule is outlined in Table 2.5.

Table 2.5 Tissue Processing

PROCESS	CHEMICAL	TIME
Fixation	10% Buffered formalin	30 min
Dehydration	50% Formal-methanol	20 min
	95% ethanol	30 min
	100% Isopropanol	20 min
	100% Isopropanol	30 min
Clearing	Xylene	20 min
	Xylene	30 min
Vacuum infiltration	Paraffin wax	20 min
	Paraffin wax	20 min
	Paraffin wax	30 min
	Paraffin wax	30 min

2.8.2 Haematoxylin and Eosin Staining

Sections were cut (3 μm) on a Leica microtome RM2135 (Leica Biosystems, Newcastle Upon Tyne, UK). The sections were floated on a water bath (Leica, Newcastle Upon Tyne, UK) at 60°C and collected onto slides. The slides were dried on a hot plate (Sakura, California, USA) at 60°C for 10 minutes, dewaxed in xylene and rehydrated. The nuclei were stained with Mayer's haematoxylin and 0.5% eosin (BDH Laboratory Supplies) was used as a counterstain. The schedule for staining is indicated in Table 2.6.

Table 2.6 Procedure for H & E staining

PROCESS	CHEMICAL	TIME
Dewax	Xylene	2 X 5 min
Rehydrate	Isopropanol	2 X 5 min
	Methanol	3 X 5 min
	Running tap water	5 min
Nuclei stain	Haematoxylin	10 min
	Lithium carbonate	Until blue colour appeared
Counterstain	Eosin	1 min
Wash	Running tap water	5 min
Dehydrate	75% methanol	2 X 5 min
	100% isopropanol	2 X 5 min
	Xylene	3 X
Mount	Entellen	

The slides were viewed on a Zeiss Axioscope A1 light microscope (Oberkochen, Germany).

2.8.3 Immunohistochemistry

Sections were cut (3 µm) on a Leica microtome RM2135 (Leica Biosystems, Newcastle Upon Tyne, UK). The sections were floated on a water bath (Leica, Newcastle Upon Tyne, UK) at 60°C and collected onto charged slides. The slides were heat fixed on a hot plate (Sakura, California, USA) at 60°C for 10 minutes, dewaxed in xylene, rehydrated and stained as indicated in the schedule in Table 2.7. The LSAB kit (catalogue number K0679, Dako, Glostrup, Denmark) was used for the immuno-staining.

2.8.3.1 Primary Antibody

The leptin antibody *Ob* (A-20) was used: (catalogue number: sc-842; Santa Cruz Biotechnology, USA). This was an affinity purified rabbit polyclonal antibody raised against a peptide mapping at the C-terminus of leptin of human origin. Initial staining trials were performed with the following antibody dilutions: 1:200, 1:100, 1:50 and 1:25. No DAB staining was observed on the 1:200, 1:100 and 1:50 dilutions whilst very pale staining was observed on the 1:25 dilution. A further trial using an antigen retrieval solution (S169984, Dako, Glostrup, Denmark) to enhance staining showed suitable staining with the 1:25 antibody dilution.

2.8.3.2 Secondary Antibody

Biotinylated anti-rabbit, anti-mouse and anti-goat secondary antibody was supplied in the LSAB kit (LSAB+System-HRP, K0679, Dako, Glostrup, Denmark).

This antibody contained biotinylated anti-rabbit, anti-mouse and anti-goat immunoglobulins diluted in phosphate buffered saline (PBS) containing stabilizing proteins and 0.015 mol/l sodium azide as a preservative.

2.8.3.3 Antigen Retrieval and Blocking

Antigen retrieval for the antibody was performed in a commercial sodium citrate retrieval solution viz., Target Retrieval Solution (S169984, Dako, Glostrup, Denmark) for 10 min at medium high power in a microwave oven with a power consumption of 1200W (LG MS2524W). Endogenous peroxidase blocking involved incubation in 3% H₂O₂ for 5 minutes and additional protein blocking was carried out with 10% bovine serum albumin (A4503, Sigma, USA) solution in phosphate buffered saline (PBS) for 30 min.

2.8.3.4 Immunohistochemical Staining Procedure

Following blocking, the sections were washed three times in PBS: 0.1% Tween 20 followed by a single PBS wash on a flatbed rocking station (catalogue number 7813; Hamilton Robotics Inc, Reno, U.S.A.). Thereafter, slides were incubated overnight in 150 μ l leptin antibody (1:25) in 2% bovine serum albumin/PBS at 4°C.

Subsequently sections were washed three times in PBS: 0.1% Tween 20 and once in PBS. A biotinylated anti-rabbit/mouse/goat secondary antibody was incubated for 30 min at room temperature (K0679, LSAB+System-HRP, Dako Glostrup, Denmark). Following PBS washes, sections were incubated for 30 min at room temperature with Streptavidin peroxidase (K0679, LSAB+System-HRP, Dako Glostrup, Denmark). The reaction was visually detected within three minutes with diamino-benzidine (DAB) as the chromogen. Nuclei were counterstained with Mayer's haematoxylin for five minutes. Sections were subsequently dehydrated and mounted in dibutylphthalate xylene (DPX). Negative controls were performed by replacing the primary antibody with non immune sera. Buffer controls were performed by replacement of the primary antibody with PBS. Breast tissue was used as a positive control. Specimens from different clinical groups were mixed in successive staining schedules. This procedure is outlined in Appendix X.

2.8.2.5 Morphometric Image Analysis of Antibody Expression

Image analysis was performed to quantify immuno-expression of leptin from archived villi types of the placenta. This immuno-expression was evaluated with the Zeiss AxioScope A1 brightfield microscope interfaced with the AxioVision Image analysis software package version 4.8.3 (auto measurement module; Carl Zeiss, Germany). Image analysis was performed by three individuals, two of whom were blinded to the clinical groups.

Regions of interest (ROI; with/without immuno-reactivity) were randomly selected and archived at an initial magnification of X20 (Figure 2.7A). Five ROIs comprising placental exchange villi/specimen and three ROIs of placental stem villi/specimen were segmented. Leptin immuno-localisation within the villi was assessed by brown chromogen precipitation (Figure 2.7B). This immuno-reactivity was expressed as a percentage of chromogen visualized within the region of interest ($\%/ \mu\text{m}^2$). The immuno-reactivity of red blood cells was excluded whilst intravillous red blood reactivity was included in the calculations (Figure 2.7C). Intensity of immuno-reaction was expressed on a densitometric grey scale (0-255) and the average value (range) was calculated for villi type/specimen (Figure 2.7D). Statistical analysis for this quantification was performed between villi types and across all study groups.

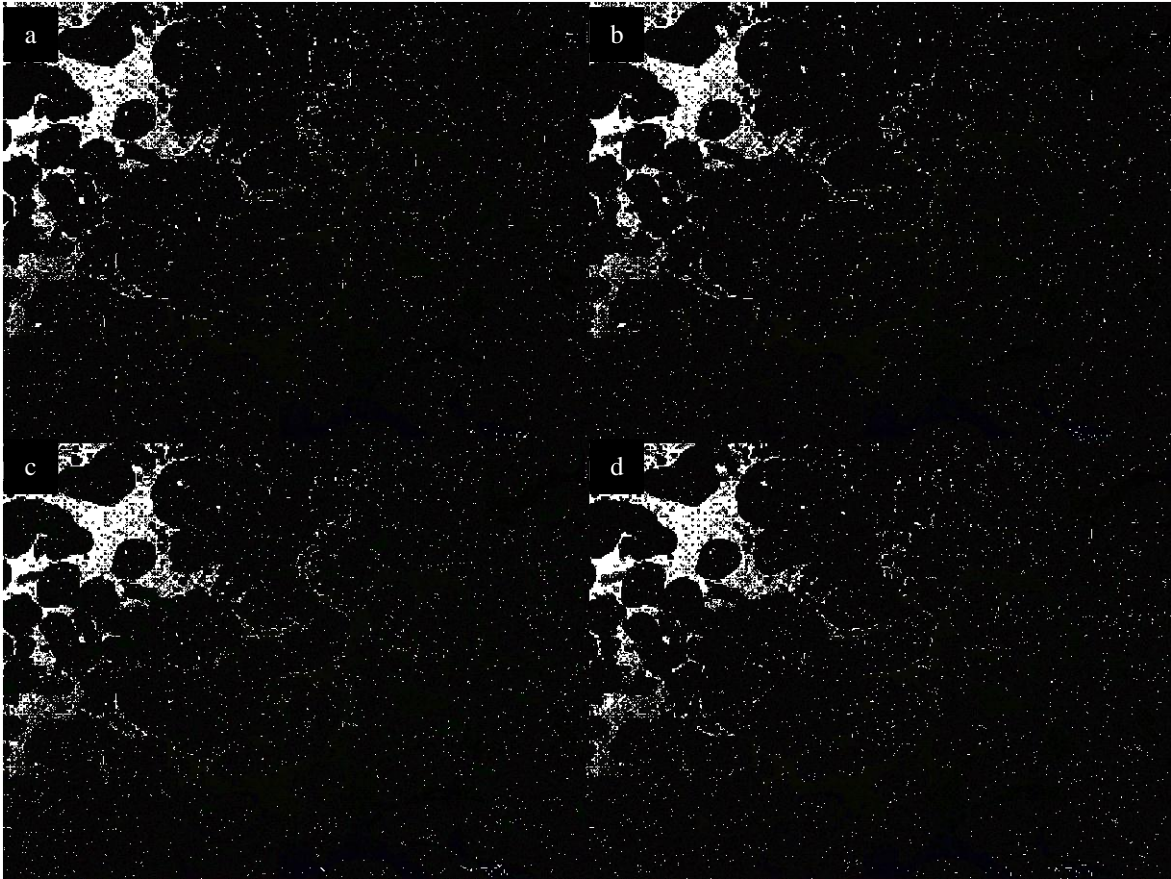


Figure 2.6 Histogram illustrating the method of assessing the percentage chromogen in the exchange villi of the placenta. (a) archived image of placental villi; (b) all immuno-positive areas were highlighted in green; (c) extravillous red blood cells were excluded; (d) different intensities of chromogen were analysed in all measured areas and the different intensities indicated in different colours in this micrograph. Initial magnification: X20

2.8 Statistical Analysis

SPSS for windows (version 20) was used for the statistical analysis. The Shapiro-Wilk test was used to assess normality of sample distribution. If the dependent variable was parametric (normally distributed), the raw data were used in the statistical analysis. When the dependent variable was non-parametric (not normally distributed), the data were transformed by either a logarithmic or square root transformation (as required) to normalised the data distribution. The transformed data were subsequently used in the analysis. Two-way ANOVA, followed by the Bonferroni post hoc test was used in the statistical analysis. When transformations were unable to correct the data distribution, the Kruskal-Wallis test, followed by Dunn's post hoc test, was used for the statistical analysis of the raw data.

The student's *t*-test was used for pair-wise comparisons of parametric data, whilst the Mann-Whitney *U* test was used for pair-wise comparisons of non-parametric data. The Fisher's exact test was used for the analysis of categorical data. Spearman's correlation test was used to analyse relationships between non-parametric data, whilst Pearson's correlation co-efficient test was used to analyse relationships between parametric data. A *p* value < 0.05 was considered statistically significant.

CHAPTER 3

RESULTS

A total of 180 Black African women were studied consisting of the following groups:

- A. Normotensive pregnant (n = 90):
 - i. Normotensive HIV positive (n = 60)
 - a. CD4 < 200 (n = 30)
 - b. CD4 > 200 (n = 30)
 - ii. Normotensive HIV negative (n = 30)

- B. Pre-eclamptic (n= 90):
 - i. Pre-eclamptic HIV positive (n = 60),
 - a. CD4 <200 (n = 30)
 - b. CD4 >200 (n = 30)
 - ii. Pre-eclamptic HIV negative (n = 30)

Fifty percent of the study population were normotensive pregnant and 50% were pre-eclamptic. In terms of HIV status within the study population (n = 180), the distribution was 66.6% HIV positive and 33.3% HIV negative. The former group was equally distributed in terms of severity of HIV infection (50% CD4 > 200 and 50% CD4 < 200).

3.1 PATIENT DEMOGRAPHICS

The clinical demographics of the study participants are shown in Table 1.

3.1.1 Maternal Age

Maternal ages of the different study groups are outlined in Table 3.1. The mean age \pm SD of the study population was 24 ± 5 years. Raw data for maternal age was not normally

distributed across the smallest comparative groups, thus a natural logarithm transformation of the data was used in the analysis.

A two-way analysis of variance test (ANOVA), indicated a main effect of HIV status on maternal age across all groups [$F(1,176) = 44.35, p < 0.001$]. This effect showed that the maternal age of the HIV positive group (mean = 25 years, CI 95%: 21 – 28) was higher than that of the HIV negative group (mean = 21 years, CI 95%: 18 – 22). There was also a significant effect in the different pregnancy categories (normotensive *vs* pre-eclamptic) on maternal age [$F(1,176) = 7.32, p = 0.007$]. More specifically maternal age was higher in pre-eclampsia (mean = 24 years, CI 95%: 20 – 28) compared to normotensive pregnancies (mean = 22 years, CI 95%: 19 – 24). Finally, there was no interaction between HIV status and type of pregnancy (normotensive *vs* pre-eclamptic) on maternal age [$F(1,176) = 0.053, p = 0.818$].

The Bonferroni post hoc test indicated that the maternal age of the HIV positive pre-eclamptic group (mean = 26 years, CI 95%: 25 - 27) was higher than that of the HIV negative pre-eclamptic (mean = 21 years, CI 95%: 20 - 23, $p < 0.001$) and HIV negative normotensive groups (mean = 20 years, CI 95%: 18 - 21, $p < 0.001$). Additionally the maternal age of the HIV positive normotensive group (mean = 24 years, CI 95%: 23 – 25) was significantly higher than the HIV negative pre-eclamptic (mean = 21 years, CI 95%: 20 - 23, $p = 0.03$) and the HIV negative normotensive groups (mean = 20 years, CI 95%: 18 - 21, $p < 0.001$).

Table 3.1 Clinical demographics across study groups

	NHIV⁻	NHIV⁺	PEHIV⁻	PEHIV⁺
	(n=30)	(n=60)	(n=30)	(n=60)
Maternal Age (years)	20±2	24±4	21±4	26±6
Gestational Age (weeks)	38.4±1.8	38.4±2.1	38.1±2.2	38.1±2.4
Systolic pressure (mm/Hg)	122±13	121±10	153±14	149±16
Diastolic Pressure (mm/Hg)	71±9	72±12	97±13	97±8
CD4 cell count (cells/μl)		257.6±181.9		253.4±206.2
Neonatal Birth weight (Kg)	3.2±0.4	3.1±0.5	3.0±0.5	2.9±0.6
Placental weight (g)	461.7±77.0	500.0±99.4	471.8±119.1	487.5±63.8

Values are given as mean±SD
Key to abbreviations: NHIV⁻ : Normotensive HIV negative
NHIV⁺: Normotensive HIV positive
PEHIV⁻: pre-eclamptic HIV negative
PEHIV⁺: pre-eclamptic HIV positive

3.1.2 Maternal Blood Pressure

Maternal blood pressure of the different study groups is outlined in Table 3.1. Two-way ANOVA found no significant difference in the systolic blood pressure between the HIV positive and negative groups [$F(1,176) = 1.824, p = 0.179$]. Based on our inclusion criteria, the systolic blood pressure was higher in the pre-eclamptic groups (mean = 151 mmHg, CI 95%: 148 - 154) compared to the normotensive groups (mean = 121 mmHg, CI 95%: 119 - 124; $F(1,176) = 199.38, p < 0.001$). There was no significant interaction between HIV status and type of pregnancy [$F(1,176) = 0.238, p = 0.627$].

Similarly, there was no significant main effect of HIV on the diastolic blood pressure across groups [$F(1,176) = 0.075, p = 0.784$]. The diastolic blood pressure was significantly higher in pre-eclampsia (mean = 97 mmHg, CI 95%: 95 - 99) compared to normotensive pregnancy (mean = 71, CI 95%: 69 - 74; $F(1,176) = 246.31, p < 0.001$). There was no significant interaction between HIV status and type of pregnancy [$F(1,176) = 0.041, p = 0.839$].

3.1.3 Maternal Oedema

Statistical analysis (Fisher's exact test) of the categorical data for oedema indicated that there was an overall difference in the presence of maternal oedema between pre-eclampsia and normotensive pregnancy ($p < 0.001$). In the HIV positive groups, 83% normotensive participants had no oedema compared to 48.3% of pre-eclamptic participants. In pre-eclampsia a greater percentage of oedema 'up to the ankle', 'up to the knee' as well as a generalized oedema that was inclusive of the face were noted ($p < 0.001$, Table 3.2). Similar comparisons were noted in HIV negative pregnancies ($p = 0.037$, Table 3.2).

The categorical clinical sign of oedema was independent of HIV status on both normotensive ($p = 0.181$) and pre-eclamptic ($p = 0.592$) pregnancies.

3.1.4 Maternal Proteinuria

Statistical analysis (Fisher's exact test) of the categorical data for proteinuria (urine dipstick assessment) indicated an overall difference in the presence of proteinuria between pre-eclampsia and normotensive pregnancy ($p < 0.001$). In the HIV positive groups, there was an absence of proteinuria in 73.3% of normotensive participants whilst all the pre-eclamptic participants presented with some degree of proteinuria ($p < 0.001$, Table 3.2). In the HIV negative groups, proteinuria was absent in the normotensive participants but of a variable level in all pre-eclamptic participants ($p < 0.001$, Table 3.2).

Based on HIV status, there were no differences in the level of proteinuria within the pre-eclamptic groups ($p = 0.480$, Table 2). However, proteinuria varied significantly within normotensive participants that were HIV positive (26.7%) compared to those that were HIV negative (0%, $p = 0.004$, Table 3.2).

Table 3.2 Categorical Maternal Data

	HIV Positive Pre-eclamptic (n = 60)		HIV positive Normotensive (n = 60)		HIV negative Pre-eclamptic (n = 30)		HIV negative Normotensive (n = 30)	
	Count	%	Count	%	Count	%	Count	%
<u>Oedema</u>								
None	29	48.3%	50	83.3%	15	50.0%	22	73.3%
Up to the ankle	16	26.7%	6	10.0%	8	26.7%	7	23.3%
Up to the knee	7	11.7%	1	1.7%	1	3.3%	1	3.3%
Generalized (inc. facial)	8	13.3%	3	5.0%	6	20.0%	0	0%
Total	60	100.0%	60	100.0%	30	100.0%	30	100.0%
<u>Proteinuria</u>								
0	0	0%	44	73.3%	0	0%	30	100.0%
1+	31	51.7%	12	20.0%	19	63.3%	0	0%
2+	19	31.7%	4	6.7%	6	20.0%	0	0%
3+	10	16.7%	0	0%	5	16.7%	0	0%
Total	60	100.0%	60	100.0%	30	100.0%	30	100.0%

3.1.5 Gestational Age

The mean gestational age for the study groups is indicated in Table 3.1. Pre-term delivery (< 36 weeks) occurred in all the study groups. Five pre-term deliveries occurred in the HIV positive pre-eclamptic group, two in the HIV negative pre-eclamptic group, four in the HIV positive normotensive and two in the HIV negative normotensive group.

Raw data for gestational age were not normally distributed across the groups, thus transformations were applied, but these did not correct the data distribution. The Kruskal-Wallis test with Dunn's post hoc test, were therefore used for the data analysis. These tests indicated that gestational age was similar amongst all the study groups (Table 3.1).

3.1.6 Maternal Anthropometric Measurements

3.1.6.1 Body Mass Index (BMI)

BMI data across all study groups is listed in Table 3.3. A two-way ANOVA found a main effect of HIV status [$F(1,176) = 7.493, p = 0.007$] with regards to BMI across all groups. This effect showed that the BMI of the HIV positive group (mean = 29.51 kg/m², CI 95%: 28.53 - 30.48) was lower than that of the HIV negative group (mean = 31.85 kg/m², CI 95%: 30.47 - 33.22). There was no main effect of pregnancy type (normotensive vs pre-eclamptic) on BMI [$F(1,176) = 1.968, p = 0.162$], indicating no statistical significance across the groups. Finally, there was no interaction between HIV status and type of pregnancy [$F(1,176) = 0.005, p = 0.941$].

Table 3.3 Anthropometric measurements across study groups

	NHIV ⁻ (n=30)	NHIV ⁺ (n=60)	PEHIV ⁻ (n=30)	PEHIV ⁺ (n=60)
Maternal BMI (kg/m²)	31.3±5.3	28.9±5.0	32.4±6.7	30.1±5.1
Maternal triceps skin-fold thickness (cm)	1.8±0.6	1.2±0.7	1.3±0.5	0.8±0.4
Maternal mid-arm circumference (cm)	39.0±8.8	31.8±8.7	34.5±8.6	30.1±5.5
Maternal mid-arm muscle circumference (cm)	33.5±7.8	28.1±7.2	30.3±7.4	27.7±4.3

Values are given as mean±SD

Key to abbreviations: NHIV⁻ : Normotensive HIV negative

NHIV⁺: Normotensive HIV positive

PEHIV⁻: pre-eclamptic HIV negative

PEHIV⁺: pre-eclamptic HIV positive

3.1.6.2 Triceps Skin-fold Thickness

Mean triceps skin-fold thickness for the study groups is listed in Table 3.3. The raw data for the triceps skin-fold thickness were not normally distributed, thus logarithmic transformed data were used in the two-way ANOVA. Triceps skin-fold thickness differed significantly between HIV positive and negative groups [$F(1,176) = 54.75, p < 0.001$]. This measurement was lower in HIV positive (mean = 0.98 cm, CI 95%: 0.87 – 1.09) compared to HIV negative participants (mean = 1.55 cm, CI 95%: 1.40 - 1.69). There was also a main effect of pregnancy type (normotensive vs pre-eclamptic) on triceps skin-fold thickness [$F(1,176) = 21.94, p < 0.001$]. This effect showed that in pre-eclampsia the triceps skin-fold thickness was lower (mean = 0.97 cm, CI 95%: 0.85 – 1.08) than in normotensive pregnancies (mean = 1.37 cm, CI 95%: 1.23 – 1.51). There was no interaction between HIV status and type of pregnancy on triceps skin-fold thickness [$F(1,176) = 0.616, p = 0.434$].

3.1.6.3 Mid-arm Circumference

Mean mid-arm circumference for the study groups is listed in Table 3.3. Raw data for mid-arm circumference were not normally distributed across the groups, thus logarithm and square root transformations were applied, but neither of these could correct the data distribution. The raw data were thus analysed by the Kruskal-Wallis test, followed by Dunn's post hoc test.

The mid-arm circumference of the HIV negative normotensive group (39.0 cm \pm 8.8, mean \pm SD) was significantly higher than that of the HIV negative pre-eclamptic (31.8 cm \pm 8.7, $p = 0.003$) and the HIV positive pre-eclamptic groups (30.1 cm \pm 5.5, $p < 0.001$)

3.1.6.4 Mid-arm Muscle Circumference

Mean mid-arm muscle circumference for the study groups is listed in Table 3.3. The raw data for mid-arm muscle circumference were not normally distributed across the groups, thus logarithm and square root transformations were applied, but neither of these could correct the data distribution. The raw data were thus analysed by the Kruskal-Wallis test, followed by Dunn's post hoc test.

The mid-arm muscle circumference of the HIV negative normotensive group ($33.5 \text{ cm} \pm 7.8$) was significantly higher than that of the HIV negative pre-eclamptic (30.3 ± 7.4 , $p = 0.014$) and the HIV positive pre-eclamptic groups (27.7 ± 4.3 , $p = 0.003$).

There was a correlation between the BMI vs mid-arm circumference ($r = 0.39$; $p < 0.01$), triceps skin-fold thickness ($r = 0.26$; $p < 0.01$) and mid-arm muscle circumference ($r = 0.40$; $p < 0.01$). A strong relationship was demonstrated between triceps skin-fold thickness and mid-arm muscle circumference ($r = 0.63$; $p < 0.01$).

3.1.7 Placental Weight

The mean placental weights for the study groups are outlined in Table 3.1. The placental weights ranged from 300g to 750g in the study population. Six placentae were below 400g, of which four were from mothers who delivered prior to 36 weeks.

A two-way ANOVA indicated that HIV status had no effect on placental weight [$F(1,176) = 3.699$, $p = 0.056$]. It also indicated that placental weight was similar between the

normotensive and pre-eclamptic groups [$F(1,176) = 0.005, p = 0.943$]. No significant interaction between HIV and type of pregnancy (normotensive vs pre-eclamptic) was noted [$F(1,176) = 0.663, p = 0.417$].

The Spearman's correlation coefficient showed a significant relationship between placental weight and gestational age ($r = 0.215, p = 0.004$).

3.1.8 Treatment Regimen

Of the HIV positive participants, 58% (n = 35) pre-eclamptic and 38% (n = 23) normotensives were on Highly Active Antiretroviral Treatment (HAART), while the remainder received prophylaxis for Prevention of Mother To Child Transmission of HIV (PMTCT). This prophylactic treatment consisted of the nucleoside reverse transcriptase inhibitor, azidothymidine (zidovudine/AZT) during pregnancy and AZT combined with nevirapine (NVP), a non-nucleoside reverse transcriptase inhibitor, during labour. Four pre-eclamptic participants were on HAART prior to onset of pregnancy with the duration of therapy ranging from three to five years. HAART was commenced during the gestation period of 22 pre-eclamptic participants. This treatment comprised of the nucleoside reverse transcriptase inhibitors, Tenofovir disoproxil fumarate (Tenofovir/TDF) and Emtracitabine, together with the non-nucleoside reverse transcriptase inhibitor, NVP. The duration of treatment was less than seven months. Two normotensive participants were on HAART prior to commencement of pregnancy, the duration of which was one and two years respectively. From this group, 15 participants commenced HAART during pregnancy, the duration of which was under seven months. No data were available for the duration of HAART for 10 pre-eclamptic and five normotensive participants.

The CD4 cell counts of the HIV positive normotensive pregnancies were lower in the HAART treated subgroup (195.5 ± 34.13 cells/ μ l) compared to the PMTCT treated subgroup (334.5 ± 37.45 cells/ μ l, $p < 0.001$, Table 3.1, Figure 3.1a). Similarly in the HIV positive pre-eclamptic group, CD4 cell counts were lower in the HAART treated subgroup (137.6 ± 15.27 cells/ μ l) compared to the PMTCT subgroup (355.7 ± 32.01 cells/ μ l, $p < 0.001$, Figure 3.1b)

The triceps skin-fold thickness in the pre-eclamptic HAART group was significantly lower compared to the normotensive PMTCT group (0.65 ± 0.03 vs 1.32 ± 0.10 cm; $p < 0.01$). All other anthropometric parameters were similar amongst the HIV positive sub-groups.

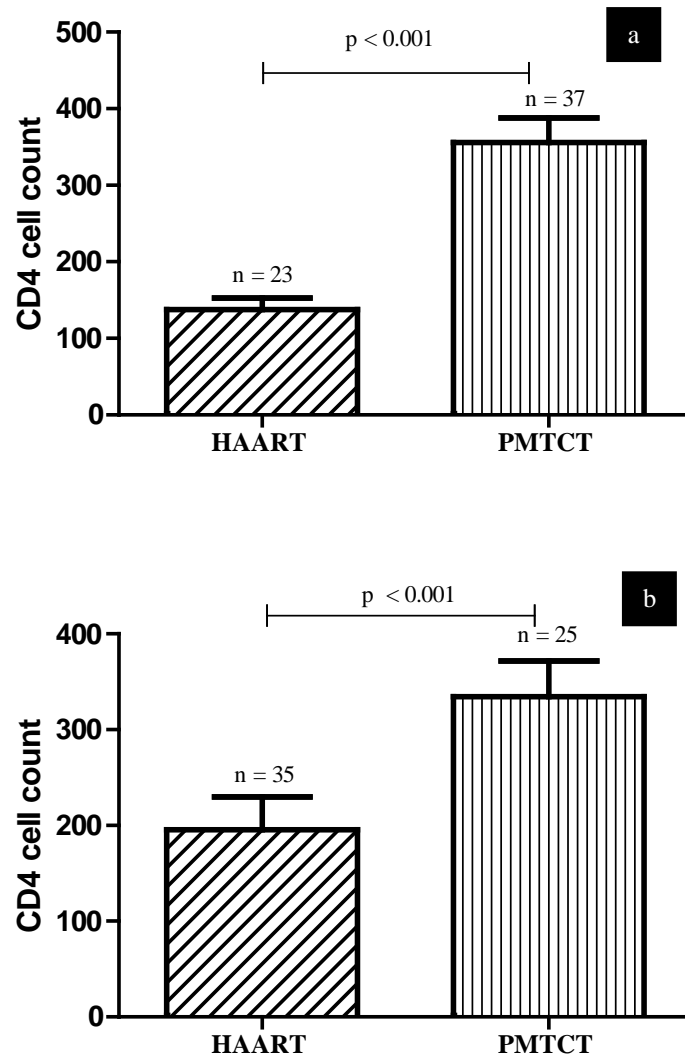


Figure 3.1 Histogram indicating CD4 cell counts in different treatment groups: (a) HIV positive normotensive and (b) pre-eclamptic participants (mean \pm SEM).

3.1.9 Neonatal Data

3.1.9.1 Neonatal Weight

A two-way ANOVA found no significant main effect of HIV [$F(1,176) = 1.673, p = 0.198$] on neonatal weight. However, a significant main effect of pregnancy type [normotensive vs pre-eclamptic; $F(1,176) = 4.481, p = 0.036$] was evident, indicating that the neonatal weight was significantly lower in babies born of pre-eclamptic pregnancies (mean = 2.97 Kg, CI 95%: 2.85 - 3.09) compared to those born of normotensive pregnancies (mean = 3.15 Kg, CI 95%: 3.03 - 3.27). There was no significant interaction between HIV status and type of pregnancy [$F(1,176) = 0.260, p = 0.611$].

3.1.9.2 APGAR Scores

The APGAR scores at one and five minutes were not normally distributed. Logarithm and square root transformations were applied, but neither could correct the data distribution. The Kruskal-Wallis test, followed by Dunn's post hoc test was used for the statistical analysis of the raw data. These tests indicated that the APGAR scores at one minute as well as at five minutes were similar amongst all the study groups (Table 3.1). The median APGAR score at 1 minute was 8/10 for all the study groups whilst the median APGAR score at 5 minutes was 9/10 for all the study groups.

Of the total study population, one baby had low APGAR scores at one and five minutes. These were 1/10 and 2/10 respectively. The neonate was born from a HIV negative pre-eclamptic mother.

3.1.9.3 Neonatal Morbidities and Mortalities

Two neonates born of HIV negative normotensive mothers had mild nappy rash at the six week hospital visit and one had a mild discharge from the eyes. Two neonates from the HIV positive normotensive group had mild, flu-like infections at six weeks. One neonate from the HIV negative pre-eclamptic group had a mild, flu-like infection as well as a discharge from the eyes at birth. The baby from the HIV negative pre-eclamptic group that displayed low APGAR scores had difficulty in breathing and died a few days after birth due to asphyxia. There were no reported morbidities in neonates born from HIV positive pre-eclamptic mothers.

3.1.9.4 Neonatal Follow-up

Sixty seven percent (n = 40) HIV negative and 33% (n = 43) HIV positive participants returned for the six week postnatal visit. A child was considered HIV positive when the HIV DNA PCR results at 6 weeks were positive. The rate of MTCT of HIV was 16% (n = 7), however this was confounded by the low rate of follow-up visits.

Of the seven HIV positive babies, four were breastfed, two of whom were delivered vaginally and two by emergency Caesarean section (C-section). One HIV-infected baby was both formula and breast fed and was delivered by elective C-section. Of the three formula fed babies, two were vaginal deliveries and one was by emergency C-section.

A total of 29 HIV positive mothers reported breastfeeding their babies at birth. Of these only 10 returned for the six week follow up visit and five (50%) of these babies were HIV-positive at six weeks. None of the mothers reported a change in feeding pattern.

Of the HIV positive participants, 81 had CD4 cell counts below 350, of which 23 (28.4%) returned for the follow up visit. Furthermore, of the 81 participants with CD4 counts below 350, 21 (30%) were not on HAART during pregnancy and 14 (67%) of these HAART eligible participants did not return for the six week follow up visit.

3.2 SERUM LEPTIN LEVELS

The data for serum leptin levels were normally distributed and are outlined in Table 3.4. A two-way ANOVA found a main effect of HIV status on serum leptin levels [$F(1,176) = 97.07, p < 0.001$]. This effect showed that serum leptin levels in the HIV positive group (mean = 12.21 ng/ml, CI 95%: 10.56 - 13.86) were lower than in the HIV negative group (mean = 26.46 ng/ml, CI 95%: 24.13 - 28.80). There was no overall main effect of pregnancy type (normotensive *vs* pre-eclamptic [$F(1,176) = 0.054, p = 0.816$]) on serum leptin levels. There was, however, an interaction between HIV status and pregnancy type on serum leptin levels [$F(1,176) = 7.85, p = 0.006$]. This effect showed that within the HIV positive groups, serum leptin levels in pre-eclampsia (mean = 10.02 ng/ml, CI 95%: 7.69 - 12.35) were significantly lower than that of normotensive pregnancies (mean = 14.41 ng/ml, CI 95%: 12.08 - 16.74).

Bonferroni post hoc analysis indicated that in the HIV negative groups, serum leptin levels were elevated in pre-eclampsia compared to normotensive pregnancies, this difference was however, not statistically significant (mean = 28.32 ng/ml, CI 95%: 25.08 - 31.56 *vs* 24.6, CI 95%: 21.37 - 27.84; $p = 0.42$; Figure 3.2, Table 3.4). In the HIV positive groups, serum leptin levels were significantly lower in pre-eclampsia (mean = 10.02 ng/ml, CI 95%: 7.73

– 12.31) compared to normotensive pregnancy (mean = 14.41 ng/ml, CI 95%: 12.11 - 16.70, $p = 0.03$, Figure 3.2, Table 3.4).

In normotensive pregnancies, serum leptin levels were significantly lower in the HIV positive group (mean = 10.02 ng/ml, CI 95%: 7.73 – 12.31) compared to the HIV negative group (mean = 14.41 ng/ml, CI 95%: 12.11 - 16.70 vs 24.61 ng/ml CI 95% 21.37 – 27.84; $p < 0.01$; Table 3.4). Similarly, in pre-eclamptic pregnancies, leptin levels were significantly decreased in the HIV positive group (mean 10.02 ng/ml, CI 95%: 7.73 – 12.31) compared to the HIV negative group (mean = 28.32 ng/ml, CI 95%: 25.08 - 31.56; $p < 0.01$; Figure 3.2, Table 3.4).

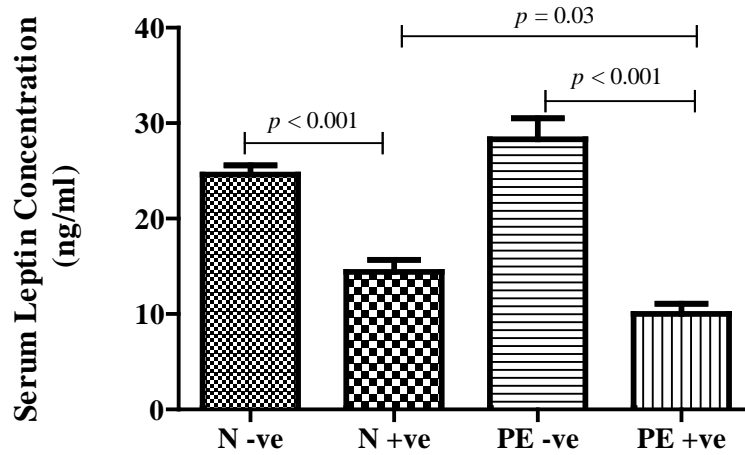


Figure 3.2 Histogram showing serum leptin levels in study groups. Leptin levels were significantly lower in HIV positive compared to HIV negative groups. Leptin levels were also decreased in HIV positive pre-eclampsia compared to the HIV positive normotensive group.

Table 3.4 Serum leptin levels in study groups

HIV	Pregnancy	Mean (ng/ml)	Std. Error	95% Confidence Interval	
				Lower	Upper
Positive	Pre-eclamptic (n = 60)	10.02	1.17	7.73	12.31
	Normotensive (n = 60)	14.41	1.17	12.12	16.70
Negative	Pre-eclamptic (n = 30)	28.32	1.65	25.08	31.56
	Normotensive (n = 30)	24.61	1.65	21.37	27.84

3.2.1 Serum Leptin Levels in HIV Positive Groups on Different Treatment Regimens

The Mann-Whitney U test was used for the analysis of serum leptin levels of HIV positive participants on different treatment regimens. In the HIV positive normotensive group, leptin levels were similar in the different treatment regimen: HAART and PMTCT (14.36 ± 2.0 vs 14.45 ± 1.6 ng/ml; $p = 1.0$, Figure 3.3a). However, in the HIV positive pre-eclamptic groups, leptin was significantly lower in HAART participants compared to those on PMTCT therapy (8.56 ± 1.4 vs 12.03 ± 1.5 ng/ml; $p = 0.04$, Figure 3.3b).

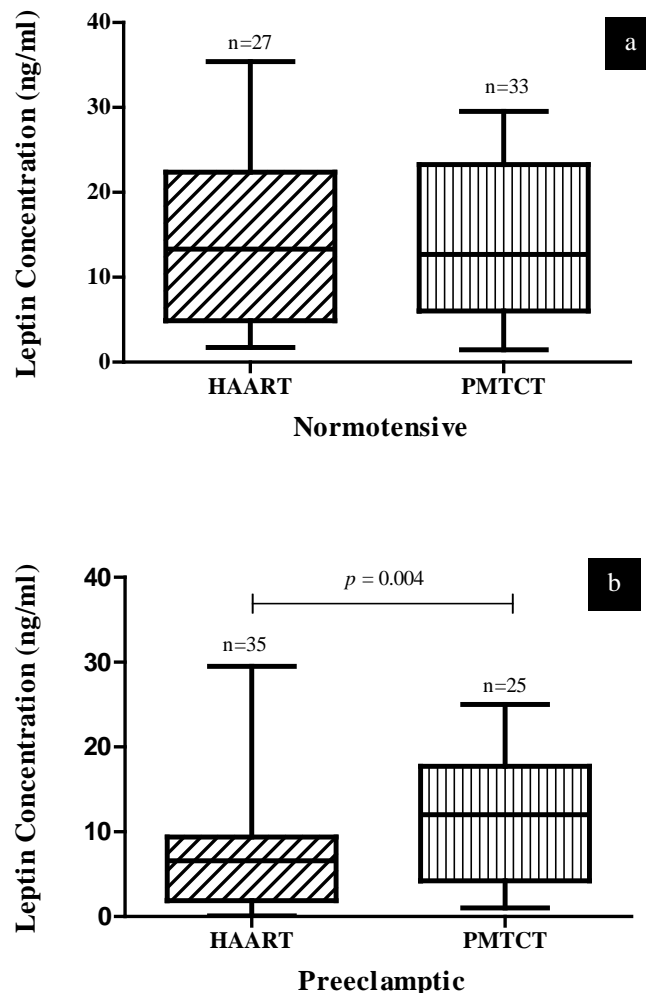


Figure 3.3 Serum leptin levels in different treatment groups of HIV positive (a) normotensive and (b) pre-eclamptic participants (mean and range). (a) The leptin levels in HIV positive normotensive pregnancies were similar in the HAART and PMTCT treatment groups. (b) In pre-eclampsia leptin levels were lower in HAART compared to the PMTCT group.

3.2.2 Correlation Studies with Leptin

A negative correlation between serum leptin and maternal age was noted ($r = -0.30$, $p < 0.001$, Figure 3.4a). There was no relationship between serum leptin and BMI ($r = 0.15$; $p = 0.51$), however a significant correlation existed between leptin and the other anthropometric measurements, namely: triceps skin-fold thickness ($r = 0.37$; $p < 0.001$, Figure 3.4b), mid-arm circumference ($r = 0.26$, $p = 0.001$, Figure 3.4c) and mid-arm muscle circumference ($r = 0.18$, $p = 0.02$, Figure 3.4d). There was no correlation between leptin levels vs gestational age ($p = 0.7$), neonatal weight ($p = 0.1$), systolic blood pressure ($p = 0.1$), diastolic blood pressure ($p = 0.1$) and CD4 cell counts of HIV positive participants ($p = 0.22$).

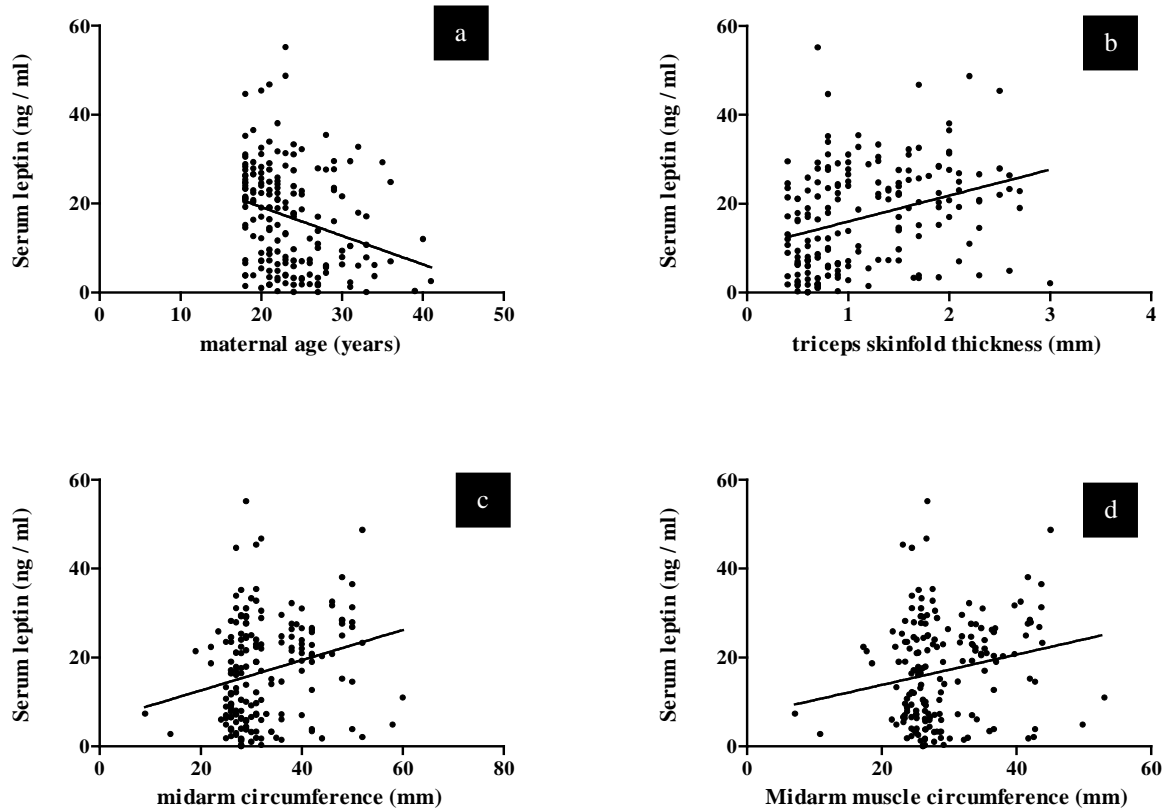


Figure 3.4 Scatterplot demonstrating correlation between serum leptin levels and maternal parameters. (a) There was a negative correlation between serum leptin levels and maternal age ($r = -0.2$, $p < 0.001$). A positive correlation was present between leptin vs (b) triceps skin-fold thickness ($r = 0.37$; $p < 0.001$), (c) mid-arm circumference ($r = 0.26$, $p = 0.001$) and (d) mid-arm muscle circumference ($r = 0.18$ $p = 0.02$, $n = 180$).

3.3 SERUM INSULIN LEVELS

Raw data for insulin were not normally distributed across the comparative groups, thus a natural logarithm transformation was applied and used in the following analysis.

A two-way ANOVA found a main effect of HIV status on serum insulin levels [$F(1,176) = 17.18, p < 0.001$]. This effect showed that insulin levels in the HIV positive group was lower (mean = 4.15 mU/l, CI 95%: 0.14 - 1.53) than in the HIV negative group (mean = 7.57 mU/l, CI 95%: 3.10 - 10.55). There was also a main effect of type of pregnancy (normotensive vs pre-eclamptic) on serum insulin levels [$F(1,176) = 5.329, p = 0.022$]. This effect showed that serum insulin levels in pre-eclampsia were lower (mean = 4.41 mU/l, CI 95%: 0.00 - 5.67) than that in normotensive pregnancies (mean = 6.16 mU/l, CI 95%: 1.27 - 7.84). Finally, there was no interaction between HIV status and type of pregnancy [$F(1,149) = 0.004, p = 0.950$].

The Bonferroni post hoc test indicated that serum insulin levels were lower in the HIV positive normotensive group (mean = 4.97 mU/l, CI 95%: 3.45 - 6.49) compared to the HIV negative normotensive group (mean = 8.55 mU/l, CI 95%: 6.39 - 10.70, $p = 0.009$, Table 3.4, Figure 3.5). Similarly in pre-eclampsia serum insulin levels were lower in the HIV positive (mean = 3.33 mU/l, CI 95%: 1.80 - 4.85) compared the HIV negative groups (mean = 6.59 mU/l, CI 95%: 4.43 - 8.74, $p = 0.032$, Table 3.4, Figure 3.5).

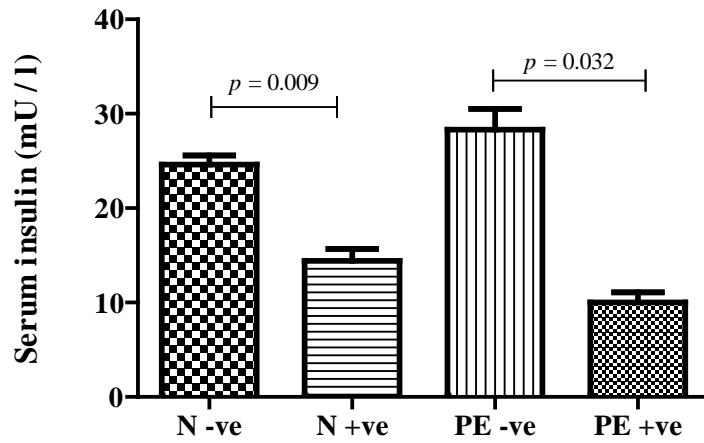


Figure 3.5 Histogram depicting serum insulin levels across study groups. Insulin levels in the serum were lower in HIV positive groups compared to HIV negative groups.

Table 3.5 Serum levels of insulin in study groups

HIV	Pre-eclampsia	Mean (mU/l)	Std. Error	95% Confidence Interval	
				Lower	Upper
Positive	Pre-eclamptic (n = 60)	3.33	0.78	1.80	4.85
	Normotensive (n = 60)	4.97	0.78	3.45	6.49
Negative	Pre-eclamptic (n = 30)	6.59	1.10	4.43	8.74
	Normotensive (n = 30)	8.55	1.10	6.39	10.70

3.3.1 Insulin in Different Treatment Regimens in HIV Positive Groups

The Mann-Whitney U test was used for the analysis of serum insulin levels of HIV positive participants on different treatment regimens. In the HIV positive normotensive groups, serum insulin levels were significantly lower in HAART ($3.16 \text{ mU/l} \pm 0.85$) compared to those on PMTCT therapy ($6.29 \text{ mU/l} \pm 1.13$, $p = 0.03$, Figure 3.6a). Similarly in the HIV positive pre-eclamptic groups, serum insulin levels were significantly lower in HAART ($1.28 \pm 0.30 \text{ mU/l}$) participants compared to those on PMTCT therapy ($4.55 \pm 1.55 \text{ mU/l}$; $p = 0.04$, Figure 3.6b).

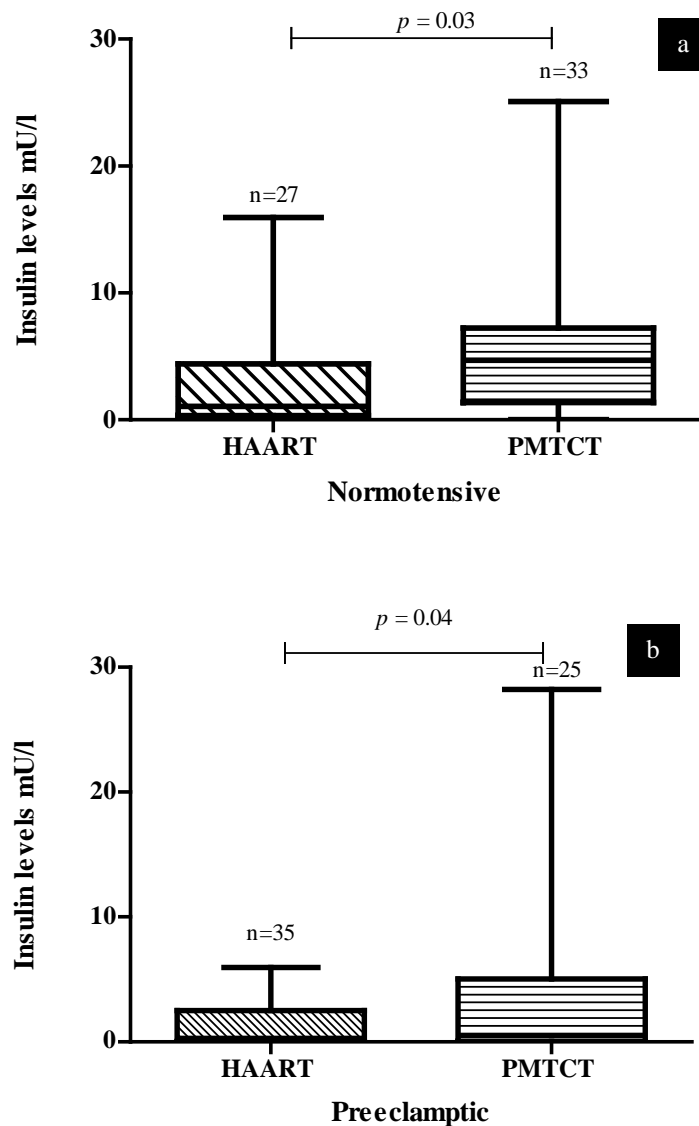


Figure 3.6 Bar graphs illustrating serum insulin levels in the different treatment groups in HIV positive (a) normotensive and (b) pre-eclamptic pregnancies. Insulin levels were lower in HAART treated participants in both normotensive and pre-eclamptic pregnancies.

3.3.2 Correlation Studies with Insulin

There was a significant correlation between serum insulin levels and the CD4 cell count in the HIV positive normotensive pregnancies (Spearman's test, $r = 0.33$, $p = 0.009$). However in HIV positive pre-eclampsia, there was no relationship between serum insulin levels and CD4 cell counts (Spearman's test, $p = 0.49$).

There was no relationship between serum insulin levels and BMI ($p = 0.89$). However, the levels of serum insulin were correlated positively with the following anthropometric parameters: triceps skin-fold thickness (Spearman's test, $r = 0.573$, $p < 0.001$), mid-arm circumference ($r = 0.46$, $p < 0.001$) and mid-arm muscle circumference ($r = 0.37$, $p < 0.001$). Serum insulin levels were correlated negatively with maternal age (Spearman's test, $r = -0.36$, $p < 0.001$), placental weight ($r = -0.19$, $p = 0.01$), systolic ($r = -0.20$, $p = 0.006$) and diastolic blood pressure ($r = -0.24$, $p = 0.001$). Notably, correlation analysis revealed a relationship between serum leptin and serum insulin levels ($r = 0.27$, $p < 0.001$, Figure 3.7).

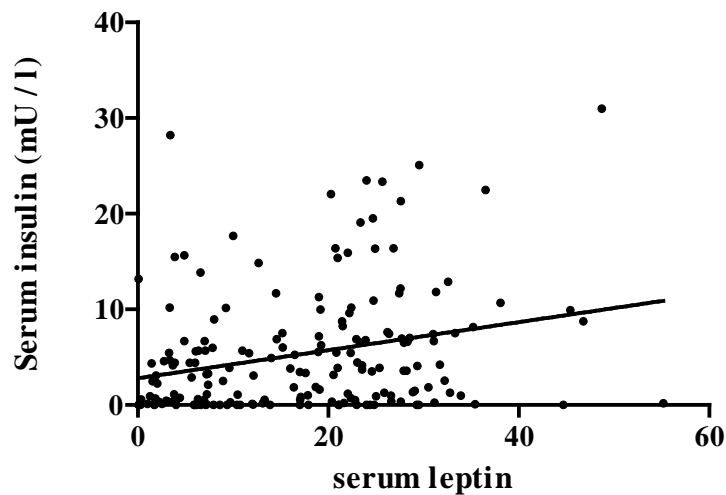


Figure 3.7 Scatterplot depicting correlation between serum leptin and serum insulin levels. A positive correlation was present between serum leptin and insulin levels ($r = 0.27$, $p < 0.001$, $n = 180$).

3.4 PLACENTAL LEPTIN CONCENTRATION BY ELISA

Raw data of placental leptin levels were not normally distributed across the comparative groups. The Mann-Whitney U test indicated that leptin levels in the placenta were similar between the central and peripheral regions of the placenta (Table 3.6, Figure 3.6). The average of the central and peripheral leptin concentration for each placenta was calculated and a natural logarithm transformation was applied to normalize the data for further analysis.

The transformed data were used in the two-way ANOVA, which found no significant main effect of HIV status on placental leptin concentration [$F(1,176) = 0.859, p = 0.36$]. There was a significant main effect of type of pregnancy [normotensive vs pre-eclamptic, $F(1,176) = 21.298, p < 0.001$]. This effect indicated significantly higher placental leptin levels in pre-eclampsia (mean = 18.15 ng/ml, CI95%: 12.90 – 23.39) compared to normotensive pregnancies (mean = 5.56 ng/ml, CI 95%: 4.43 – 6.69). Finally, there was no significant interaction between HIV and type of pregnancy [$F(1,176) = 0.010, p = 0.92$].

Table 3.6 Placental leptin values from the central and peripheral regions of the placenta

	Normotensive						Pre-eclampsia					
	HIV ⁻			HIV ⁺			HIV ⁻			HIV ⁺		
	Central	Peripheral	<i>p</i>	Central	Peripheral	<i>p</i>	Central	Peripheral	<i>p</i>	Central	Peripheral	<i>p</i>
ELISA leptin concentration in placenta (ng/ml)	3.84±0.70 (n=30)	4.67±0.71 (n=30)	0.42	6.72± 1.15 (n=60)	5.51±0.85 (n=60)	0.84	14.38±4.47 (n=30)	11.00±3.10 (n=30)	0.82	18.88±2.95 (n=60)	22.87±5.61 (n=60)	0.06
Placental leptin mRNA	1.84± 0.48 (n= 23)	2.21±0.81 (n = 23)	0.21	4.10±0.73 (n = 42)	2.54±0.75 (n = 42)	0.26	8.29±2.05 (n= 18)	6.23±2.24 (n= 18)	0.22	3.93±0.78 (n = 43)	4.86±1.25 (n = 43)	0.16
Field area percentage of leptin immuno-staining in exchange villi (%/µm)	11.22±4.91 (n=30)	12.53±4.72 (n=30)	0.50	13.96±4.85 (n=60)	14.76±5.66 (n=60)	0.75	29.16±1.31 (n=30)	31.63±5.71 (n=30)	0.08	25.30±0.91 (n=60)	26.49±1.08 (n=60)	0.42
Intensity of leptin immuno-staining in exchange villi (grey scale 0 – 255 densitometric arbitrary units)	47.33±2.8 (n=30)	55.29±2.8 (n=30)	0.07	54.99±2.7 (n=60)	61.17± 3.1 (n=60)	0.15	74.96±3.6 (n=30)	84.08±2.8 (n=30)	0.07	130.7±5.0 (n=60)	74.96± 3.6 (n=60)	0.86
Field area percentage of leptin immuno-staining in conducting villi (%/µm)	20.65±1.4 (n=30)	20.19±1.3 (n=30)	0.81	20.39±1.2 (n=60)	19.72±1.2 (n=60)	0.86	19.28±1.0 (n=30)	18.39±0.7 (n=30)	0.35	17.83±0.7 (n=60)	17.21±0.6 (n=60)	0.89
Intensity of leptin immuno-staining in conducting villi (grey scale 0 – 255 densitometric arbitrary units)	115.0±2.3 (n=30)	122.8±3.3 (n=30)	0.60	109.6±4.5 (n=60)	111.9±4.8 (n=60)	0.49	111.1±2.7 (n=30)	111.3±2.9 (n=30)	0.60	121.6±5.6 (n=60)	127.5±5.0 (n=60)	0.39

Values are given as mean ± SEM

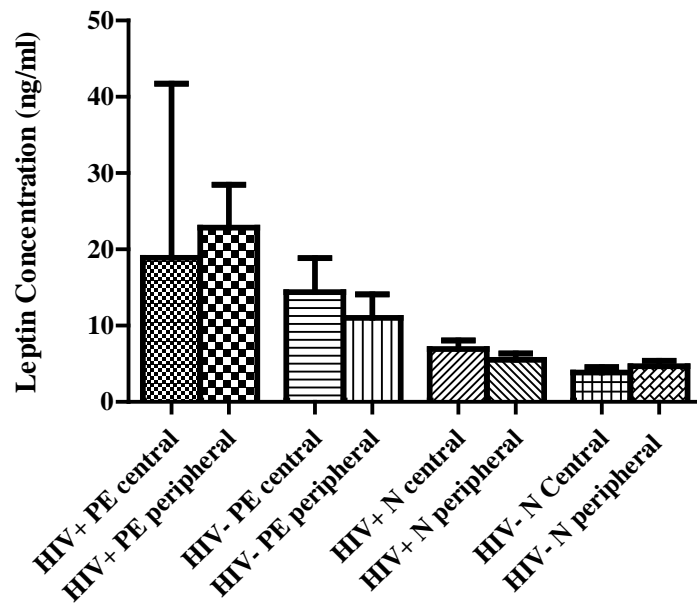


Figure 3.8 Histogram depicting leptin concentration in central and peripheral regions of the placenta (mean \pm SEM). Leptin levels were similar in the central and peripheral regions of the placenta within all study groups.

3.5 PLACENTAL GENE EXPRESSION OF LEPTIN

Gene expression was monitored by semi-quantitative real time polymerase chain reaction (RT PCR). This measured the change in leptin mRNA expression levels relative to the change in GAPDH mRNA expression levels.

The threshold cycle (Ct) reflected the cycle number at which the fluorescence generated in the reaction crossed a pre-selected threshold and this was inversely related to the concentration of cDNA in the sample prior to amplification. Both GAPDH and leptin amplified within 15 to 40 cycles (Appendix XI), 45 cycles were thus considered sufficient for product amplification.

The specificity of the GAPDH and leptin in the placental tissue total RNA were ascertained using melting curve analysis. The fluorescent dye, SYBR Green which binds specifically to double stranded DNA, was used to detect the accumulation of PCR products of the specific amplicons. With the Light Cycler, the accumulation of amplicons were visualised in real time, and the specific product was determined by its characteristic melting temperature (T_m) as all PCR products for a particular primer pair have the same T_m (Appendix XII). The T_m for GAPDH was 83°C whilst that for leptin was 80°C. Samples that had a lower T_m (Appendix XII) were not specific for the gene of interest and were excluded from the study.

The T_m of the genes of interest was visualized more clearly as a peak by plotting the negative derivative of fluorescence emitted by each sample during the increase of temperature by which PCR products were slowly denatured in a first derivate plot shown in Appendix XIII. The non-specific amplification products melted at lower temperatures and

over a broader range. Agarose gel electrophoresis confirmed the specificity of the genes of interest (Figure 3.9).

Raw data for leptin/GAPDH mRNA were not normally distributed across the comparative groups. The Mann-Whitney U test indicated that the ratio of leptin/GAPDH mRNA was similar in the central and peripheral regions of the placenta across all the study groups (Table 3.6, Figure 3.10). The natural logarithm transformation was applied to normalize the data. The average leptin/GAPDH mRNA from the central and peripheral regions of each placenta was calculated for use in further analysis.

The log transformed data were used in a two-way ANOVA that demonstrated no significant main effect of HIV status on leptin mRNA expression [$F(1,129) = 2.218, p = 0.14$]. There was a significant main effect of type of pregnancy (normotensive vs pre-eclamptic [$F(1,129) = 3.730, p = 0.04$]. This effect indicated significantly higher leptin mRNA expression in pre-eclampsia (mean = 4.12, CI 95%: 0.41 – 6.89) compared to normotensive pregnancies (mean = 2.94, CI 95%: 0.58 – 3.65). Finally, a significant interaction between HIV and type of pregnancy (normotensive vs pre-eclamptic) was shown [$F(1,129) = 9.735, p = 0.002$].

The Bonferroni post hoc test indicated that leptin mRNA expression was up-regulated in HIV negative pre-eclampsia (mean = 6.93, CI 95%: 5.06 - 8.81) compared to the HIV negative normotensive group (mean = 2.11, CI 95%; 0.36 - 3.85, $p = 0.012$, Table 3.5, Figure 3.11). Leptin mRNA expression was also significantly up-regulated in HIV negative pre-eclampsia (mean = 6.93, CI 95%: 5.06 - 8.81) compared to HIV positive pre-

eclampsia (mean = 2.99, CI 95%: 1.80 - 4.18, $p = 0.007$, Table 3.7, Figure 3.14). In the HIV positive groups, leptin was similar in pre-eclamptic (mean = 2.99 CI 95%: 1.80 - 4.18) and normotensive participants (mean = 3.36 CI 95%: 2.14 - 4.57, $p = 1.00$)

Table 3.7 Ratio of Leptin/GAPDH in placenta of study groups

PREGNANCY	HIV	Mean	Std. Error	95% Confidence Interval	
				Lower	Upper
Normotensive	Negative (n = 30)	2.11	0.89	0.36	3.85
	Positive (n = 60)	3.36	0.62	2.14	4.57
Pre-eclamptic	Negative (n = 30)	6.93	0.96	5.06	8.81
	Positive (n = 60)	2.99	0.61	1.80	4.18

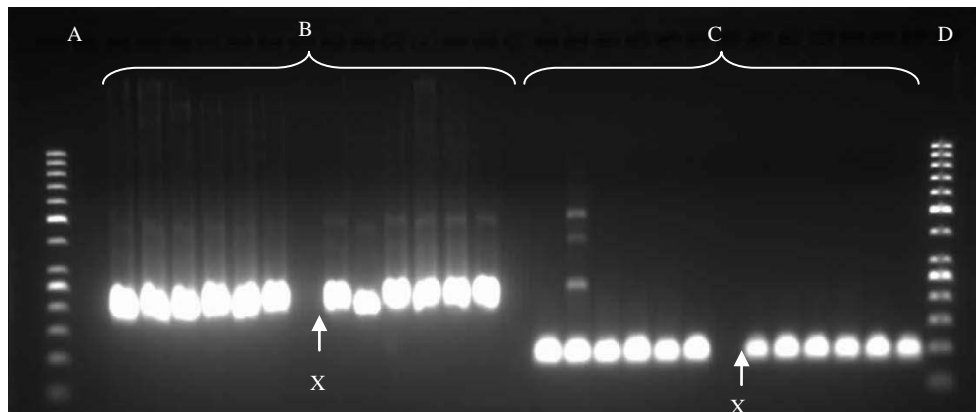


Figure 3.9 Photomicrograph of an agarose gel electrophoresis plate. Lanes (A) and (D) represent a commercial ladder of 100 base pairs. Lanes (B) and (C) indicate the results for GAPDH and leptin respectively. No product was present at X.

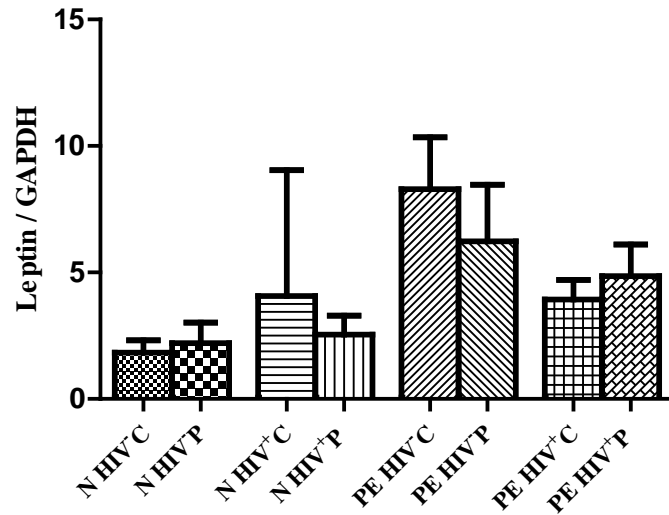


Figure 3.10 Histogram depicting RT-PCR analysis of leptin expression across the study groups indicating leptin/GAPDH (mean ± SD) in the central and peripheral regions of the placenta. The ratio of leptin/GAPDH was similar in the central and peripheral regions of each group.

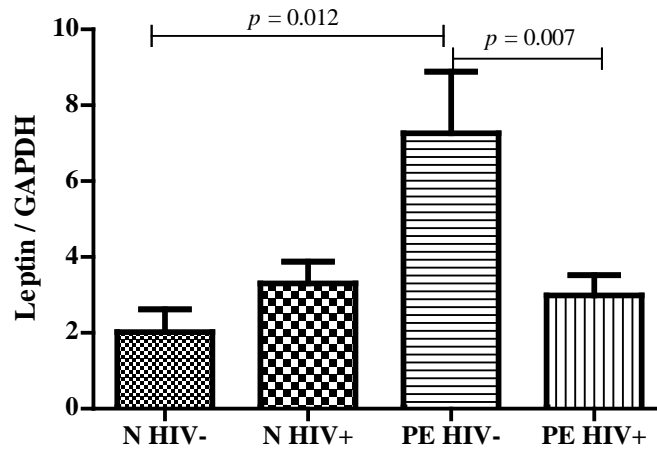


Figure 3.11 Histogram indicating ratio of leptin/GAPDH in placenta of study groups. Leptin mRNA was significantly up-regulated in HIV negative pre-elampsia compared to HIV negative normotensives and HIV positive pre-eclampsia.

3.6 GENERAL HISTOLOGICAL EVALUATION OF THE PLACENTA ACROSS ALL STUDY GROUPS

Histological analysis of 3 – 5 μm sections of placental tissue revealed an amnion (Figure 3.12a & b) that comprised of a single layer of epithelium, the cells of which varied from a simple cuboidal (Figure 3.12b) to a simple columnar tissue type. This epithelium rested on a thick basement membrane. The amnion was not observed in all sections viewed. Beneath this epithelium was a spongy layer (Figure 3.12a & b) made up of connective tissue. This spongy layer connected the amnion to the layer below, the chorion which was a fibrous tissue layer, containing the fetal blood vessels (Figure 3.12a).

Three types of chorionic villi were observed viz., stem (Figure 3.13), intermediate (Figure 3.14a) and terminal villi (Figure 3.14b). These villi were surrounded by an uninterrupted outer multinucleated syncytiotrophoblast layer beneath which were occasional unicellular cytotrophoblasts (also called Langhans cells). This syncytiotrophoblast layer was surrounded by the intervillous space. Each villus contained a mesenchymal core containing connective tissue cells, fibres and ground substance as well as capillaries, arteries and veins that varied according to villi type. These features were observed across all study groups.

Large stem villi were observed (Figure 3.13a). The stroma of these villi contained arteries and veins, the lumina of which were surrounded by a layer of endothelium (Figure 3.13a & b). Both arteries and veins displayed a tunica media composed of smooth muscle cells in concentric layers. The tunica media was thicker in the arteries than in the veins (Figure

3.13b). The tunica adventitia varied in thickness and merged with the surrounding mesenchymal stroma which contained fibroblasts (Figure 3.13a & b).

Immature intermediate villi contained capillaries, sinusoids and occasional Hofbauer cells (macrophages). These villi contained a reticular stroma, and fetal vessels such as capillaries, arterioles and venules, interspersed with few collagen fibres. The stroma of mature intermediate villi consisted of loose bundles of connective tissue fibres surrounding numerous capillaries that were positioned peripherally within these villi (Figure 3.14a).

The terminal villi were characterized by large dilated sinusoids and fetal capillaries, which comprised more than half the stromal volume of terminal villi. These blood vessels often adjoined to the adjacent syncytiotrophoblast layer (Figure 3.14b). The stromal core displayed connective tissue cells. A microvillous brush border was occasionally clearly evident, projecting into the intervillous space.

Placental septa and extravillous trophoblast (EVT) cells were noted within the basal plate (Figure 3.15).

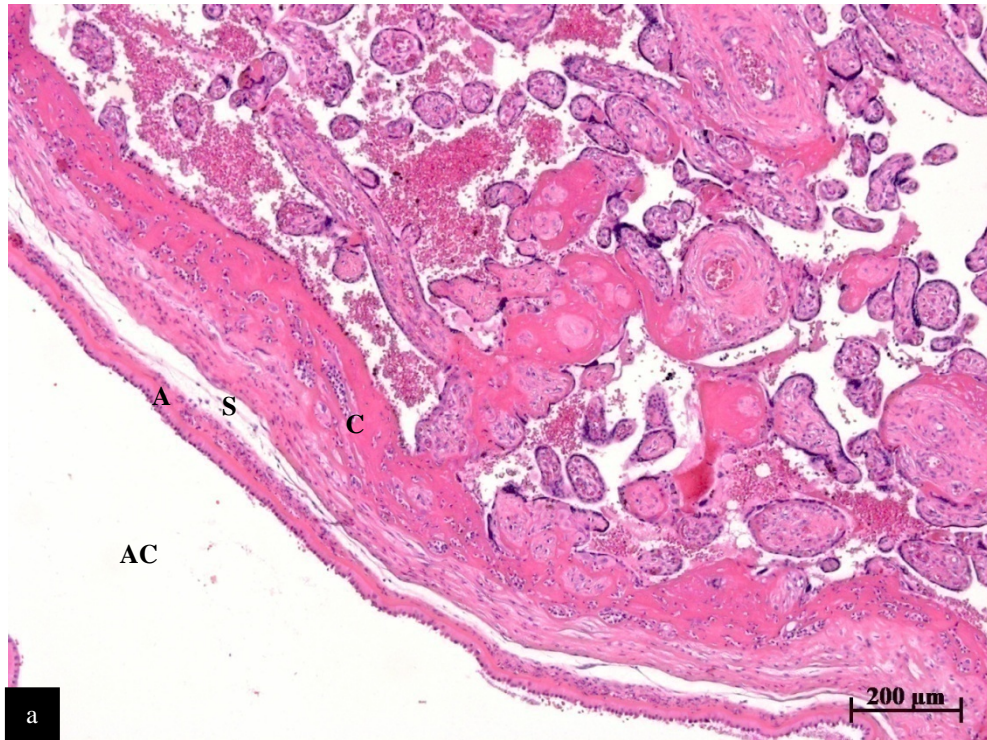


Figure 3.12 Light micrograph of H&E stained placental membranes. (a) Low magnification showing amnion (A) and chorion (C) sandwiched by a spongy layer (S) in between. (b) Higher magnification of amnion showing cuboidal epithelium (E) lining the amnion. Note amniotic cavity (AC).

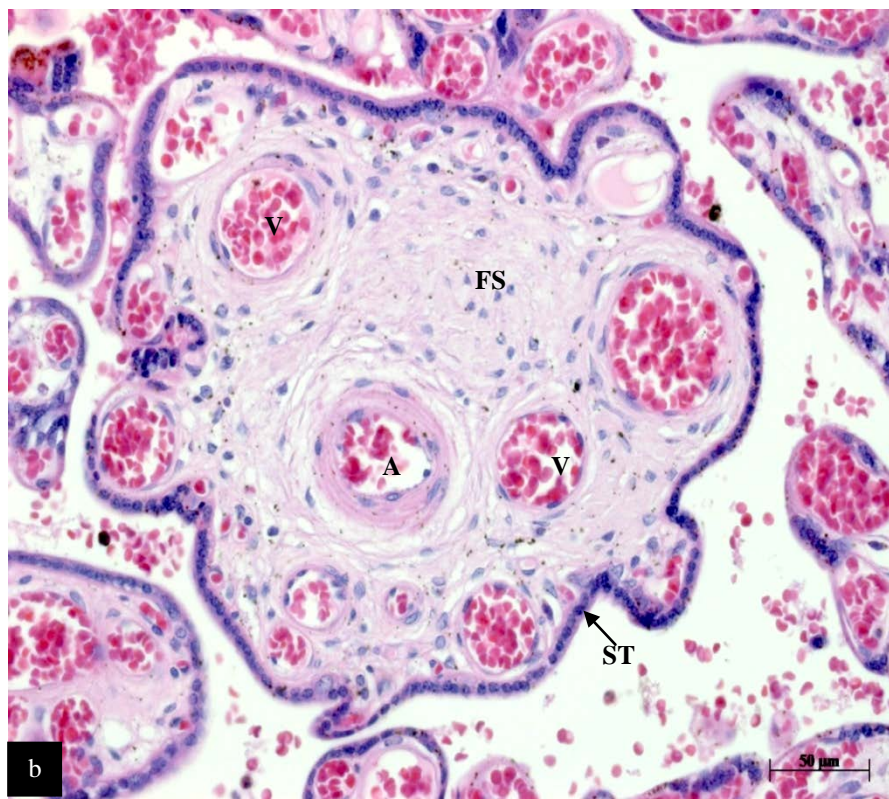
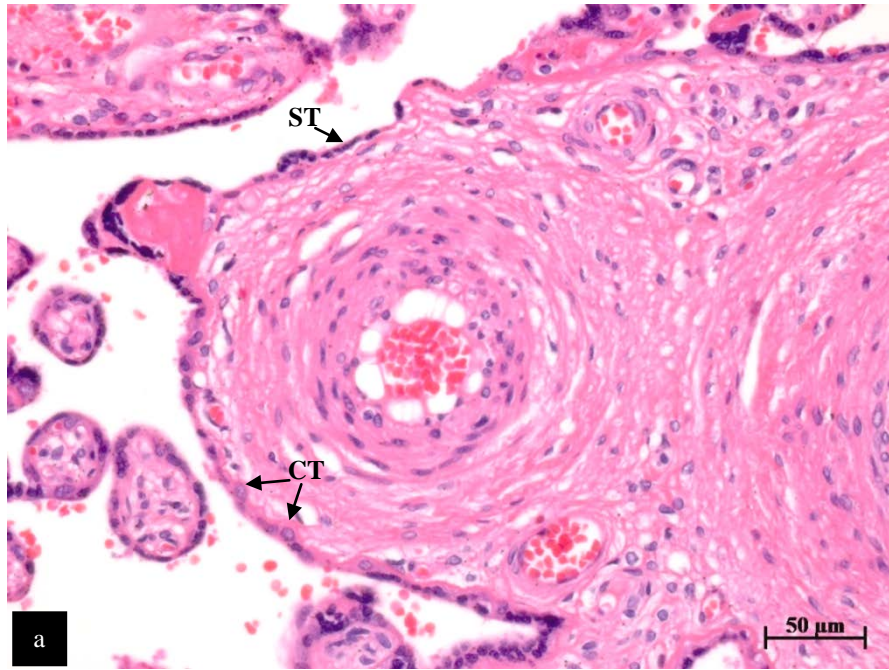


Figure 3.13 Light micrographs (a & b) depicting H&E stained stem villi. Note syncytiotrophoblast (ST), cytotrophoblast (CT), artery (A), vein (V), fibrous stroma (FS).

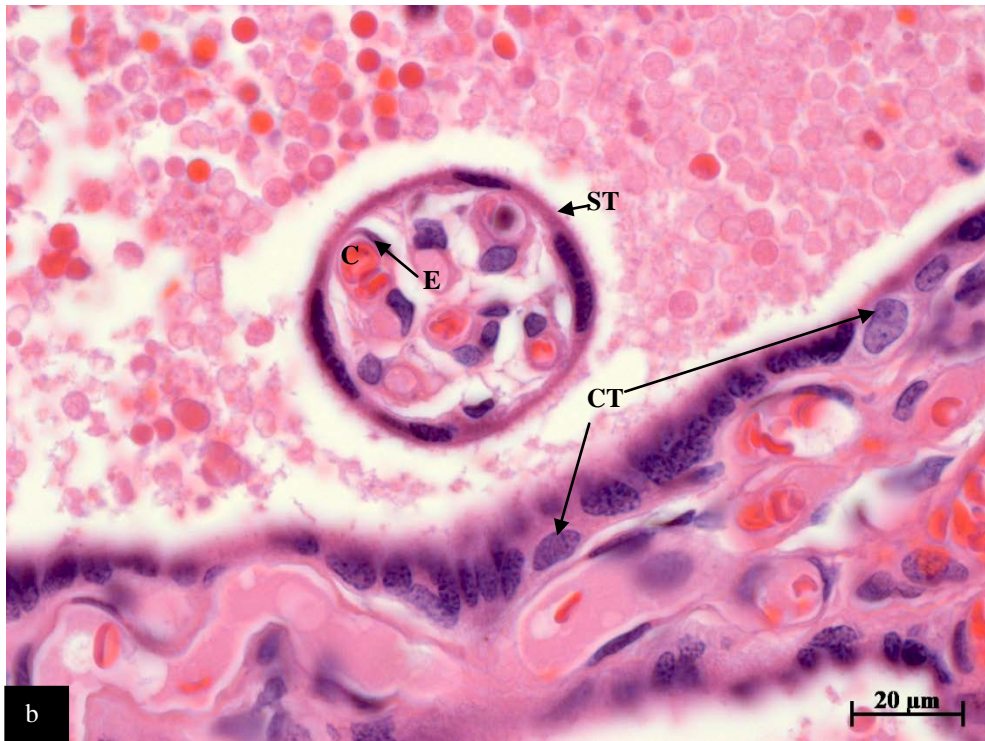
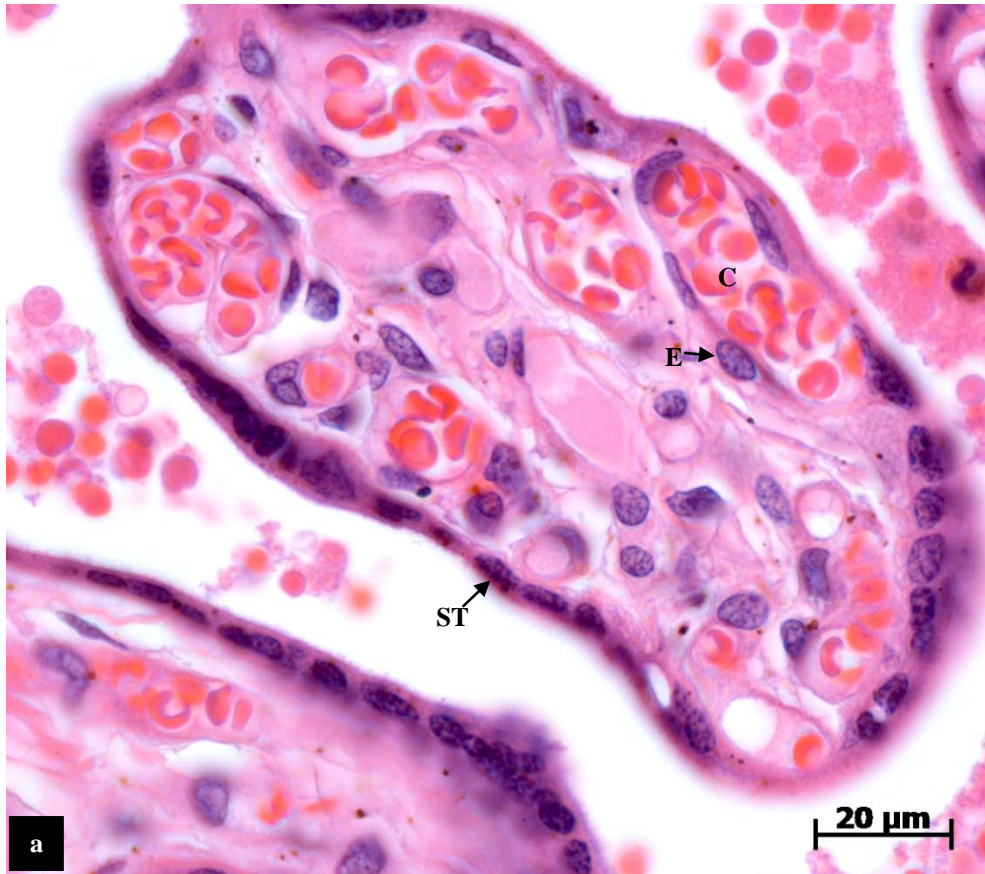


Figure 3.14 Light micrograph of H&E stained villi illustrating. (a) intermediate villus and (b) terminal villus with a portion of intermediate villus. Note syncytiotrophoblast (ST), cytotrophoblasts (CT), capillary (C) and endothelium lining of capillaries (E).

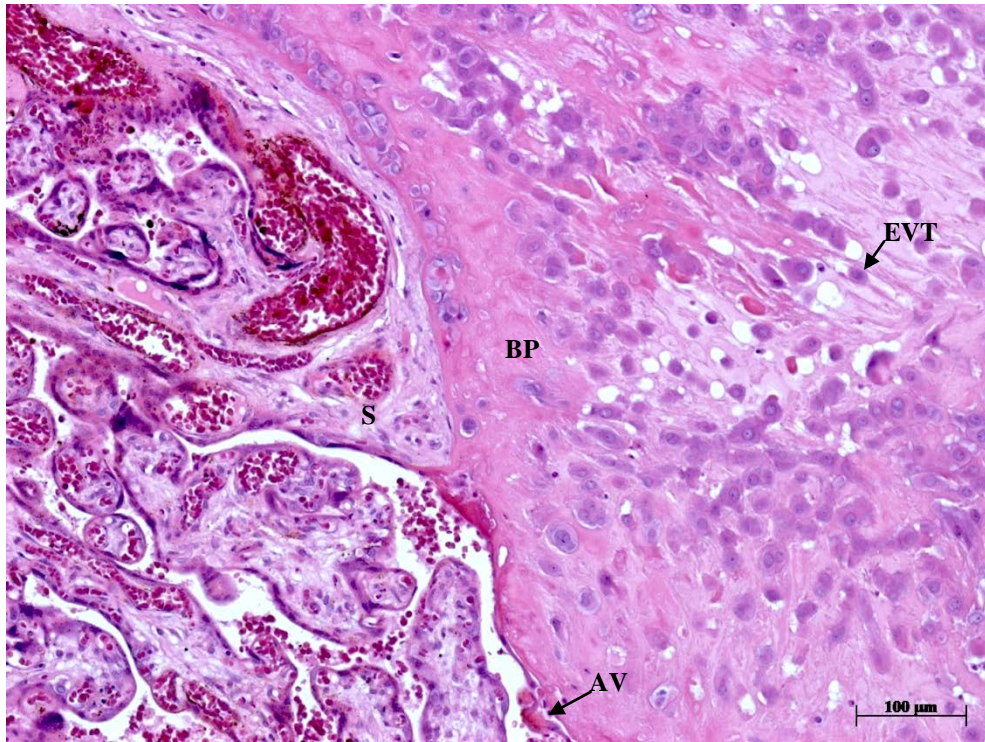


Figure 3.15 Light micrograph of H&E stained placental septum (S) resting on the basal plate (BP). Note anchoring villi (AV) and streams extra-villous trophoblasts (EVT)

3.6.1 Pathological Observations

An acellular eosinophilic, perivillous fibrinoid type, material was observed in the region of the syncytiotrophoblasts. This was referred to as perivillous fibrin (Figure 3.16a). In some instances the fibrinoid replaced the stroma beneath an intact trophoblast and was then referred to as an intravillous fibrinoid (Figure 3.16b). Both type of fibrinoid were present in all study groups but were much more prevalent in pre-eclamptic compared to the normotensive group. This fibrinoid was also more prevalent in HIV positive compared to HIV negative placentae. Villi obliterated by intravillous fibrinoid lacked vascularisation (Figure 3.16b). Additionally, a large number of Hofbauer cells were observed in the areas with fibrin deposits.

Peripheral clustering of syncytiotrophoblast nuclei, called syncytial knots were noted in all the study groups but occurred less frequently in the normotensive groups. Excessive syncytial knotting was present in pre-eclampsia. The knots comprised of a densely clumped agglomeration of syncytial nuclei (Figure 3.17). The knots often connected adjacent nuclei, a feature referred to as syncytial bridges (Figure 3.18).

Marked thickening of the basement membrane of the syncytiotrophoblast was observed in the pre-eclamptic (HIV positive and negative) groups (Figure 3.19a). There was a qualitative increase in cytotrophoblasts in all villus types in the pre-eclamptic groups (Figure 3.19b, Figure 3.20a & b). Additionally, there appeared to be a predominance of immature intermediate villi displaying a reticular type stroma (Figure 3.21a).

Qualitative assessment revealed that the exchange villi in both the central and peripheral regions of the placenta were more densely packed in pre-eclamptic compared to normotensive placentae, irrespective of HIV status and CD4 T cell counts (Figure 3.21a and b).

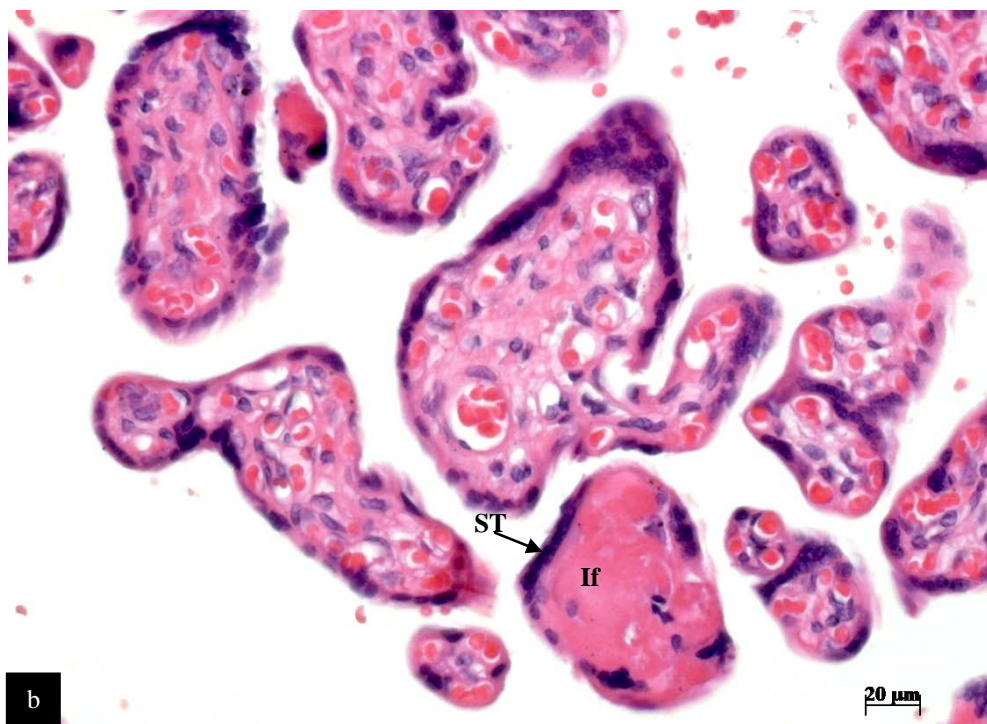
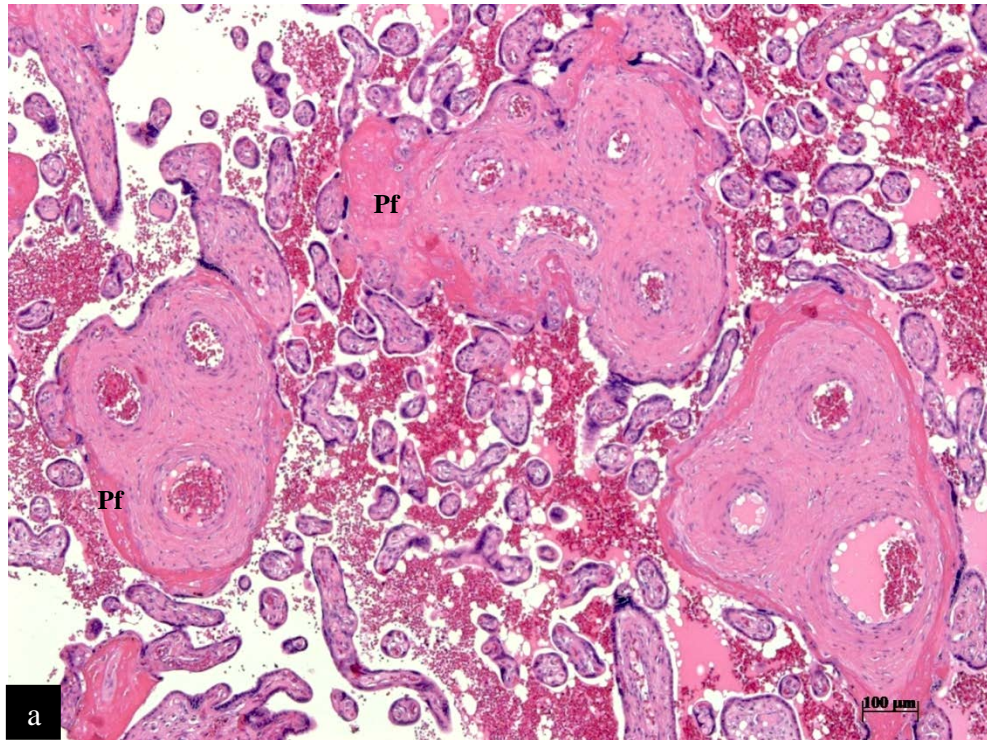


Figure 3.16 Light micrograph of H&E stained villi from a pre-eclamptic placenta showing (a) stem villi surrounded by perivillous fibrin (Pf) and (b) villous stroma obliterated by intravillous fibrin (If). Note syncytiotrophoblast (ST) surrounding intravillous fibrin.

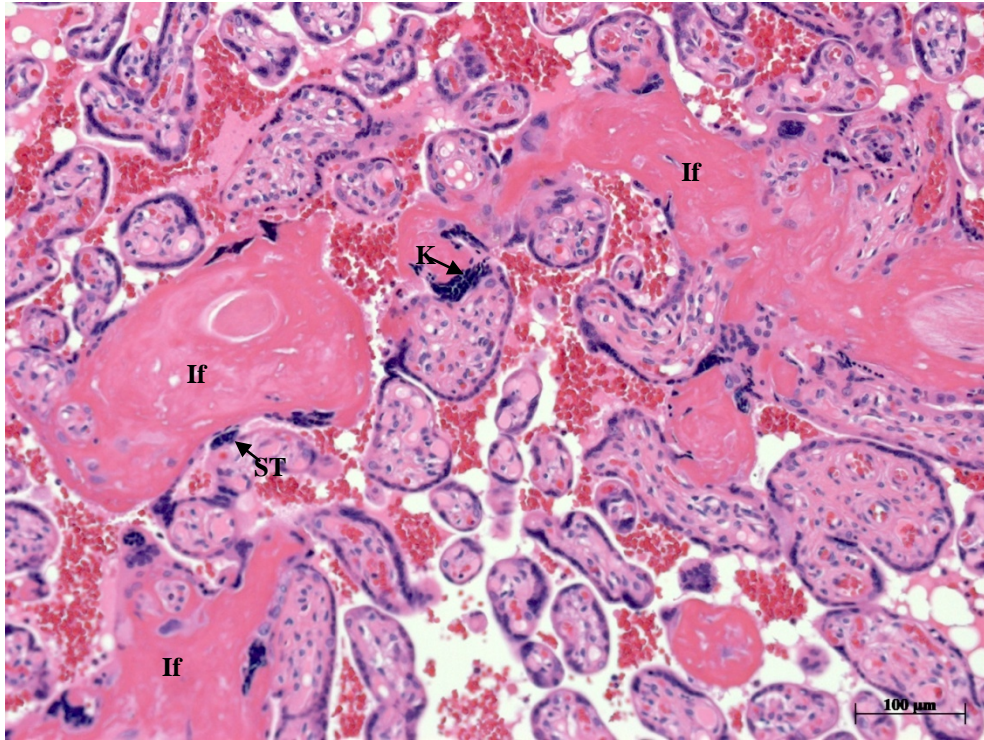


Figure 3.17 Light micrograph of H&E stained villi from pre-eclamptic placenta denoting syncytiotrophoblast (ST), extensive intravillous fibrin (If) and syncytial knots (K).

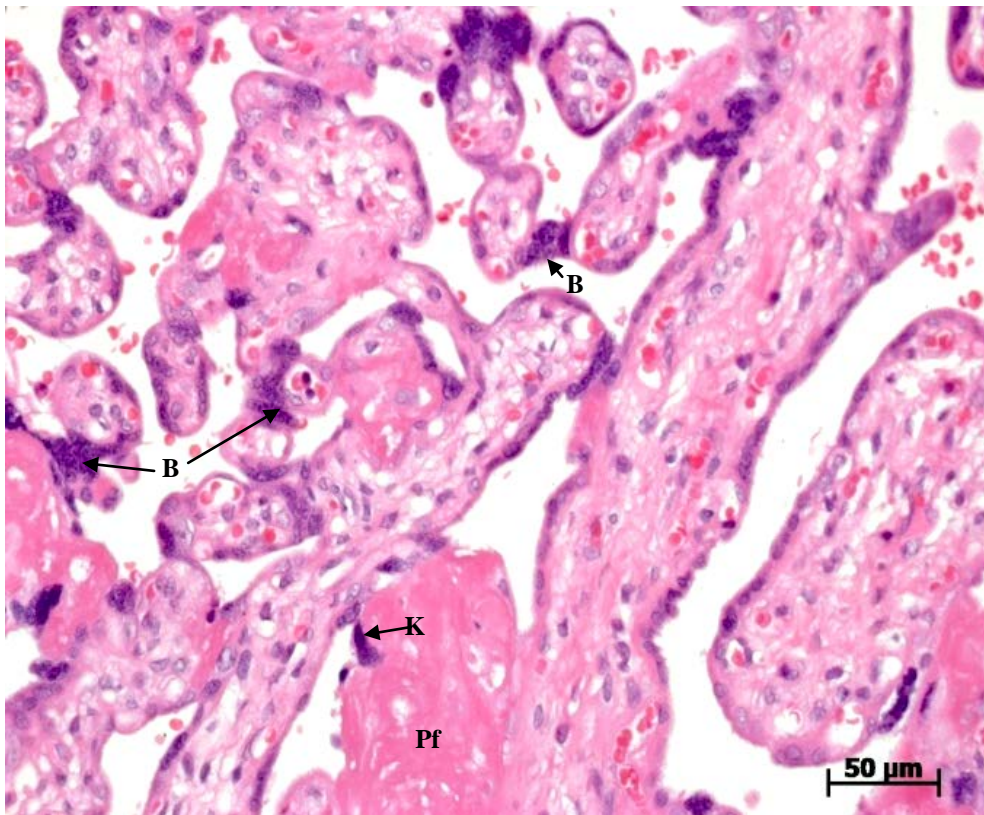


Figure 3.18 Light micrograph of H&E stained placenta featuring syncytial knots (K), bridges (B) and perivillous fibrin (Pf).

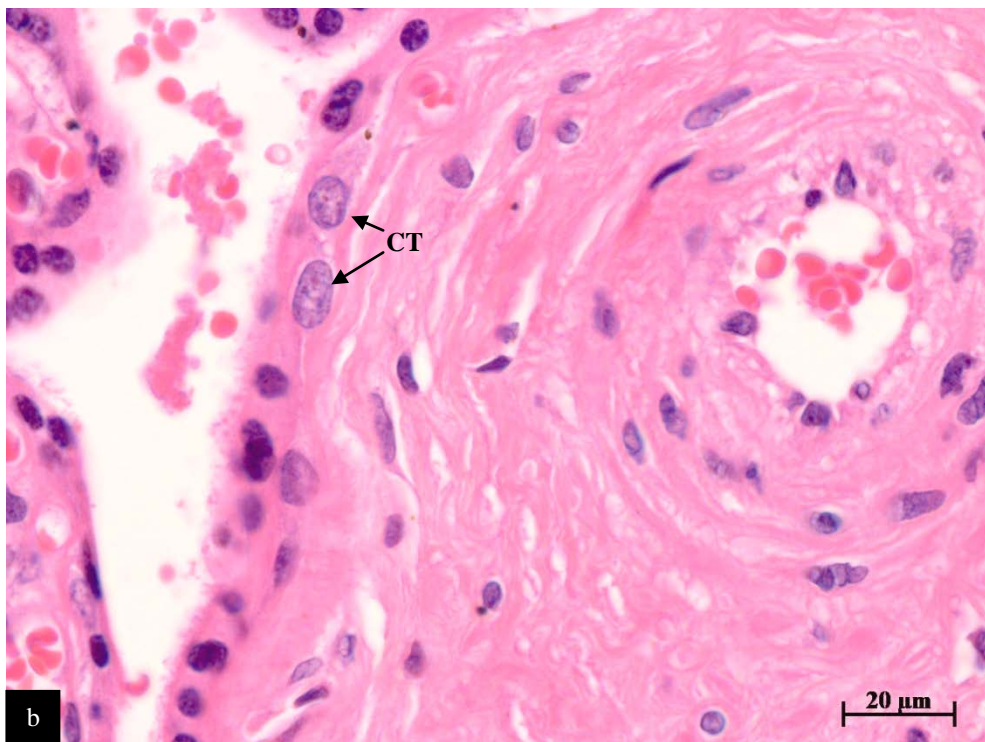
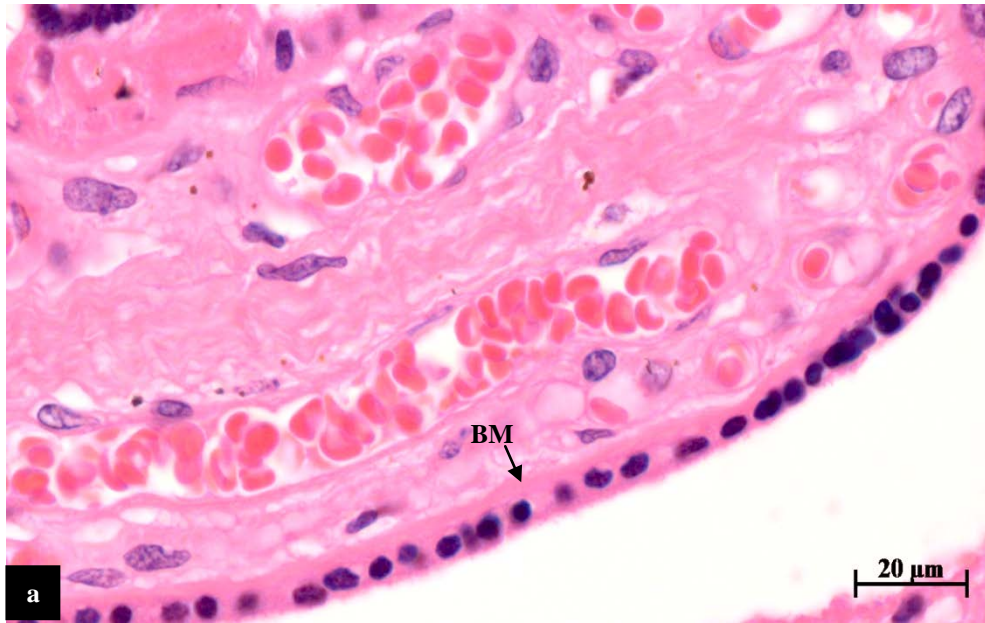


Figure 3.19 Light micrograph of H&E stained pre-eclamptic placenta denoting (a) marked thickening of the basement membrane (BM) of the syncytiotrophoblast and (b) cytotrophoblast (CT) proliferation.

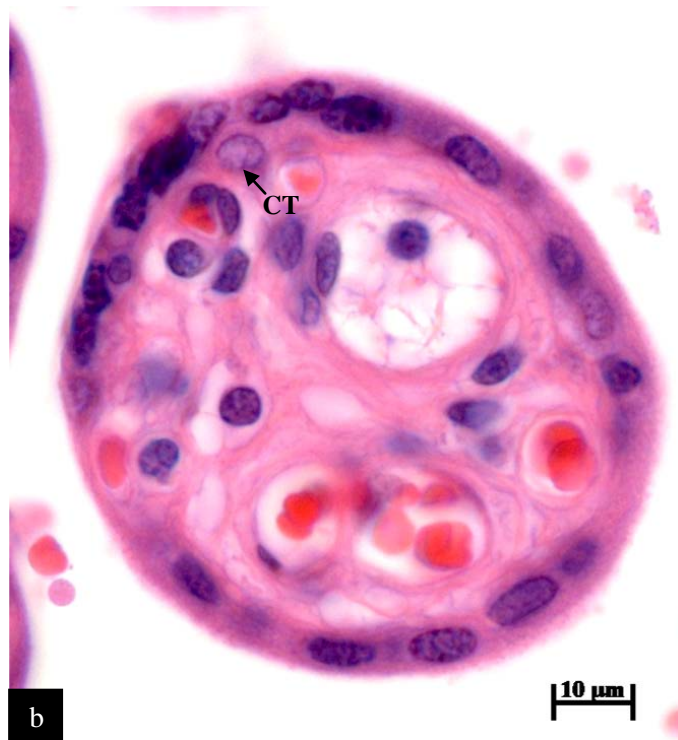


Figure 3.20 Light micrograph of H&E stained pre-eclamptic placenta illustrating cytotrophoblasts (CT) in (a) intermediate and (b) terminal villi.

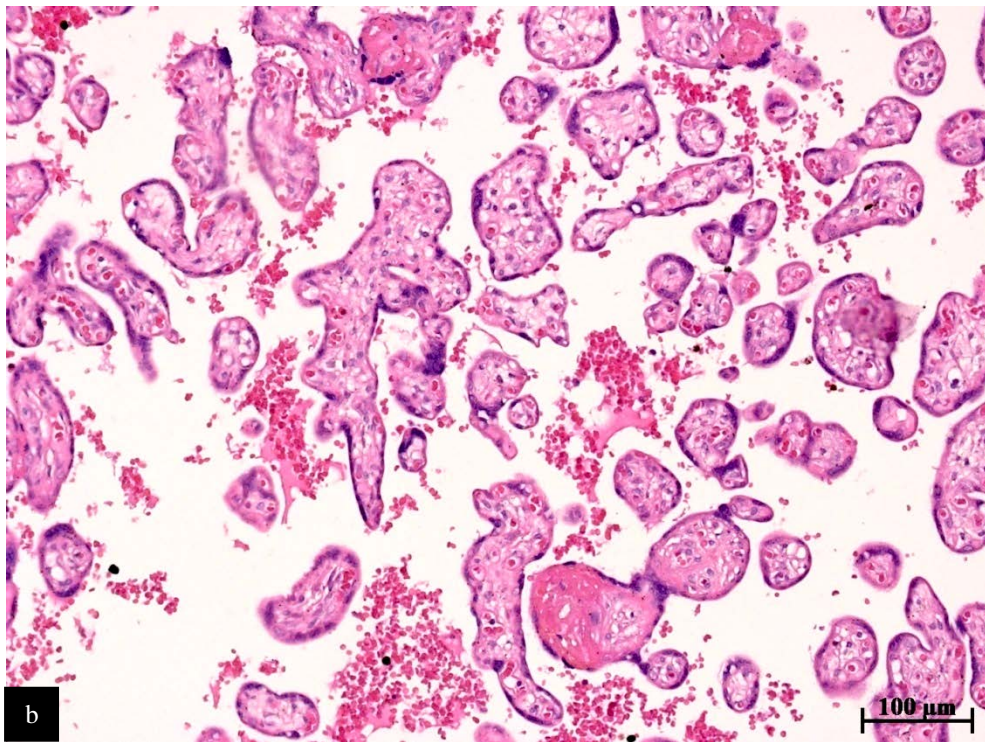
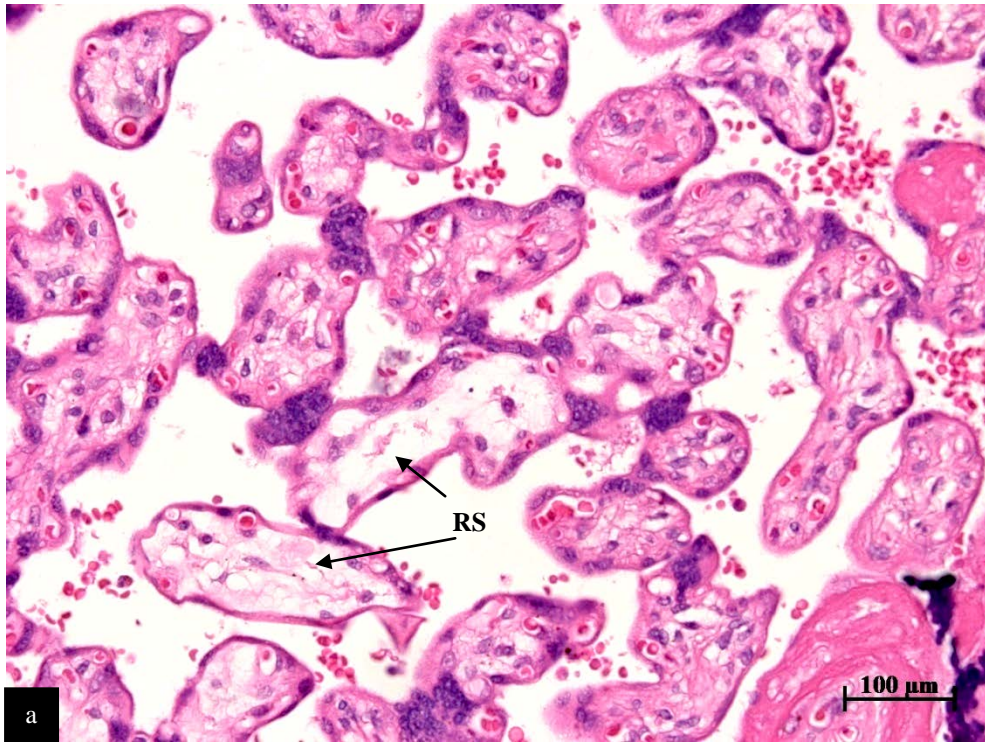


Figure 3.21 Light micrograph of H&E stained (a) pre-eclamptic and (b) normotensive placenta illustrating immature intermediate villi with reticular stroma (RS) in pre-eclampsia. Note the more densely packed villi in (a) pre-eclampsia compared to (b) normotensive pregnancy.

3.7 LEPTIN IMMUNO-LOCALISATION

Breast tissue was used as a positive control for leptin immuno-staining. Adipose tissue within the breast tissue stained positive for leptin (Figure 3.22a). In the negative control, both replacement of the primary antibody with buffer and with a non-immune serum of the same IgG class produced an absence of immuno-reactivity (Figure 3.22b and c, respectively).

The amniotic epithelium, when present, was immuno-positive for leptin (Figure 3.23a). Beneath this layer, fibroblasts were immuno-positive in the mature chorionic plate (Figure 3.23b). Extra villous cytotrophoblasts were immuno-positive in the Langhans' fibrinoid of this plate (Figure 3.23b)

In all study groups, leptin immuno-reactivity was observed within the different trophoblast cell population viz., extravillous trophoblasts (EVT), cytotrophoblasts and the syncytiotrophoblasts. Leptin immuno-precipitation was similar in the syncytiotrophoblast and cytotrophoblast cells (Figure 3.24).

Within the stem villi, the endothelial cells lining arteries (Figure 3.25a) and veins (Figure 3.25a and b) were immuno-reactive. Additionally, smooth muscle fibres within the medial layer of the vessel walls were immuno-positive for leptin. Furthermore, fibroblasts and Hofbauer cells were immuno-positive (Figure 3.25b). In the HIV positive groups, peripheral blood mono-nucleocytes were observed (Figure 3.25b). The mesenchymal stroma did not contain leptin.

Intermediate villi displayed strong immuno-reactivity of the syncytiotrophoblast layer (Figure 3.26a). Syncytial knots and syncytial bridges also showed positive staining for leptin (Figure 3.26b). The endothelium lining of capillaries and sinusoids of these villi were also immuno-positive (Figure 3.26a). Similarly, the syncytiotrophoblast and cytotrophoblast of terminal villi were immuno-reactive for leptin (Figure 3.27a).

Avascular fibrinoid villi displayed immuno-reactive syncytiotrophoblast (Figure 3.27b), however the fibrin displayed no immuno-staining. Similarly, perivillous and intravillous fibrin displayed no immuno-reactivity but trophoblast cells within this fibrin were immuno-positive (Figure 3.28a and b).

Extravillous trophoblast cells within the basal plate (maternal aspect, Figure 3.29a) and chorionic plate (fetal aspect), when present, were immuno-positive for leptin (Figure 3.29b & c).

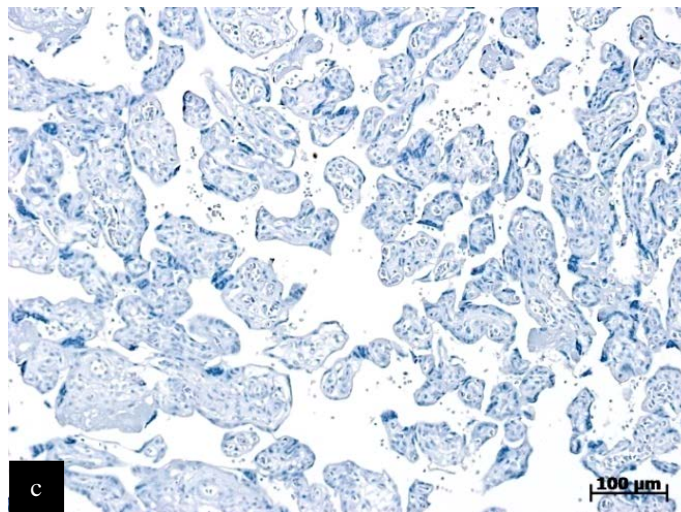
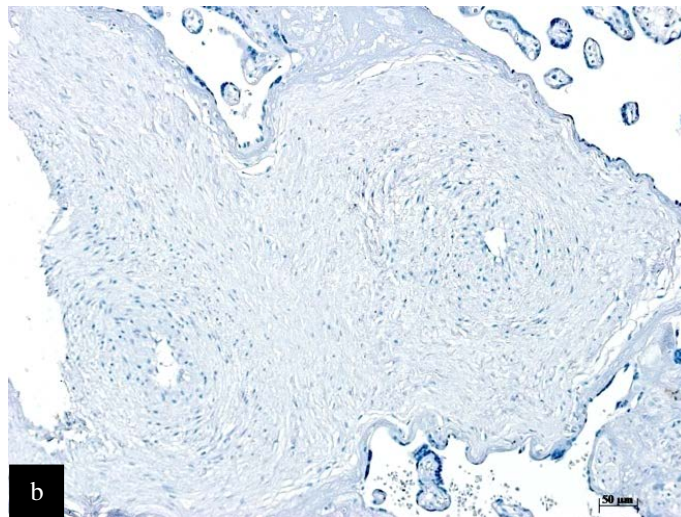
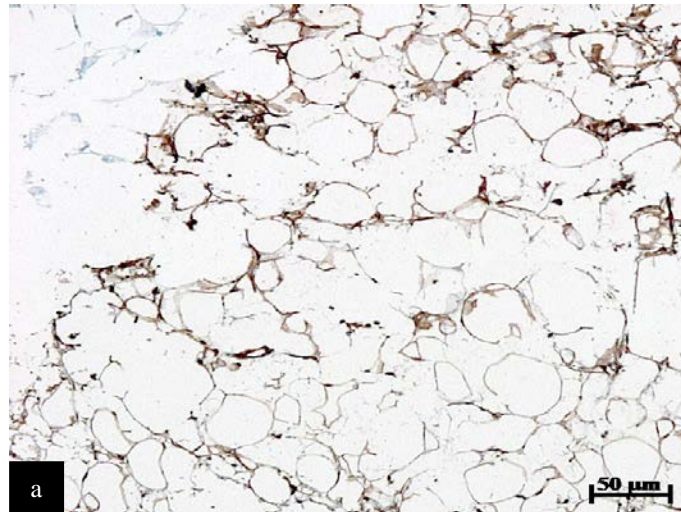


Figure 3.22 Light micrographs indicating (a) positive and (b & c) negative controls. (a) Positive control: breast tissue adipocytes stained positive for leptin. Immuno-staining is absent in (b) negative buffer control and (c) non-immune IgG control.

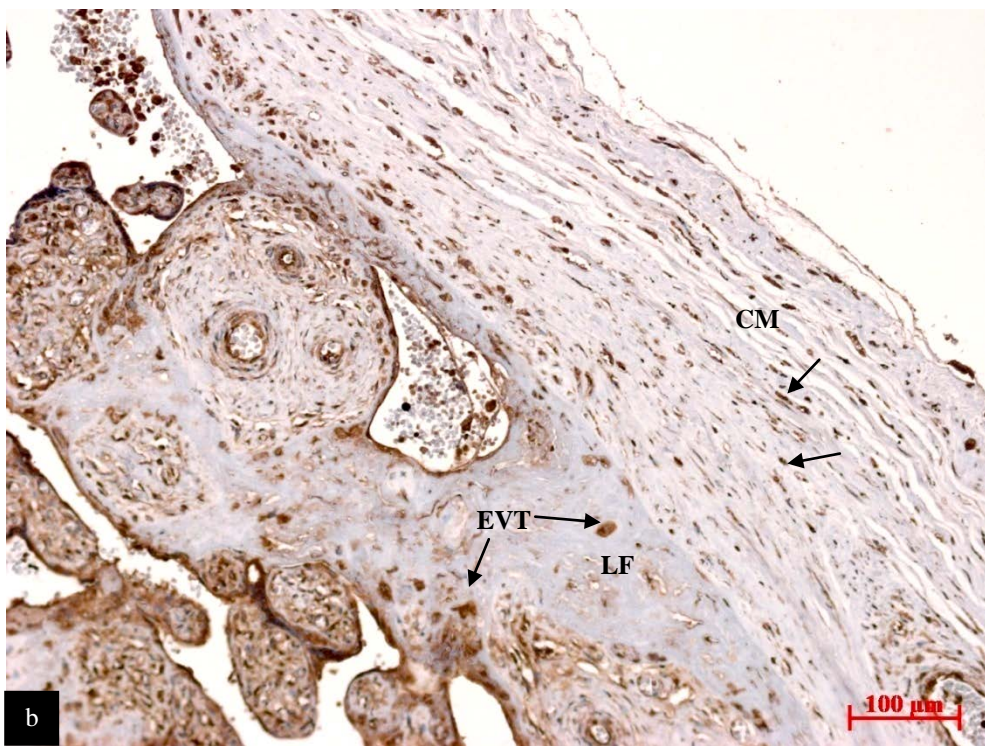
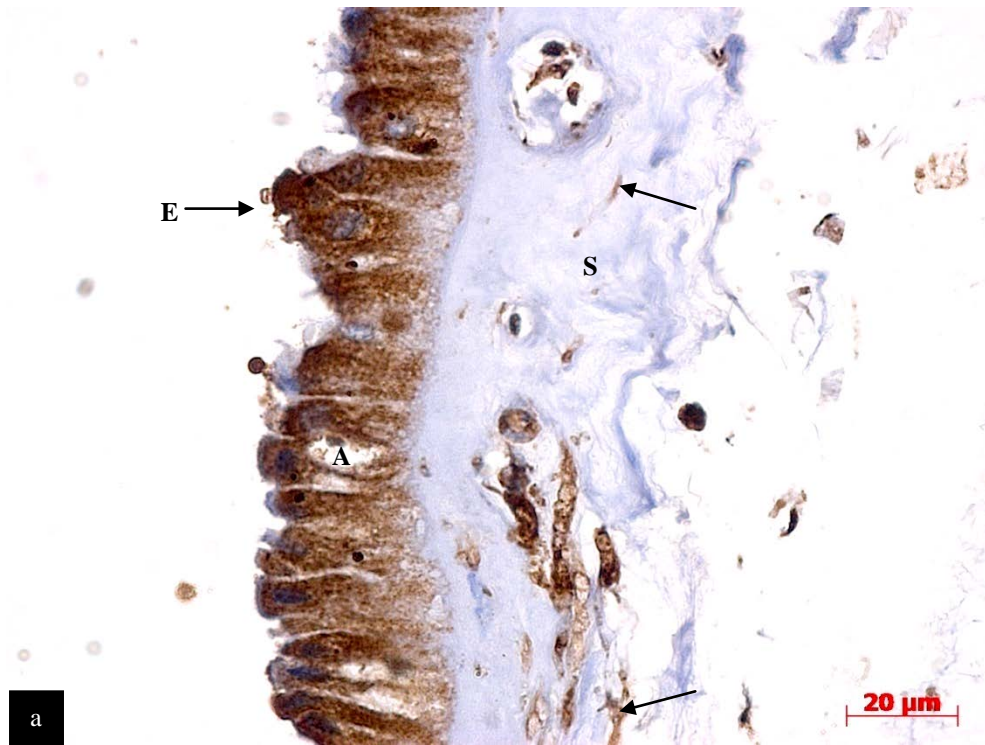


Figure 3.23 Light micrograph of immuno-stained placental membranes illustrating (a) amnion and (b) chorion. Strong immuno-staining is present within amniotic epithelial cells (E) as well as immuno-reactive fibroblast like cells in spongy layer [arrow, (a)]. Chorionic mesenchyme with distinct immuno-positive fibroblasts [(arrow, (b))]. Note extra villous trophoblast (EVT) in Langhans' fibrinoid (LF).



Figure 3.24 Light micrograph depicting intense leptin immuno-reactivity within syncytiotrophoblasts (ST) and cytotrophoblast (CT).

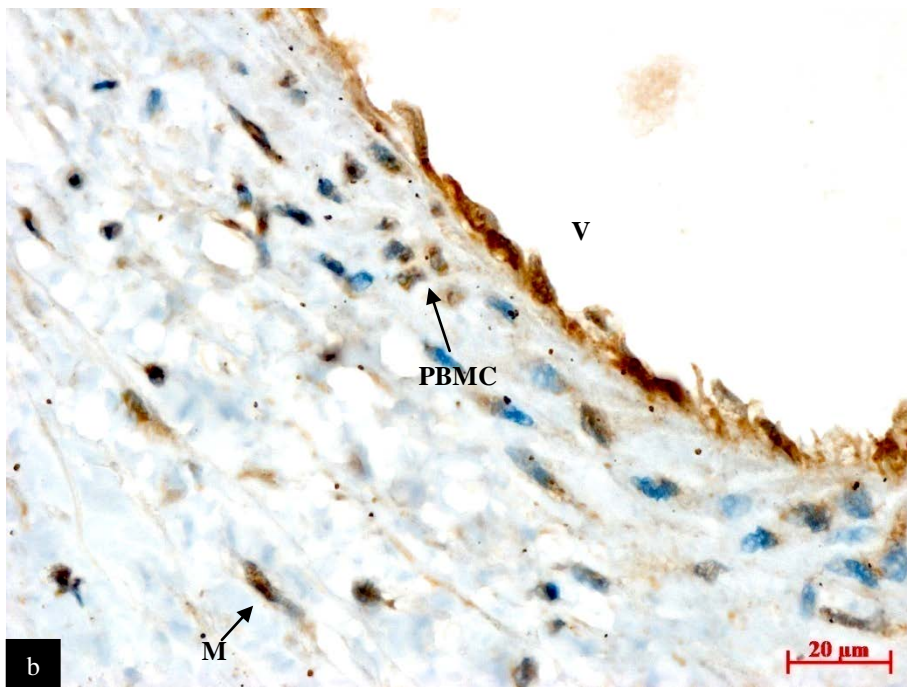
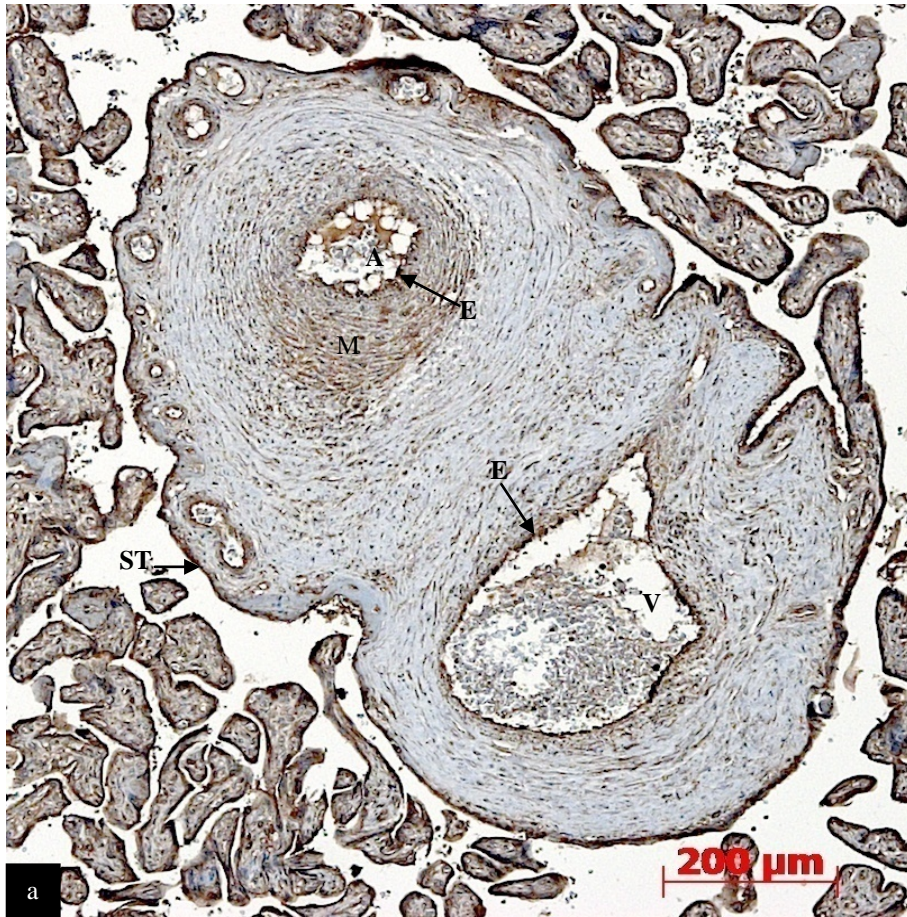


Figure 3.25 Light micrographs illustrating immuno-staining pattern within stem villus at low (a) and high (b) magnification. Note positive stain for leptin in syncytiotrophoblast (ST), endothelial cells (E) of artery (A) and vein (V), smooth muscle cells (M) and peripheral blood mono-nucleocytes (PBMC).

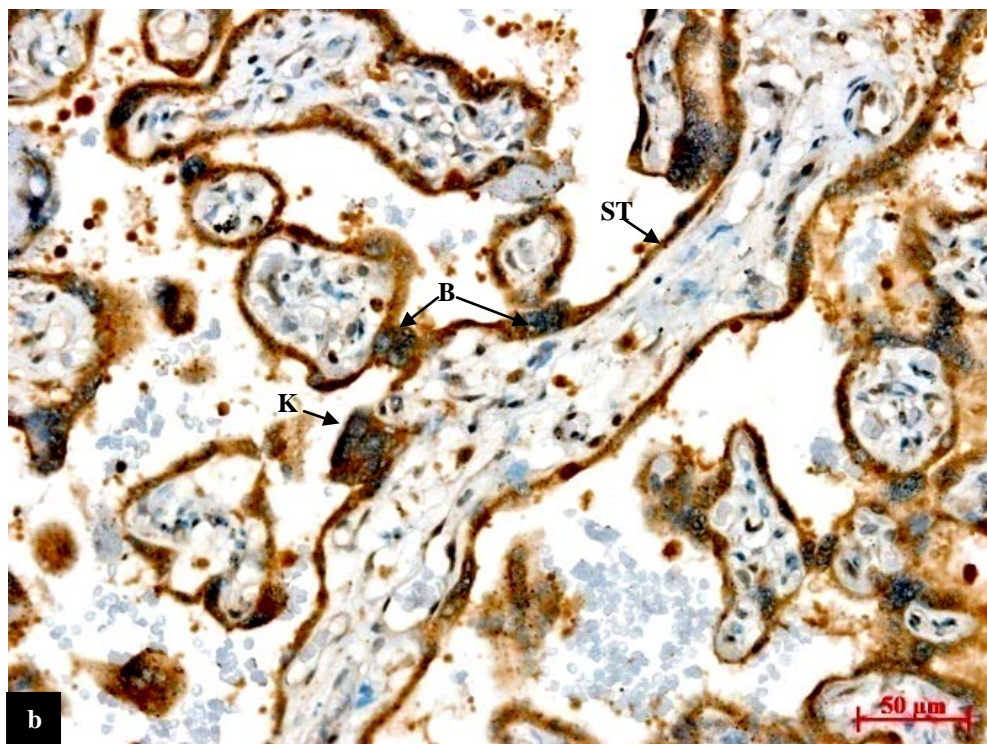
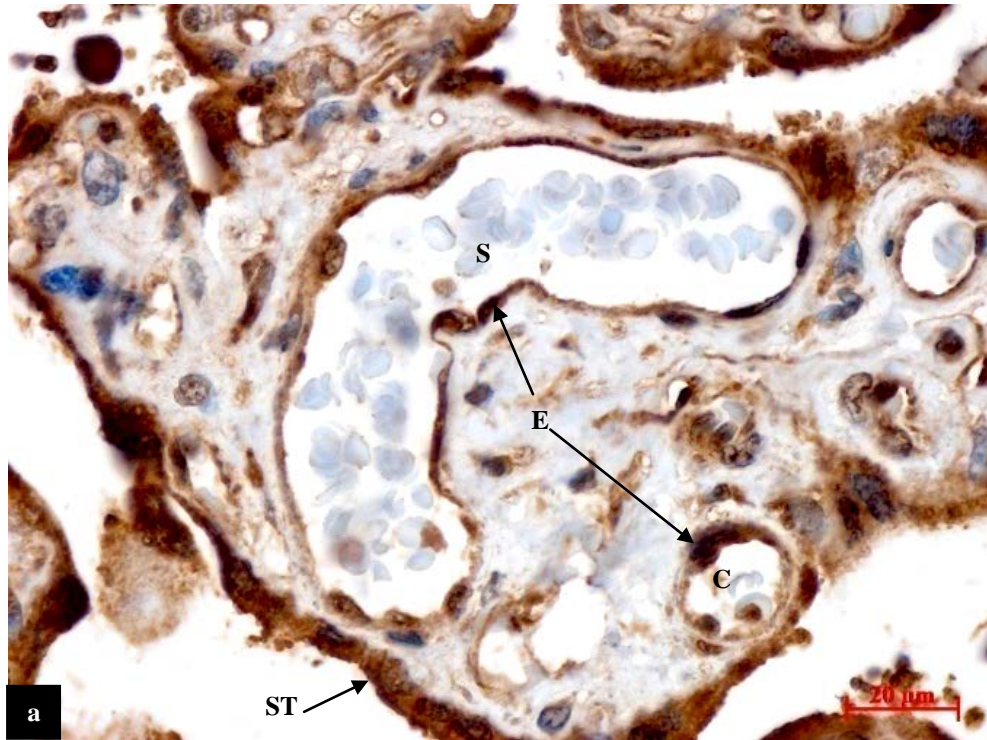


Figure 3.26 Light micrograph of immuno-stained intermediate villi. (a) Note immuno-reactivity within syncytiotrophoblast (ST), endothelial cell (E) of capillaries (C) and sinusoids (S). (b) Note the extensive knots (K) and syncytial bridges (B) in pre-eclampsia.

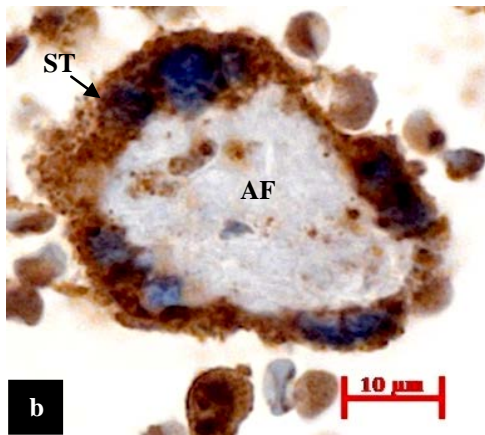
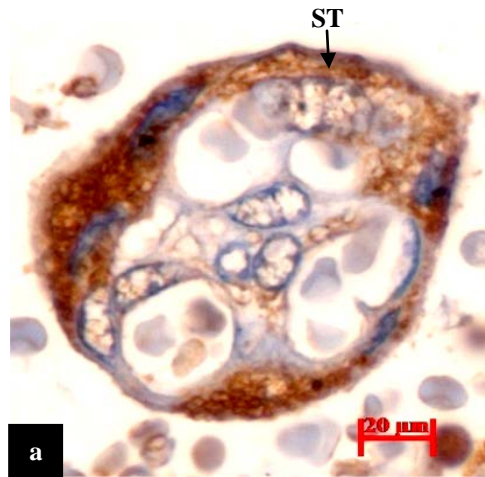


Figure 3.27 Light micrograph of immuno-stained terminal villi. (a) Note immuno-reactivity within syncytiotrophoblast (ST). (b) Note immuno-reactive syncytiotrophoblast (ST) in avascular fibrinoid (AF).

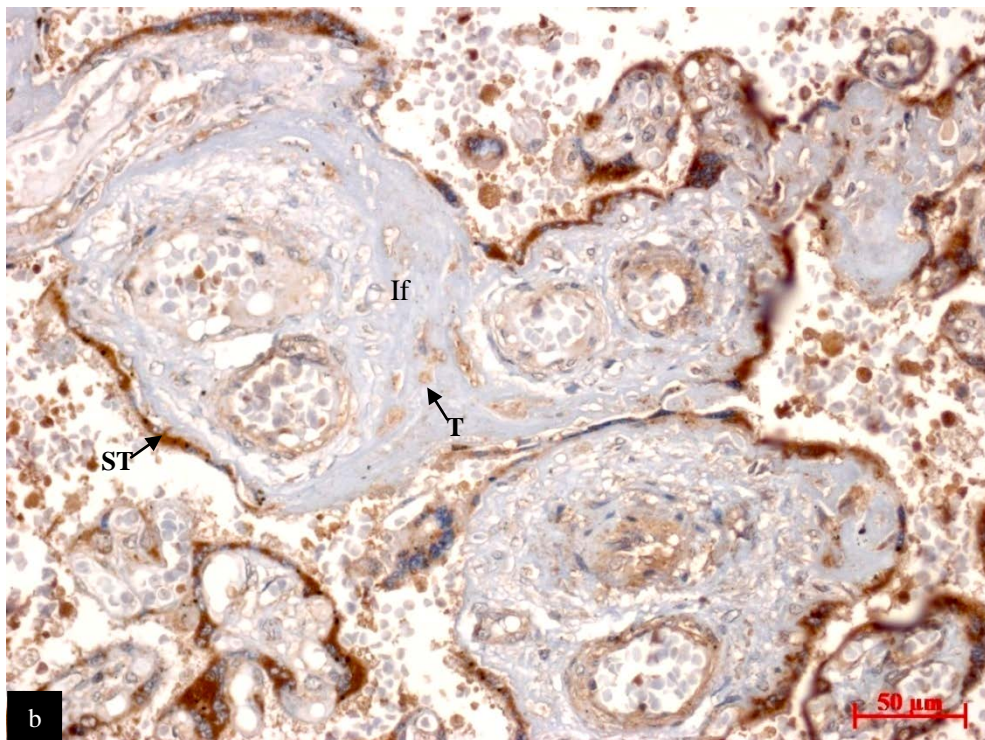
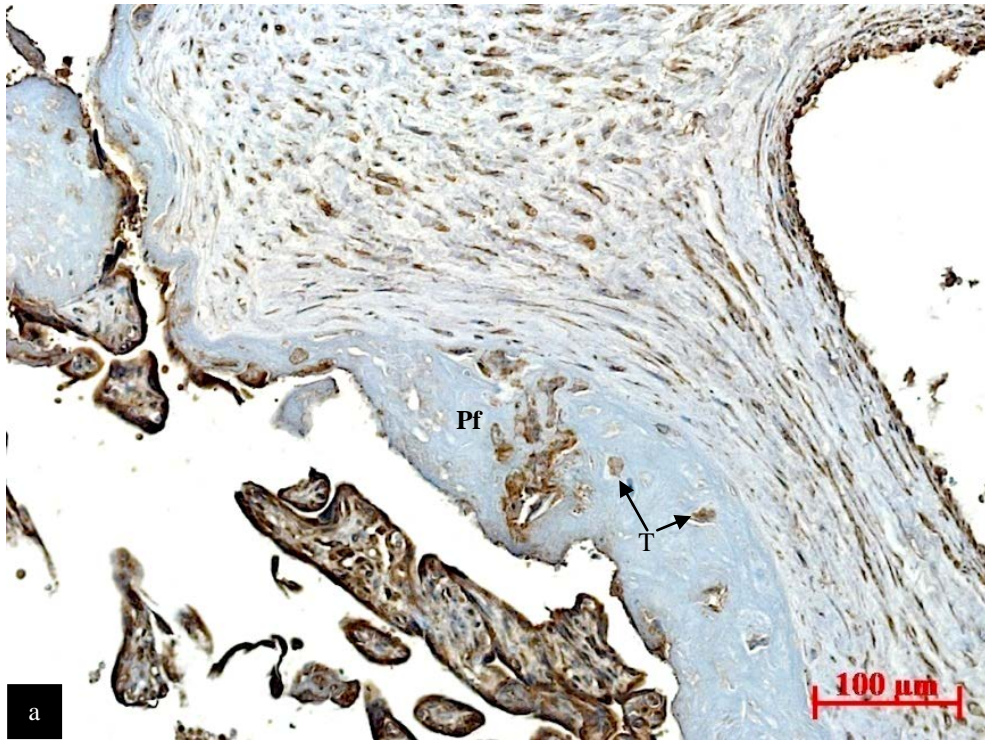


Figure 3.28 Light micrograph depicting positive immuno-reactivity of trophoblast cells (T) embedded within (a) perivillous fibrin and (b) intravillous fibrin. Note the fibrin displays an absence of immuno-staining.

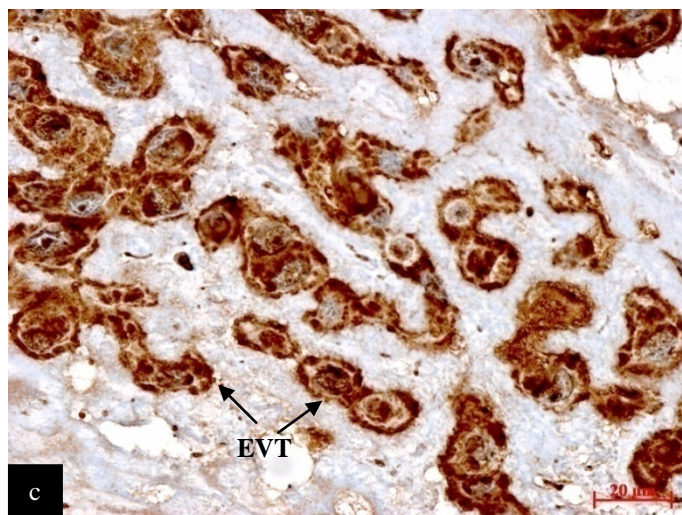
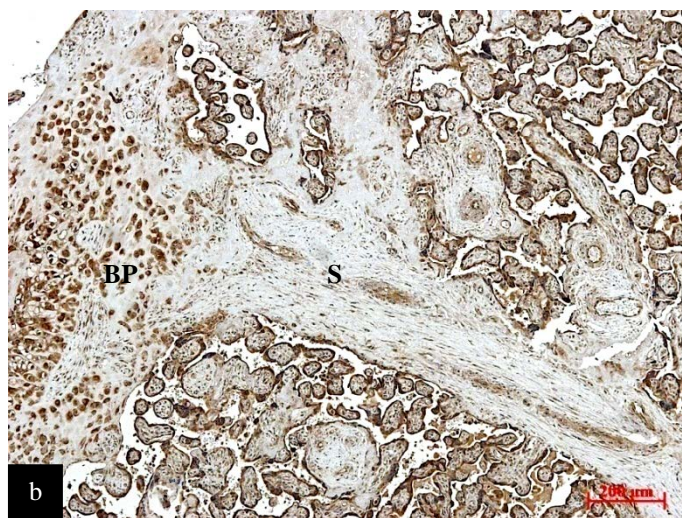
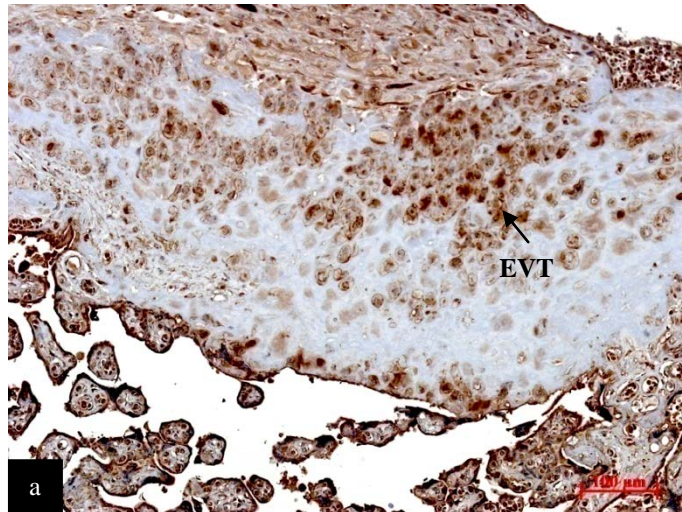


Figure 3.29 Light micrograph illustrating immuno-staining of extra villous trophoblasts (EVT) within (a) basal plate and chorionic plate at (b) low and (c) high magnification. Note septa (S).

3.8 MORPHOMETRIC IMAGE ANALYSIS OF LEPTIN

Immuno-reactive expression of leptin as a mean field area percent of the field of view as well as the intensity of the immuno-staining (expressed as a densitometric grey scale of 0 – 255) of leptin was quantified within both peripheral and central zones of the placenta. These results were normally distributed. Student t-tests were used to compare the intensity and the percentage area of immuno-staining of the central and peripheral regions of the placenta within study groups. Both the intensity and the percentage area of immuno-staining were similar in the central and peripheral regions of the placenta within all the study groups. This was observed in the exchange (intermediate and terminal) as well as the conducting (stem) villi (Figures 3.30 & 3.31, Table 3.6). Therefore the average levels of leptin in the central and peripheral regions of each placenta were calculated for inter-group analysis.

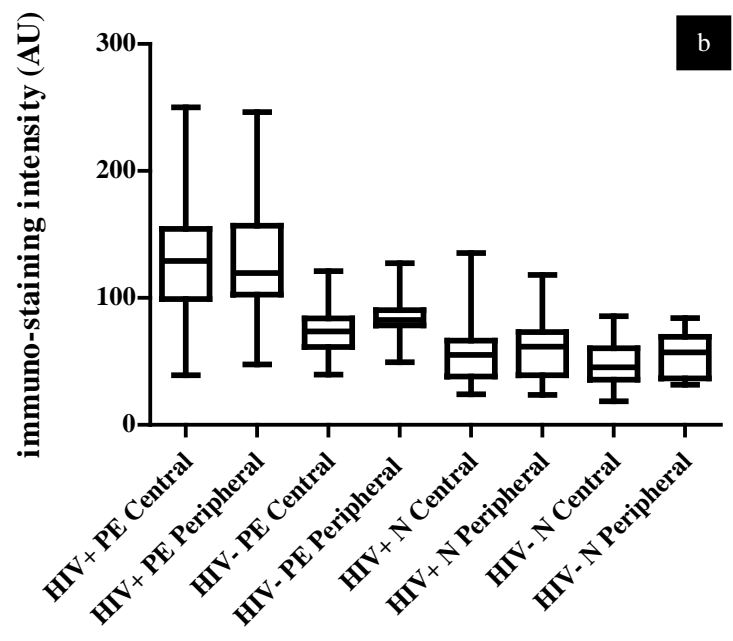
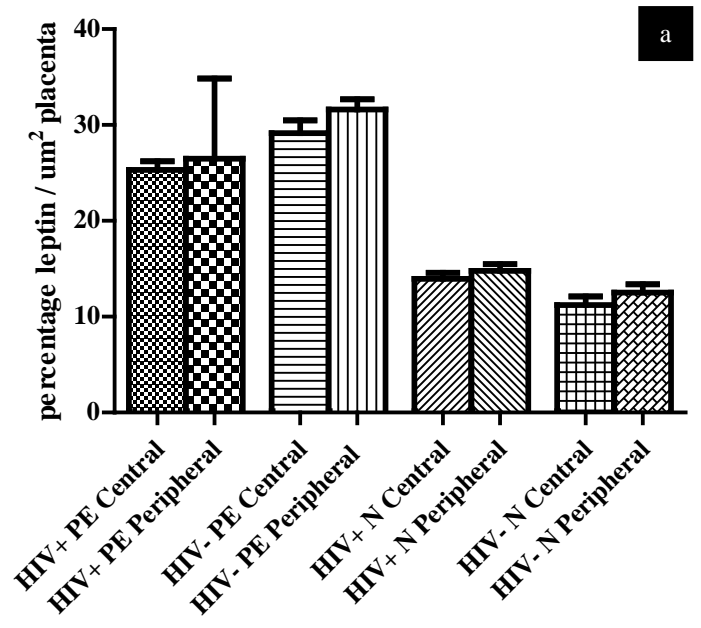


Figure 3.30 Leptin immuno-reactivity within the exchange villi of central and peripheral regions of the placenta. (a): Histogram indicating field area percentage of placental leptin immuno-reactivity (mean \pm SD) and (b): box plot indicating intensity of leptin immuno-reactivity (mean and range of staining intensity). Field area percentage and intensity of immuno-staining were similar in the central and peripheral regions of the placenta within each of the study groups.

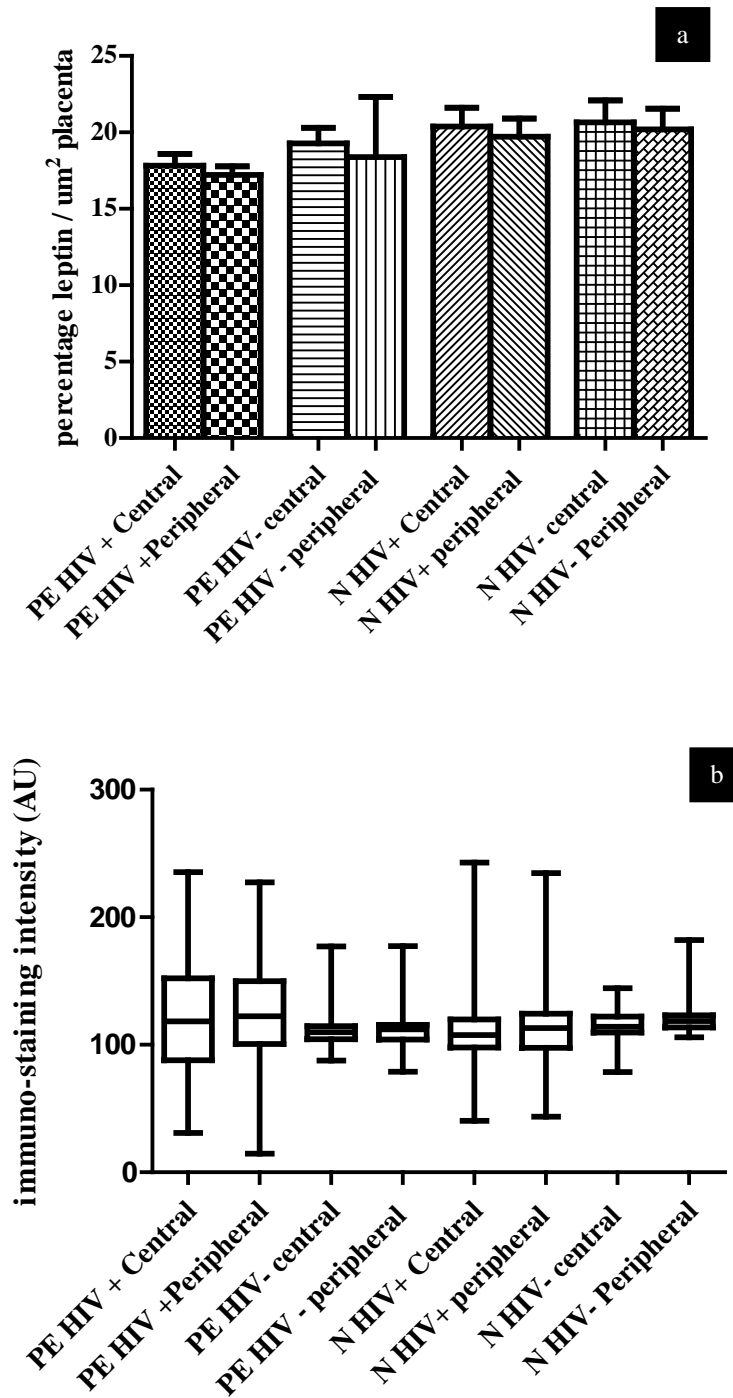


Figure 3.31 Leptin immuno-reactivity within the conducting villi of the central and peripheral regions of the placenta across study groups. (a): Histogram indicating field area percentage of placental leptin (mean \pm SD) and (b): box plot indicating intensity of leptin immuno-reactivity (mean and range of staining density). The central and peripheral regions of the placentae of each group had similar percentage area (a) and intensity (b) of immuno-staining.

3.8.1 Morphometric Image Analysis of Leptin in the Exchange Villi

3.8.1.1 Percent area immuno-staining in exchange villi

The data were normally distributed. The raw data were used in the two-way ANOVA, which indicated no significant effect of HIV status on the percentage of leptin immuno-reactivity within exchange villi [$F(1,176) = 1.459, p = 0.229$]. It however, indicated a significant effect of leptin immuno-reactivity between normotensive (mean = 13.12 $\%/\mu\text{m}^2$, CI 95%: 11.96 - 14.28) and pre-eclamptic pregnancy (mean = 28.15 $\%/\mu\text{m}^2$, CI 95%: 26.98 - 29.31; $F(1,176) = 1324.99, p < 0.001$). There was also a significant interaction between HIV status and type of pregnancy [$F(1,176) = 17.58, p < 0.001$].

The Bonferroni post hoc test showed that within HIV positive groups, there was a greater area of placental leptin immuno-staining in pre-eclampsia (mean = 25.89 $\%/\mu\text{m}^2 \pm 0.67$, mean \pm SEM) compared to normotensive pregnancy (14.36 $\%/\mu\text{m}^2 \pm 0.67, p < 0.001$). Similarly, within HIV negative groups, pre-eclampsia also had higher percentage of leptin immuno-staining (mean = 30.40 $\%/\mu\text{m}^2$, 95% CI: 28.53 - 32.26) compared to normotensive pregnancies (mean = 11.88 $\%/\mu\text{m}^2$, 95% CI: 10.01 - 13.74, $p < 0.001$). The percent area of immuno-staining was also significantly higher in HIV negative pre-eclampsia (mean = 30.40 $\%/\mu\text{m}^2$, 95% CI: 28.53 - 32.26) compared to HIV positive pre-eclampsia (mean = 25.90 $\%/\mu\text{m}^2$, 95% CI: 24.58 - 27.21, $p = 0.001$, Table 3.8, Figure 3.32).

Table 3.8 Percent area immuno-reactivity in exchange villi of study groups

PREGNANCY	HIV	Mean (%/μm ²)	Std. Error	95% Confidence Interval	
				Lower	Upper
Normotensive	Negative (n = 30)	11.88	0.95	10.01	13.74
	Positive (n = 60)	14.36	0.67	13.05	15.68
Pre-eclamptic	Negative (n = 30)	30.40	0.95	28.53	32.26
	Positive (n = 60)	25.90	0.67	24.58	27.21

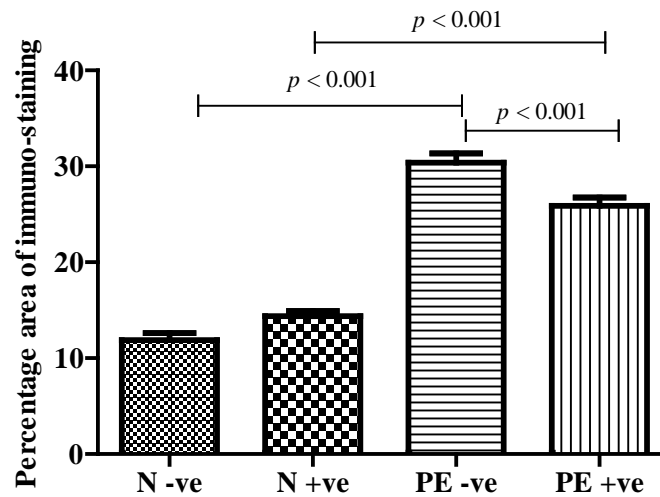


Figure 3.32 Histogram illustrating mean field area percentage of leptin immuno-reactivity in the exchange villi of study groups. Immuno-precipitation of leptin was greater in pre-eclampsia compared to normotensive pregnancies in both HIV positive and negative groups. Immuno-precipitation was also enhanced in HIV negative pre-eclampsia compared to HIV positive pre-eclampsia.

3.8.1.2 Intensity of immuno-staining in exchange villi

The data were normally distributed. The raw data were used in the two-way ANOVA, which indicated a main effect of HIV status on the intensity of immuno-staining in the exchange area of the placentae ($F(1,176) = 49.96, p < 0.001$). There was a significantly higher intensity of immuno-staining in HIV positive [mean = 95.07 arbitrary units (AU), CI 95%: 90.29 - 99.84] compared to HIV negative groups (mean = 65.42 AU, CI 95%: 58.66 - 72.17). There was also a main effect of type of pregnancy (normotensive vs pre-eclamptic, $F(1,176) = 148.39, p < 0.001$). The intensity of placental leptin immuno-staining was higher in pre-eclampsia (mean = 105.79 AU, CI 95%: 99.93 - 111.64) compared to normotensive pregnancy (mean = 54.69 AU, CI 95%: 48.84 - 60.55). Finally, there was a significant interaction between HIV and pregnancy status [$F(1,176) = 29.77, p < 0.001$].

The Bonferroni post hoc test showed that within HIV positive groups, the intensity of immuno-staining was higher in pre-eclampsia (mean = 132.05 AU, CI 95%: 125.42 - 138.69) compared to normotensives (mean = 58.07 AU, CI 95%: 51.44 - 60.71, $p < 0.001$, Table 3.37, Figure 3.33). Similarly within HIV negative pregnancies, the intensity of immuno-staining was higher in pre-eclampsia (mean = 79.52 AU, CI 95%: 70.14 - 88.91) compared to normal pregnancies (mean = 51.31 AU, CI 95%: 41.93 - 60.69, $p < 0.001$). Within the pre-eclamptic groups, the intensity of immuno-staining was significantly higher in the HIV positive pregnancies (mean = 132.05 AU, CI 95%: 125.42 - 138.69) compared to the HIV negative pregnancies (mean = 79.52 AU, CI 95%: 70.14 - 88.91, $p = 0.002$, Table 3.37, Figure 3.33).

Table 3.9 Intensity of immuno-staining in exchange villi of placenta

PREGNANCY	HIV	Mean (AU)	Std. Error	95% Confidence Interval	
				Lower (AU)	Upper (AU)
Normotensive	Negative (n = 30)	51.31	4.79	41.93	60.69
	Positive (n = 60)	58.07	3.39	51.44	60.71
Pre-eclampsia	Negative (n = 30)	79.52	4.79	70.14	88.91
	Positive (n = 60)	132.05	3.39	125.42	138.69

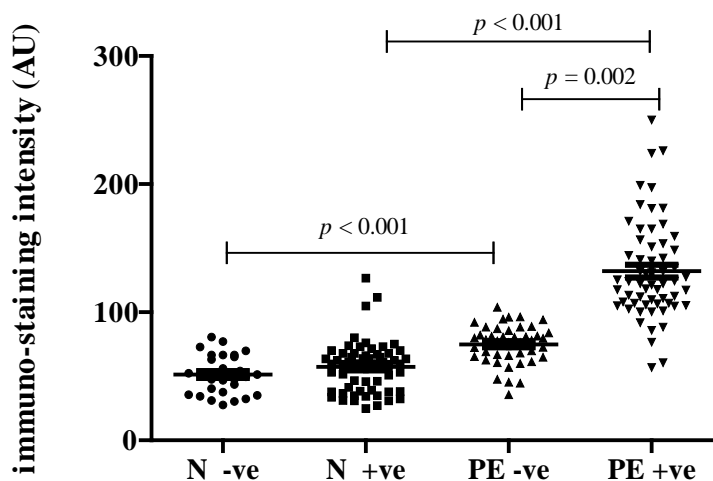


Figure 3.33 Scatter plot illustrating mean intensity of leptin immuno-staining in the exchange area of the placenta in the study groups. The staining intensity for leptin was enhanced in pre-eclampsia compared to normotensive pregnancy in both HIV positive and negative groups. In pre-eclampsia, the staining intensity was greater in the HIV positive group compared to the HIV negative group.

3.8.2 Morphometric Image Analysis of Leptin in the Conducting Villi

3.8.2.1 Percent area immuno-staining in the conducting villi

The data were normally distributed. The raw data were used in the two-way ANOVA, which indicated no significant effect of HIV status on the percentage of leptin immuno-reactivity within conducting villi [$F(1,176) = 0.738, p = 0.39$]. There was a main effect of pregnancy status (normotensive vs pre-eclamptic) on the percent area of leptin immuno-reactivity [$F(1,76) = 4.441, p = 0.03$]. This effect showed that the percent area that was immuno-stained was lower in pre-eclampsia (mean = 17.96, CI 95%: 17.12 - 18.80) compared to normal pregnancy (mean = 20.17, CI 95%: 18.50 - 21.85). Finally, there was no interaction between HIV status and type of pregnancy, $F(1,176) = 0.237, p = 0.627$ (Table 3.4, Figure 3.29a). Bonferroni post tests indicated that the area of immuno-staining within the conducting placental villi was similar in all study groups (Table 3.10).

Table 3.10 Area of immuno-staining of conducting placental villi

PREGNANCY	HIV	Mean	Std.	95% Confidence Interval	
		(%/μm ²)	Error	Lower	Upper
Normotensive	Negative (n = 30)	20.42	1.15	18.17	22.66
	Positive (n = 60)	20.05	0.80	18.48	21.63
Pre-eclamptic	Negative (n = 30)	18.84	1.09	16.71	20.97
	Positive (n = 60)	17.52	0.77	16.02	19.03

3.8.2.2 Intensity of immuno-staining in the conducting villi

The data were not normally distributed across the comparative groups, thus transformations (logarithmic and square root) were applied, but neither transformation could correct the data distribution. The Kruskal-Wallis test, with Dunn's post hoc test was thus used for the statistical analysis. These tests indicated that the intensity of immuno-staining of the conducting villi was similar in all the study groups ($p = 0.06$, Table 3.11).

Table 3.11 Leptin staining intensity in the conducting placental villi

PREGNANCY	HIV	Mean	Std.	95% Confidence Interval	
		(AU)	Error	Lower	Upper
Normotensive	Negative (n = 30)	118.89	5.55	108.02	129.76
	Positive (n = 60)	110.74	3.89	103.12	118.36
Pre-eclamptic	Negative (n = 30)	111.21	5.26	100.89	121.53
	Positive (n = 60)	124,51	3.75	117.15	131.86

3.8.3 Comparison of Morphometric Analysis of Leptin Immuno-expression Between the Exchange and Conducting Villi

In the normotensive groups, the field area percentage of leptin immuno-staining was greater in the conducting villi compared to the exchange villi in both the HIV positive (20.05 ± 0.80 vs 14.36 ± 0.67 , $p < 0.001$) and negative groups (20.42 ± 1.15 vs 11.88 ± 0.95 , $p < 0.001$, mean \pm SEM, Figure 3.36). In contrast, the area of immuno-staining was greater in the exchange villi compared to the conducting villi in HIV positive (25.90 ± 0.67 vs 17.52 ± 0.77 , $p < 0.001$) and negative pre-eclampsia (30.40 ± 0.95 vs 18.84 ± 1.09 , $p < 0.001$, mean \pm SEM, Figure 3.34).

The intensity of immuno-staining was greater in the conducting villi compared to the exchange villi in the HIV negative (118.89 ± 5.55 vs 51.31 ± 4.79 , $p < 0.001$) and positive normotensive groups (110.74 ± 3.89 vs 58.07 ± 3.39 , $p < 0.001$ mean \pm SEM, Figure 3.35). This enhanced staining intensity was also present in the conducting villi compared to the exchange villi of the HIV negative pre-eclamptic group (111.21 ± 5.26 vs 79.52 ± 4.79 , $p < 0.001$, Figure 3.35).

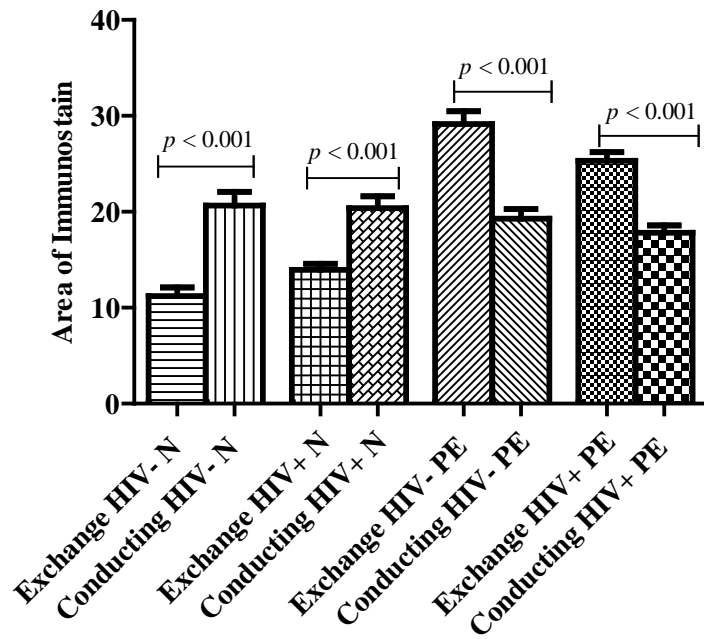


Figure 3.34 Histogram illustrating mean field area percentage of leptin immuno-reactivity in the exchange and conducting villi of study groups. Leptin immuno-reactivity was greater in the conducting villi compared to the exchange villi in the normotensive groups. In contrast, leptin immuno-reactivity was greater in the exchange compared to conducting villi in pre-eclampsia.

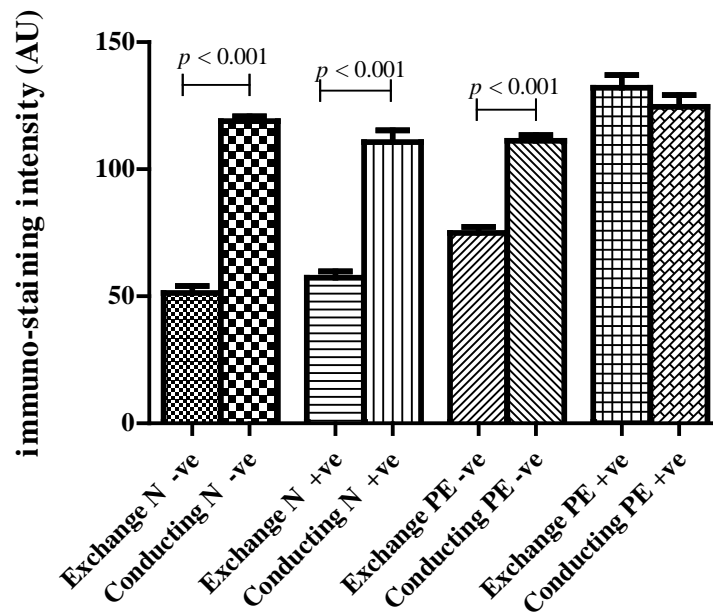


Figure 3.35 Histogram indicating the intensity of immuno-staining in the exchange and conducting villi of different study groups. The intensity of immuno-staining was greater in the conducting villi compared to the exchange villi in the normotensive groups. The staining intensity was also higher in the conducting villi compared to the exchange villi of the HIV negative pre-eclamptic group.

CHAPTER 4

DISCUSSION

The levels of the adipose derived hormone, leptin, are additionally augmented by placental production implicating a direct role in pregnancy (Haugen *et al.*, 2006; Mise *et al.*, 2007). The placenta is an important site of leptin production as well as a target for it's action, as it expresses both the *Ob* gene and it's receptor gene, *Ob-R* (Masuzaki *et al.*, 1997).

In abnormal pregnancies such as pre-eclampsia, a condition associated with new onset proteinuric hypertension, leptin levels are reported to be increased (Laivuori *et al.*, 2000; Laivuori *et al.*, 2006). As far as we are aware, there are no reports on leptin production in pregnancies compromised by HIV infection. The province of KwaZulu-Natal (KZN) in South Africa represents the epicentre of the global HIV pandemic. Notably, the highest proportion of maternal deaths in South Africa occurs within KZN (UNAIDS, 2012). The main cause of maternal deaths is non-pregnancy related infections associated with HIV/AIDS, followed by obstetric haemorrhage and hypertension (Saving mother's Report: 2008-2010, 2012).

It may be plausible to hypothesise that the immune-hyperactivity associated with pre-eclampsia may be counteracted by the lowered immunity of HIV infection. This study is novel in that it is the first investigation examining leptin expression in HIV associated pre-eclampsia. This study compares leptin protein and leptin mRNA expression between HIV infected and uninfected pre-

eclamptic pregnancies. Hence placental leptin mRNA expression, placental leptin protein concentration and placental immuno-reactivity for leptin will be sequentially discussed below. In addition, serum leptin protein concentration is compared between normotensive and pre-eclamptic HIV positive and negative pregnancies. The effect of antiretroviral therapy on HIV associated pre-eclampsia is also discussed. As the secretion of leptin by adipose tissue is affected by insulin levels, this study also evaluates the insulin levels in HIV infected and uninfected pregnancies with and without pre-eclampsia.

4.1 LEPTIN LEVELS IN THE PLACENTA (ELISA)

Using enzyme linked immunosorbent assay (ELISA), this study establishes that leptin levels are increased in the pre-eclamptic placenta irrespective of the HIV status of the study participants ($p < 0.001$). Furthermore, we establish that HIV does not have an effect on placental leptin levels ($p = 0.36$).

These findings of an elevation in the concentration of placental leptin in pre-eclampsia corroborates with the findings of previous studies (Laivuori *et al.*, 2000; Laivuori *et al.*, 2006). This placental leptin amplification may be directly extrapolated to the reduced placental perfusion and consequential placental hypoxia associated with pre-eclampsia (Mise *et al.*, 1998). Evidence supporting this hypothesis is that cultured trophoblast cells have demonstrated an increase in leptin production under hypoxic conditions (Grosfeld *et al.*, 2001).

Additionally, a reduction in intervillous space compensated by highly branched terminal villi as well as the presence of syncytial knots, sprouts and bridges, are all features of hypoxia (Benirschke *et al.*, 2006). The latter hypoxic features are well illustrated in this study suggesting that the hypoxic conditions in the pre-eclamptic placenta are linked with the augmented leptin production.

Leptin has previously been shown to induce trophoblast apoptosis (Liu *et al.*, 2009). Deficient placentation is classically associated with pre-eclampsia development. The lack of physiological conversion of the spiral artery in pre-eclampsia is associated with reduced blood flow hence the resultant hypoxia (Redman and Sargent, 2003). Thus in pre-eclampsia, the increased expression of leptin may be linked to deficient trophoblast invasion brought about by increased trophoblast apoptosis. It should also be noted that spiral arteries in pre-eclampsia may be obstructed due to acute arteriosclerosis with an accumulation of lipid laden macrophages (Redman and Sargent, 2003). Albeit in a small way, this lipid derived from the macrophages may contribute to the elevation of leptin in pre-eclampsia.

Inflammatory stimuli have also been implicated in the up-regulation of leptin expression (Sarraf *et al.*, 1997). A generalized systemic maternal inflammatory response occurs in normal pregnancy but is further exacerbated in pre-eclampsia (Redman *et al.*, 1999). The elevation of leptin concentration in the pre-eclamptic groups is thus directly linked to the maternal inflammatory response.

4.2 PLACENTAL LEPTIN mRNA

This study demonstrates an overall elevation of leptin expression by RT-PCR in the placenta of pre-eclampsia compared to normotensive pregnancy ($p = 0.04$). This up-regulation corresponds to the elevated leptin concentration observed by the ELISA tests. Leptin gene expression has previously been reported to be higher in a pro-inflammatory response (La Cava and Matarese, 2004; Otero *et al.*, 2005). As previously eluded to, pre-eclampsia is an excessive maternal immune response hence leptin gene expression would be up-regulated.

Whilst we report no overall effect of HIV on placental leptin mRNA levels ($p = 0.14$), post hoc analysis reveals that the upregulated expression of leptin by the placenta occurs only in the HIV negative pre-eclampsia ($p = 0.012$). Unpredictably, in the HIV positive groups, leptin expression using RT-PCR was similar in pre-eclampsia and normotensive pregnancies ($p = 1.00$). These results differ from the leptin concentration obtained by ELISA. This discrepancy may be attributed to the short half life of leptin which approximates 10 hours (Yang *et al.*, 2003). This half-life constraint would play a regulatory role in the transcription of this protein (Yang *et al.*, 2003). An alternate explanation has demonstrated that when leptin concentration in the trophoblast cells is high, then the further secretion of leptin by these cells is decreased (Liu *et al.*, 2009).

It must also be noted that the range of leptin mRNA across the different placentae is large and thus a possible confounding factor. The leptin levels in the different groups overlap with each

other possibly reflecting a continuum of immune response of the mother from normal to pre-eclamptic pregnancies. As reported previously, pre-eclampsia is not a separate condition but an extreme end of responses engendered by pregnancy itself (Redman and Sargent, 2003).

4.3 IMMUNO-LOCALISATION OF LEPTIN

In this study, leptin was immuno-localised within different trophoblast cell populations namely cytotrophoblasts, syncytiotrophoblasts and extra-villous trophoblasts. Additionally leptin was observed within endothelial cells, smooth muscle cells of the medial layer of blood vessel walls, fibroblasts and PBMCs. Stromal Hofbauer cells and amniotic cells were also immuno-reactive. These observations have also been previously reported by Liu *et al.* (2009). The latter study reports that leptin is sub-cellularly distributed around the plasmalemma, cytoplasm and on the nuclear membrane of cytotrophoblast cell populations only (Liu *et al.*, 2009). This immuno-localisation is supported by our observations, however in both cytotrophoblasts as well as in syncytiotrophoblast cell populations. Similarly, Castellucci *et al.* (2000) also report that leptin is expressed in cytotrophoblasts, yet its receptor is strongly expressed in extravillous trophoblast cell columns. Whilst these studies showed this distribution in first trimester pregnancies, the current study demonstrates immuno-localisation at term. The importance of leptin occurrence in the first trimester must be noted as it has an autocrine function in influencing the secretion of matrix metalloproteinases (MMPs) and hence cytotrophoblast migration (Castellucci *et al.*, 2000). In the present study, morphometric analysis of leptin demonstrated a similar expression within the syncytiotrophoblasts, cytotrophoblasts and extra-villous trophoblasts.

Reports indicate that leptin mRNA is present within the syncytiotrophoblast and endothelial cells but that the leptin receptor is found only in the syncytiotrophoblast cells (Lea *et al.*, 2000). The binding of leptin with its receptor plays a role in trophoblast invasion (Liu *et al.*, 2009). Whilst the possibility for leptin to be released into both maternal and fetal circulation exists, the focal presence of its receptor in syncytiotrophoblasts indicates an autocrine role for leptin at the maternal interface (Lea *et al.*, 2000). Placental leptin promotes placental growth and angiogenesis and regulates immune function in the placenta (Hoggard *et al.*, 2001; Mise *et al.*, 2007). The release of leptin into the fetal circulation promotes fetal growth and development. Studies have shown decreased placental leptin associated with intra uterine growth restriction (Lea *et al.*, 2000). Li *et al.* (2004) demonstrated an upregulated expression of leptin and leptin receptors in gestational trophoblastic disease. The latter study showed a much higher intensity of leptin expression in the cytotrophoblast than in syncytiotrophoblasts. In our study, a qualitative evaluation of the intensity of immuno-reactivity, reveals a uniform intensity within syncytiotrophoblasts and cytotrophoblasts. Cytotrophoblasts are the most proliferative and invasive population of trophoblast cells. However, it must be noted that trophoblast migration occurs in the first 20 weeks of gestation and this explains the differential intensity in the previous study which examined first trimester placenta.

4.3.1 Image Analysis

4.3.1.1 Image Analysis of the Exchange Villi of the Placenta

We demonstrate that irrespective of HIV status, leptin immuno-reactivity expressed as a field mean area percentage within the exchange area of the placenta was significantly higher in pre-eclampsia compared to normotensive pregnancies ($p < 0.001$). Furthermore, the intensity of

immuno-reactivity was more pronounced in the exchange villi of pre-eclamptic compared to normotensive placentae in both HIV positive and negative pregnancies ($p < 0.001$). These results corroborate our findings obtained by ELISA.

Placental villous density has previously been shown to be increased in pre-eclampsia, women who smoke, in maternal anaemia and in women living at high altitudes, all of which are conditions associated with hypoxic stress (Mayhew, 1996; Ducray *et al.*, 2011). A qualitative assessment in our study indicates that the surface area of exchange villi is greater in pre-eclampsia than in normotensive pregnancies. This implies that the surface area occupied by the syncytiotrophoblasts in the exchange villi is greater in pre-eclampsia, with the consequential enhanced leptin expression.

This densely packed intermediate and terminal villi in the pre-eclamptic groups, also observed by Ducray *et al.* (2011), would be associated with less maternal blood perfusion of the placenta which in turn leads to hypoxia. Compromise in maternal and fetal exchange is detrimental to pregnancy outcome. The increased density of these villi in both the central and peripheral regions of the placenta together with the elevated leptin levels, indicates that the hypoperfusion and resultant hypoxia which subsequently leads to increased leptin production is present throughout the placenta and not only in localized regions. According to Benirschke *et al.* (2006), pre-eclampsia without obvious intra-uterine growth restriction (IUGR) usually shows normal villous branching. In the current study however, pre-eclampsia without IUGR also exhibits the features of extensive villous branching.

4.3.1.2 Image Analysis of the Conducting Villi of the Placenta

Leptin immuno-reactivity expressed as a field mean area percentage within the conducting (stem) villi was lower in pre-eclampsia compared to the normotensive groups ($p = 0.037$). It is well established that pre-eclampsia is associated with an absence of physiological conversion of the maternal spiral arteries, hence decreased blood supply to the fetus (Naicker *et al.*, 2003; Lorquet *et al.*, 2010). More recently Ducray *et al.* (2011) have shown a decrease in lumen diameter of conducting villous arteries. The lower leptin expression in the pre-eclamptic conducting arteries reflects the reduction in endothelial cell surface area surrounding the lumen of arteries present in these villi.

Additionally our study demonstrates an increased immuno-expression of leptin in the exchange villi compared to the conducting villi in both HIV positive and negative pre-eclamptic placentae ($p < 0.001$). The observed greater villous density of the exchange area in pre-eclampsia with the consequential larger surface area occupied by syncytiotrophoblasts explains this increased leptin expression in the exchange villi of the pre-eclamptic placenta.

In contrast, in normotensive pregnancies, leptin expression is greater in the conducting villi than in the exchange villi ($p < 0.001$). The lower density of exchange villi in these pregnancies would explain this differential expression.

4.4 PATHOLOGICAL ASSESSMENT

A pathological assessment of placentae within our study groups revealed widespread syncytiotrophoblast degeneration, extensive areas of fibrinoid and larger numbers of syncytial

knots in the pre-eclamptic group. These observations were also noted by Brunori *et al.* (2005), who in addition also reported damage to the endoplasmic reticulum and microvilli of syncytiotrophoblast cells. Although only at a light microscopy level, our study also noted marked microvillous projections in the HIV positive pre-eclamptic villi.

In this study, the occurrence of syncytial knots (Tenney-Parker changes), sprouts and bridges were observed in all groups albeit more prominently in the pre-eclamptic groups. The syncytial knots are a group of aggregated nuclei that bulge on the surface of the trophoblast, often containing apoptotic nuclei. This degeneration occurs in the placenta of normal pregnancy as a cell cycle turnover of syncytiotrophoblast cells (Benirschke *et al.*, 2006). The knots are thus sites of extrusion of the apoptotic nuclei surrounded by a thin cytoplasm (Huppertz *et al.*, 1998). As the knots are sites of accumulated apoptotic nuclei they could also be the result of trophoblast degeneration (Huppertz *et al.*, 1998).

The syncytial sprouts are larger aggregates of syncytial nuclei that protrude into the intervillous space. In this study, the observation of increased syncytial sprouts in pre-eclampsia may be attributed to local hyperplasia of the syncytium and the result of widespread trophoblast degeneration, features characteristic of hypoxia (Kadyrov *et al.*, 1998; Todros *et al.*, 1999; Benirschke *et al.*, 2006). Alternatively, inter villous sprouts are also believed to occur as a result of flat sectioning across many irregular and highly branched villous surfaces (Benirschke *et al.*, 2006).

Syncytial bridges occur when adjacent villous surfaces fuse with each other. The cell membranes disintegrate following syncytial fusion (Cantle *et al.*, 1987). The observation of bridges in our study may be due to the extensive villous branching that occurs in pre-eclampsia. This is also a common feature in the placentae of women with anaemia and would hence be due to hypoxia (Benirschke *et al.*, 2006).

The extensive placental fibrin deposition observed in pre-eclampsia in this study, may be due to the poor circulation and subsequent local hypoxia that occurs in hypertensive disorders of pregnancy (Benirschke *et al.*, 2006). This observation of increased fibrin deposition in pre-eclampsia has been morphometrically analysed in the same geographic area as the current study (Ducray *et al.*, 2011).

Perivillous fibrin is a normal finding in term placenta whilst intravillous fibrin is a pathological feature that is increased in pre-eclampsia (Benirschke *et al.*, 2006). With intravillous fibrin deposition, the syncytium degenerates secondarily to the stromal region (Benirschke *et al.*, 2006). In this study, both types of fibrin deposition were noted in excess in pre-eclampsia and in HIV positive pregnancies. Hypoxia which occurs in pre-eclampsia may lead to syncytial degeneration and eventually result in replacement of the syncytium by fibrinoid. These infarcts are formed after platelet activation and aggregation which induces the formation of fibrin to stabilise the platelet thrombus which may in turn occlude maternal blood flow (Dekker and Sibai, 1999). The intermittent blood flow in the intervillous space could have caused the enhanced development of exchange villi which in turn would exacerbate the problem of hypoxia.

Excessive trophoblast shedding will result in an increase in the trophoblastic debris containing fetal DNA and protein into the maternal circulation, which would cause an inflammatory response and endothelial damage (Stepan *et al.*, 2004; Zhong *et al.*, 2004).

A further qualitative observation of the present study is an increase in the number of capillaries in the terminal villi of pre-eclampsia. This corroborates with the finding of Challier *et al.* (2001). Thus local ischemia due to hypoperfusion of intervillous space leads to an increase in vascularisation as a compensatory measure.

4.5 SERUM LEPTIN IN PRE-ECLAMPSIA

Raised serum leptin levels have previously been reported in pre-eclampsia (Laivuori *et al.*, 2000; Laivuori *et al.*, 2006). The present study also demonstrates raised serum leptin levels in HIV negative pre-eclamptics compared to HIV negative normotensive pregnant women. Although the difference was not statistically significant ($p = 0.42$), this trend confirms previous reports (Kafulafula *et al.*, 2002). In the present study, leptin levels in the HIV negative pre-eclamptics are similar to those reported by Kafulafula *et al.* (2002, 28.3 ± 2.2 vs 26.66 ng/ml). The latter study enrolled Black African women from the same geographical region as our study and they also reported elevated, albeit non significant, leptin levels in pre-eclampsia. Studies on Caucasian populations show statistically significant upregulation of leptin in pre-eclampsia compared to normotensive pregnancies (Laivuori *et al.*, 2000; Laivuori *et al.*, 2006). In contrast, whilst leptin levels in the South African studies are raised in pre-eclampsia, they are not statistically significant. A plausible explanation for this non-significant but raised leptin may be

a reduction in the range of leptin levels between normotensive and pre-eclamptic pregnancies in Black South African women. It must be borne in mind that the factors that contribute to the development of pre-eclampsia are an extreme end of a range of factors common to all pregnancies and that these factors always overlap with those of normal pregnancy (Redman and Sargent, 2003).

4.5.1 Serum Leptin HIV Positive Pregnancies

In this study serum leptin levels were lowered by HIV immune status ($p < 0.001$). The decreased level of this hormone was observed in normotensive as well as pre-eclamptic pregnancies associated with HIV infection. The HIV infected groups had lower BMI ($p = 0.007$) and triceps skin-fold thickness ($p < 0.001$) than the HIV uninfected groups. Triceps skin-fold thickness is a good indicator of the amount of adipose tissue in the body (Steinberger *et al.*, 2005). A direct translation of this association to the current study indicates less adipose tissue in these study groups with the consequent lower leptin production.

Previous studies in non-pregnant individuals indicate that a correlation exists between serum leptin levels and BMI (Maffei *et al.*, 1995). However, it must be noted that whilst the overall BMI was lower in the HIV positive groups and that these groups had lower leptin levels, there was no direct correlation between leptin levels and BMI ($p = 0.51$). A possible explanation for this lack of correlation is that pregnant BMI reflects total body weight inclusive of the weight of the fetus, placenta, amniotic fluid and enlarged uterus as well as water retention. We report a

correlation between leptin and triceps skin-fold thickness ($p < 0.001$), which represents body adiposity (Zhang *et al.*, 1997). Other studies also suggest that leptin is better correlated with absolute fat mass than BMI (Prolo *et al.*, 1998). Thus the lower triceps skin-fold thickness in the HIV positive group indicates decreased adiposity in HIV infection, which could account for the lower leptin levels.

The lower mid-arm muscle circumference of HIV positive participants observed in this study indicates that the loss of body weight in HIV infection is due to both lipotrophy as well as loss of muscle mass. Previous studies have indicated that weight loss in HIV infection reflects depletion of both lean and fat tissue. The loss of fat and lean tissue is related to the severity of the HIV infection and also to initial body composition changes before weight loss (Grinspoon and Mulligan, 2003).

To date, there is a paucity of data on leptin levels in HIV compromised normotensive and pre-eclamptic pregnancies. This study is novel as it examines leptin levels in normotensive and pre-eclamptic pregnancies compromised by HIV infection. An interesting finding of this study is that serum leptin levels are significantly decreased in HIV positive pre-eclamptic pregnancies compared to normotensive pregnancies ($p = 0.03$). Previous studies have linked low leptin levels to decreased inflammatory responses (Lord, 2002). Additionally, leptin promotes the release of cytokines such as TNF- α and IL-6 (La Cava and Matarese, 2004), which may result in the increased immune response associated with pre-eclampsia (Baker and Kingdom, 2004). Leptin also activates macrophages and T1 helper cells and in this way promotes inflammation which is a feature of pre-eclampsia (Redman and Sargent, 2009). HIV infection is associated with

immune suppression, whilst pre-eclampsia is linked with an exaggerated immune response thus an amelioration of the immune hyper-reactivity will occur in pre-eclampsia in HIV infection. In the current study, there was no relationship between leptin and severity of HIV disease.

4.5.1.1 Serum Leptin Levels in HIV Positive Pregnancies with Different Treatment Regimen

Leptin is a pro-inflammatory hormone and stimulates the TH-1 inflammatory response by promoting the release of cytokines that cause inflammation (La Cava and Matarese, 2004). One would therefore expect leptin levels to be decreased in untreated HIV patients but raised in those receiving highly active antiretroviral therapy (HAART). However, leptin levels in HIV positive normotensive participants were similar in the two treatment sub-groups ($p = 1.0$). This indicates that the mechanism for the improvement of immune response of HIV positive women on HAART occurs independently of leptin. Other studies also indicate that plasma levels of leptin were similar between patients on HAART and those not on this treatment (Wunder *et al.*, 2005).

Nucleoside analogues have also been linked to the development of lipodystrophy as they may inhibit mitochondrial DNA activity within adipocytes (Grinspoon and Carr, 2005). In the current study, the prophylactic treatment for the prevention of mother to child transmission of HIV (PMTCT) included zidovudine (AZT), a nucleoside reverse transcriptase inhibitor which could thus also have an effect on the development of lipodystrophy. Adipose tissue loss and leptin deficiency may contribute to the metabolic abnormalities associated with lipodystrophy (Gavrilova *et al.*, 2000; Grinspoon and Mulligan, 2003).

On the other hand, we demonstrate that leptin levels in HIV positive pre-eclampsia are lower in women on HAART compared to those not on this treatment ($p = 0.04$). This may be attributed to peripheral lipoatrophy. Lipodystrophy, comprising of an increase in abdominal fat, and peripheral lipoatrophy, has been shown in patients receiving HAART (Flint *et al.*, 2009). These changes in fat distribution have previously been linked to low leptin levels (Reitman *et al.*, 2000).

The prevalence of lipodystrophy in patients on HAART is around 40%, but the frequency of lipodystrophy varies with the drug being used. Nucleoside reverse transcriptase inhibitors like AZT are associated with fat loss in the extremities while protease inhibitors are associated with biochemical derangements of glucose and lipids as well as localised accumulation of fat (Mallewa *et al.*, 2008). Lipodystrophic HIV patients have reduced adipocyte expression of leptin and a decreased plasma level of this protein (Mallewa *et al.*, 2008). Down-regulation of leptin has also been reported in patients not on treatment for HIV, suggesting that HIV itself may play a role in causing lipodystrophy (Giralt *et al.*, 2006).

4.6 SERUM INSULIN

In this study, the insulin levels of the HIV positive groups were significantly lower than those of the HIV negative groups ($p < 0.001$). Decreased exocrine pancreatic function has been reported in HIV infection (Price *et al.*, 2005). Additionally, opportunistic infections may also cause dysfunction to endocrine organs with infection of the pancreas and beta cell destruction (Brown, 2011). Treated HIV infection has also been associated with increased systemic inflammation and immune dysfunction but at lower levels than without treatment (Al-Harthi *et al.*, 2004). The

occurrence of lipid disorders, insulin resistance and diabetes are increased in patients receiving antiretroviral treatment (Flint *et al.*, 2009).

A further explanation for the low insulin as well as low leptin levels in HIV infection, is a possible link to decreased incidence of metabolic syndrome, which has previously been reported to be lower than in the general population (Samaras *et al.*, 2007). Metabolic syndrome includes disturbances in insulin and lipid metabolism (Grundy *et al.*, 2004). Since metabolic syndrome, is associated with an increase in leptin and insulin (Samaras *et al.*, 2007), the lower rates of metabolic syndrome in HIV infection could also account for the lower insulin and leptin observed in HIV positive pregnancies in our study. We also demonstrate a correlation between insulin and CD4 cell counts in HIV positive normotensive pregnancies ($p = 0.009$). With HIV disease progression, CD4 cell count decreases, hence the lower lipid and insulin levels.

Normal pregnancy can be considered a state of insulin resistance and fasting insulin concentrations double during the course of gestation. Insulin levels peak in the third trimester (Yen, 1973). The current study demonstrates that insulin levels are lower in pre-eclampsia with or without HIV infection. The literature on relationship between insulin resistance and pre-eclampsia is controversial and conducted on different ethnic groups. It is probable that the different insulin levels reflect their genetic profiles (Sowers *et al.*, 1995; Kaaja *et al.*, 1999; Parretti *et al.*, 2006). A study on Caucasian populations indicates both low glucose and insulin levels in pre-eclampsia (Caruso *et al.*, 1999). In contrast, other studies report elevated insulin levels in pre-eclampsia (Laivuori *et al.*, 2000; Seely and Solomon, 2003; Suy *et al.*, 2006; Carpenter, 2007). Moreover, a study performed on African American women demonstrates

decreased insulin levels during the postpartum elevation of blood pressure, thus indicating that blood pressure elevation is not associated with insulin resistance (Jacobson *et al.*, 1994).

Elevated leptin and insulin levels are associated with an increase in inflammatory responses, which in turn is associated with vascular dysfunction (Redman *et al.*, 1999; Carpenter, 2007). The low levels of both these hormones observed in the present study, in HIV positive pre-eclampsia implicates an underlying vascular dysfunction that may occur independently of insulin resistance. Kaaja *et al.* (1996) proposed that in pre-eclampsia there is a state of insulin resistance which is possibly present prior to pregnancy. Insulin resistance and concomitant changes in lipid and lipoprotein on endothelial cell function could result in vasoconstriction (Kaaja *et al.*, 1999; Suy *et al.*, 2006).

It should be noted that the present study measured random insulin levels. Insulin sensitivity quantifies the ability of insulin to lower blood glucose concentration by stimulating the uptake of glucose. The latter is measured either by the glucose clamp or the euglycemic hyperinsulinaemic clamp techniques (Pacini and Mari, 2003). Both these techniques require the measurement of fasting glucose levels and require additional blood samples. Furthermore, the tests take between two and three hours to perform (Pacini and Mari, 2003). In the current study, participants were recruited at presentation to the hospital maternity unit, after the onset of labour. Late presentation during labour to hospital is a common occurrence in our geographic setting. It was thus not feasible to perform the glucose clamp or the euglycemic hyperinsulinaemic clamp tests on patients, neither was it practical to determine fasting insulin and leptin levels. We therefore determined random serum insulin and leptin levels. In addition to the experimental complexity of both the clamp techniques, a further disadvantage of these methods is that they do

not provide information on the effects of insulin on glucose uptake in the basal condition and this is physiologically important (Pacini and Mari, 2003). Previous studies report that insulin resistance is accompanied by high insulin levels (Joffe *et al.*, 1992; Saltiel, 2001). The low insulin levels obtained in the present study thus indicate the absence of insulin resistance. Other studies support this finding, indicating that circulating levels of leptin are not associated with insulin sensitivity (Mynarcik *et al.*, 2006).

Additionally, in this study, insulin levels are lower in both HIV positive and negative pre-eclamptic compared to normotensive pregnancies suggesting that insulin resistance is not the underlying mechanism for the development of pre-eclampsia. Furthermore, the HIV positive normotensive and pre-eclamptic participants on HAART had lower insulin levels than those not on HAART ($p = 0.03$ and $p = 0.04$, respectively). This indicates that HAART does not necessarily cause insulin resistance as reported by Suy *et al.* (2006).

Treatment naïve or treated HIV infected patients are associated with increased systemic inflammation and immune dysfunction (Al-Harhi *et al.*, 2004). Additionally, in HIV infection, opportunistic infections may affect endocrine organs causing dysfunction. Autopsy records indicate infection of the pancreas and beta cell destruction (Brown, 2011).

It must also be noted that the low insulin levels observed in the present study reflect data obtained from a Black African population attending an urban regional health referral centre. The participants of this study were derived from a low socio-economic background (both urban and rural), which raises the possibility of inadequate nutrition influencing leptin and insulin levels.

Nutritionally poor diets have previously been reported among low-income population groups in South Africa (Bourne *et al.*, 2002).

4.7 BIRTH AND PLACENTAL WEIGHT

In the present study, we find that the neonatal birth weights are similar in HIV positive and negative pregnancies ($p = 0.198$), but are lower in babies born of pre-eclamptic pregnancies ($p = 0.036$). Pathological features of hypoxia (syncytial knots, sprouts and bridges) were widespread in the pre-eclamptic placentae. In addition, the decreased intervillous space in these placentae would lead to reduced placental blood flow which would in turn result in decreased fetal growth. Placental ischemia has previously been implicated in fetal malnutrition, hypoxemia and asphyxia (Redman and Sargent, 2003).

Leptin plays a role in fetal growth and development (Souren *et al.*, 2008). We however, report no relationship between serum leptin levels and neonatal birth weight ($p = 0.1$). Despite the low maternal leptin levels in HIV positive mothers, the lack of correlation between birth weight and HIV status may be linked to the sequestration of placental leptin into the fetal circulation allowing for normal fetal growth.

In the present study, there was no correlation between serum leptin levels and placental weight. This is in contrast to the negative correlation previously reported between maternal serum leptin levels and placental weight (Schubring *et al.*, 1997). The latter study attributes this correlation to the involvement of leptin in regulating placental weight. Our study indicates a correlation

between placental weight and gestational age. This corroborates previous findings of lower placental weight at smaller gestational ages (de Luca Brunori *et al.*, 2005).

Trophoblast deportation in maternal circulation has been linked to placental size. Syncytiotrophoblast microfragments in the maternal circulation, which occur in normal pregnancy, are increased in pre-eclampsia (Redman and Sargent, 2003). These are pro-inflammatory and directly damaging to the endothelium. The shedding increases as the placental size increases. On the other hand, oxidative stress and intra uterine growth restriction could result in severe pre-eclampsia accompanied by very small placentae (Redman and Sargent, 2003). This explains the occurrence of pre-eclampsia with larger placentae or oxidatively stressed smaller placentae. Additionally, other studies of pre-eclampsia have also reported birth and placental weights within the normal range (Hung and Burton, 2006). Another study showed an association of pre-eclampsia with large for gestational age babies (Hung and Burton, 2006). Furthermore, leptin levels in umbilical cord blood correlate with both birth weight and placental weight, whereas maternal leptin levels do not differ (Schubring *et al.*, 1997).

4.8 MATERNAL TO CHILD TRANSFER OF HIV

Vertical transmission from mother to infant, accounts for 90% of childhood HIV infections (Coutsoudis *et al.*, 2010). HIV is able to pass through the placental barrier from maternal to fetal blood. Given that fetal syncytiotrophoblasts are in direct contact with maternal blood, non-receptor mediated movement across epithelial cells is possible (Arias *et al.*, 2003). In our study, the histological observation of micro-abrasions in the trophoblast layer may also promote the movement of the virus across the placenta to the fetus.

In the current study, the rate of maternal to child transfer of HIV (MTCT) was 16%, which is higher than that reported in other studies. Sherman *et al.* (2004) reported an HIV transmission rate of 8.7% in Johannesburg, South Africa. The high transmission rate in the current study conforms to the fact that our recruitment occurred in KwaZulu-Natal, which is the epicentre of the global HIV pandemic (UNAIDS, 2012). A limitation of this study is the poor follow-up rate of only 33% in HIV positive mothers. Nevertheless these results do highlight that a large percentage of vertical transmission occurs at this early stage of life either across the placenta or via breast milk. Moreover, South African studies estimate that at six weeks of age, breast feeding contributes an additional 3% to the HIV transfer rate (Moodley *et al.*, 2003; Sherman *et al.*, 2004).

Poor follow-up indicates that all HIV-infected infants are not identified and therefore not on antiretroviral treatment. One of the goals of the PMTCT programme is to identify and recommend all HIV-infected infants for antiretroviral treatment. Furthermore, infants who are not HIV positive at six weeks need to continue with post-exposure prophylaxis until the cessation of breastfeeding (Department of Health, 2010). The majority of participants reported formula feeding their infants at birth. However, for those that failed to return for the follow up visit, it is unknown if there was a change in feeding pattern once they returned to their homes. Previous studies report that the avoidance of breastfeeding is often not a realistic option in developing countries and that a large proportion of HIV positive women breastfeed their infants due to social pressure (Coutsoudis *et al.*, 2001; Becquet *et al.*, 2005). At least a third of children acquire infection in the postnatal period through breastfeeding (Newell *et al.*, 2004). These

infants are thus at risk for vertical transfer of HIV via breast milk. Additionally, they are deprived of antiretroviral prophylactic treatment due to failure of follow up. Without prophylactic treatment, the risk of vertical transmission is approximately 35% in breastfeeding and approximately 20% in non-breastfeeding women (Coutsoudis *et al.*, 2010). In South Africa, only 30% of babies are reviewed after birth (Department of Health, 2011). Low rates of adherence to infant HIV testing are also reported in other countries in Sub-Saharan Africa (Chen *et al.*, 2010; Nyandiko *et al.*, 2010).

Mother to child prevention in high income countries has been successful by initiating HAART early in pregnancy and instituting formula feeding from birth, instead of breastfeeding (Zhou *et al.*, 2010). However, in Africa, water sources are of a poor quality, milk is mixed in impure water and bottles are not sterilized (Coutsoudis *et al.*, 2001). Hence, formula feeding is not the solution to the problem. It is thus imperative that follow up is ensured.

Women who are not on HAART need to be assessed for lifelong HAART eligibility by testing their CD4 cell count. In this study, 28.4% of the HIV infected participants with CD4 cell counts below 350 cells/ μ l returned for the follow up visit. Thirty percent were not on HAART during pregnancy but were eligible for this treatment. Of these participants, only 33% (n = 7) returned for the six week follow up visit and received HAART. These data are similar to that obtained in other studies. The WHO (2009) report estimated that in 2009 there were 1.4 million HIV-infected pregnant women requiring antiretroviral treatment to prevent vertical transmission in low- or middle-income countries, and that only 35% of these women received the treatment. Previous studies have indicated that the rate of HIV transmission was highest in infants of

mothers with low CD4 counts and in mothers who were HAART-eligible but untreated (Thorne and Newell, 2004; Coutsooudis *et al.*, 2010). Furthermore, lifelong HAART benefits maternal health and contributes to maternal survival and reduces mother-to-child transmission. A low CD4 count allowed the progression to AIDS or death before the introduction of HAART (Kawado *et al.*, 2006).

An objective of the PMTCT programme in South Africa is also to strengthen community-based household and door to door activities to educate and enhance the utilization rates and effectiveness of health programmes (Department of Health, 2010). Mothers need to be informed of the infant's right to receive protection from acquiring HIV. Such community based activities should also be used to deliver the required treatment to mothers and infants who are unable or unwilling to access primary health care centres.

4.9 LIMITATIONS OF THE STUDY

We were unable to sub-categorise early and late onset pre-eclampsia as it is difficult to ascertain the time of onset of the disease due to late presentation at maternity units by patients. This is a common occurrence in developing countries, including South Africa.

Fasting serum levels of leptin and insulin would provide a better insight into these levels. However, this was not possible in our setting due to late presentation of patients at the maternity unit, after the onset of labour.

4.10 SUMMARY

This is the first study examining leptin levels in pregnancies compromised by HIV infection. Utilizing immunocytochemistry, this study demonstrates immuno-reactivity in different trophoblast cell populations, endothelium, and in some stromal cells. We also demonstrate ischaemic changes in the pre-eclamptic placenta as evidenced by syncytial knots, sprouts and bridges. Morphometric image analysis demonstrates elevated leptin in the exchange villi of pre-eclamptic compared to normotensive placentae, irrespective of HIV status. Supporting this finding, ELISA also demonstrated elevated leptin levels in the pre-eclamptic placenta. Similarly, the placental leptin mRNA expression was increased in HIV negative pre-eclampsia compared to normotensive pregnancy. However, the latter expression was not elevated in HIV positive pre-eclampsia.

Circulating leptin levels as determined by ELISA were lower in HIV positive compared to HIV negative groups. Whilst these levels were elevated in pre-eclampsia compared to normotensive HIV negative pregnancies (albeit non-significantly), they were significantly decreased in HIV positive pre-eclampsia. Serum leptin levels did not correlate with BMI, in contrast to triceps skin-fold thickness. This novel study also demonstrates that insulin levels are decreased in pre-eclampsia, irrespective of HIV status, signifying that insulin resistance does not contribute to the

development of pre-eclampsia in this population. We did however note that a correlation exists between serum leptin and insulin levels.

In addition, we demonstrate widespread qualitative pathology such as cytotrophoblast proliferation, increased basement membrane thickness, fibrin deposition, Tenney-Parker changes and an increase in density of exchange villi in the pre-eclamptic placenta, irrespective of HIV status.

Our findings indicate that post natal follow up visits are poor, therefore education and/or other interventions are required so that interventions to reduce maternal and infant deaths by HIV/AIDS are delivered to the mothers and children who need them.

4.11 CONCLUSION

In conclusion, placental leptin levels are elevated in pre-eclampsia, irrespective of HIV status. On the other hand serum leptin levels are elevated in HIV negative pre-eclampsia but decreased

in HIV positive pre-eclampsia. Serum insulin levels are decreased in HIV positive normotensive and pre-eclamptic groups compared to the respective HIV negative groups.

4.12 FUTURE RECOMMENDATIONS

Determination of leptin concentration in umbilical cord blood and neonates born to HIV positive pre-eclamptic women would enhance the knowledge base of the functions of leptin in fetal growth and development. Further studies investigating leptin levels in HIV pregnancies in the first trimester are required to determine whether leptin levels are raised in maternal serum, in order to elucidate whether leptin could be used as a biomarker for predicting the onset of pre-eclampsia.

Investigations on the location and levels of leptin receptors in HIV associated pre-eclampsia would provide further insight into leptin usage in placental tissue. Additionally, further studies are warranted to unravel the role played by other adipokines such as adiponectin, resistin and TNF- α in the deficient trophoblast invasion and subsequent hypoxia associated with pre-eclamptic pregnancies. A combination of HIF-1 α and leptin expression is also required to confirm the association between leptin and hypoxia.

CHAPTER 5

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APPENDIX I

APPROVAL OF PROTOCOL

UNIVERSITY OF KWAZULU NATAL
COLLEGE OF HEALTH SCIENCES
Nelson R. Mandela School of Medicine

MEMORANDUM

TO Professor T. Naicker Optics & Imaging DDMRI Building	FROM: Professor P. Moodley Dean's Assistant : MMedSc & PhD Postgraduate Education Committee Nelson R. Mandela School of Medicine 4 November 2008
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PROTOCOL : The Role of Leptin in HIV associated pre-eclampsia F Haffejee –
Optics and Imaging, PhD, Student number 821820476 (Ref. PhD 005/08)

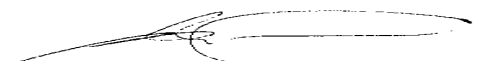
The Postgraduate Education Committee ratified the approval of the abovementioned study on 4 November 2008.

Please note :

- the Postgraduate Education Committee must review any changes made to this study.
- the study may not begin without the approval of the Biomedical Research Ethics Committee.

May I take this opportunity to wish the student every success with the study.

Many thanks
Yours sincerely


Professor P. Moodley
Assistant Dean : MMedSc & PhD
Postgraduate Education Committee
HD/Haffejee

cc F. Haffejee
Optics & Imaging
DDMRI Building

Dr M. Singh
Dept. of Biochemistry
Westville Campus

APPENDIX II

ETHICAL CLEARANCE



UNIVERSITY OF
KWAZULU-NATAL

BIOMEDICAL RESEARCH ETHICS ADMINISTRATION
Research Office, Westville Campus
Govan Mbeki Building
Private Bag X 54001
Durban
4000

KwaZulu-Natal, SOUTH AFRICA
Tel: 27 31 2604789 - Fax: 27 31 2604609
Email: BREC@ukzn.ac.za

Website: <http://research.ukzn.ac.za/ResearchEthics11415.950x>

30 March 2009

Ms Firoza Haffejee
C/o Prof T. Naicker (Supervisor)
Department of Optics and Imaging Centre
Nelson R. Mandela School of Medicine
University of KwaZulu- Natal

Dear Ms Haffejee

PROTOCOL: The role of Leptin in HIV associated Pre-eclampsia. Ms Firoza Haffejee, Optics and Imaging Centre. Ref No: BF155/08.

The Biomedical Research Ethics Committee (BREC) has considered the abovementioned application.

The study was approved by a quorate meeting of BREC on 09 December 2008 pending appropriate responses to queries raised. Your responses dated 25 March 2009 to queries raised on 20 February 2009 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 today; 30 March 2009.

The Informed Consent and Information to Participants submitted have also been reviewed and approved.

This approval is valid for one year from (today's date, 30 March 2009). 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/ResearchEthics11415.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 following Committee Members were present at the meeting held on 09 December 2008:

Prof D R Wassenaar (Chair)
Prof S Collings (Psychology)
Ms M de Haas (Social Anthropologist)
Dr U Govind (Private Practice - General Practitioner)
Ms J Hadingham (HEARD)
Dr T Hardcastle (Surgery - Trauma)
Dr Z Khumalo (KZN Health, External)
Prof T E Madiba (General Surgery)
Ms T Makhanya (External - lay person)
Prof R E Mhlanga (Obstetrics and Gynaecology)
Ms P Naidoo (Oncology and Radiology, External)
Dr S Paruk (Psychiatry)
Prof D Pudifin (Medicine)
Prof V Rambiritch (Pharmacology)
Dr M Titus (Obstetrics and Gynaecology, Greys Hospital Pmb)

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

Yours sincerely



PROFESSOR D R WASSENAAR
Chair: Biomedical Research Ethics Committee

APPENDIX III

APPROVAL FROM DEPARTMENT OF HEALTH



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

Reference : HRKM076/09
Enquiries : Mrs G Khumalo
Telephone : 033 – 395 3189

13 July 2009

Dear Ms Haffejee

Subject: Approval of a Research Proposal

1. The research proposal titled '**The role of Leptin in HIV associated pre-eclampsia**' was reviewed by the KwaZulu-Natal Department of Health.

The proposal is hereby **approved** for research to be undertaken at **Prince Mshiyeni Hospital**.

2. You are requested to undertake the following:
 - a. Make the necessary arrangement with identified facility before commencing with your research project.
 - b. Provide an interim progress report and final report (electronic and hard copies) when your research is complete.
3. Your final report must be posted to **HEALTH RESEARCH AND KNOWLEDGE MANAGEMENT, 10-102, PRIVATE BAG X9051, PIETERMARITZBURG, 3200** and e-mail an electronic copy to hrkm@kznhealth.gov.za

For any additional information please contact Mrs G Khumalo on 033-3953189.

Yours Sincerely


Dr S.S.S. Buthelezi
Chairperson, Health Research Committee
KwaZulu-Natal Department of Health

APPENDIX IV

HOSPITAL MANAGERS PERIMSSION



HEALTH
KwaZulu-Natal

PRINCE MSHIYENI MEMORIAL HOSPITAL
Private Bag X07, MOBENI 4060
Mangosuthu Highway
OFFICE OF THE MEDICAL MANAGER
DR ISMAIL JAJBHAY
Tel: 031-9078304/17, Fax: 0866060372
E mail: ismail.jajbhay@kznhealth.gov.za
www.kznhealth.gov.za

Reference: EC 12.2009A
Enquiries: Dr. IMS Jajbhay
Telephone: 031 907 8304
Date: 2009.09.11

TO: Ms. Firoza Haffejee

RE: LETTER OF PERMISSION/ APPROVAL TO CONDUCT RESEARCH AT PMMH

I have pleasure in informing you that PMMH has considered your application to conduct research on **The Role Of Leptin In HIV Associated Pre-Eclampsia** in our Institution. We hereby support your research subject to DOH KZN guidelines.

Please note the following:

1. Please ensure that you adhere to all the policies, procedures, protocols and guidelines of the Department of Health with regards to this research.
2. This research MAY commence as this office has received confirmation from the Provincial Health Research Committee in the KZN Department of Health.
3. Please ensure this office and the relevant Departments are informed before you commence your research.
4. The institution will not provide any resources for this research.
5. You will be expected to provide feedback on your findings to the institution.

Sincerely

MR. N. B. GWALA
HOSPITAL MANAGER

uMnyango Wezempilo . Department of Health
Fighting Disease, Fighting Poverty, Giving Hope

APPENDIX V

INFORMATION DOCUMENT

Study title: The Role of Leptin in HIV associated Pre-eclampsia

Greeting: Good day Ms. _____.

I am a research midwife and my name is Thank you for giving me the time to speak to you.

Introduction:

I am doing research on high blood pressure in pregnancy. Research is just the process to learn the answer to a question. Some women get very high blood pressure during pregnancy. In this study we want to learn more about why high blood pressure occurs in pregnancy.

I'm sure you know that when a baby is developing inside your womb, it gets its nutrition and oxygen from a piece of tissue (the after birth) that is attached to your womb. The after birth is called the placenta. People studying in this field believe that it is something inside the placenta that causes the high blood pressure. We want to see whether the hormones called leptin and insulin affect high blood pressure in pregnancy. We also would like to see whether babies' weights and health are affected if the mother had high blood pressure while she was pregnant.

When the baby is born, the after birth is also delivered. In order to learn more about high blood pressure in pregnancy, we need a small piece of your "after birth" and one tube of your blood. We would also like to examine your hospital records and those of your baby.

Invitation to participate: We are inviting you to participate in this research study. We need your permission to take a small piece of your after birth (placenta) after it has been delivered. We also need a small amount of your blood for testing.

We also want to see whether high blood pressure during pregnancy affects the growth of the baby in the womb. Some of the data from the baby's chart will also be used. We therefore request permission to access and study your hospital/clinic records.

What is involved in the study – We will be collecting samples from 90 women whose blood pressure is normal and from 180 women whose blood pressure was high. All these samples are being collected at this hospital from South African women and will be studied at the medical school. We require nothing more from you than to take a piece of your placenta and a blood sample and some of your baby's data from his hospital chart.

The piece of after birth will be labelled, without writing your name on it and then processed for experimental procedures. After the study, the sample will be kept in wax at the medical school. The remainder of the after birth that is not used for the study will be incinerated at the hospital.

Risks: The sample will be taken from the after birth that would have been discarded any way and therefore it will not harm you in any way. Taking one tube of blood from you will not harm you in any way. You will only feel a small pin prick. The injection site will be

cleaned with germ killing medicine (antiseptic) to minimize this risk of infection at the site of the needle prick. Any infection at this site will be treated with antibiotics.

Benefits of being in the study. You will not personally benefit from the study, but you will be contributing to a medical study that will be published and used for a PhD degree.

Participation is voluntary. Please note that participation is completely voluntary. You are not forced to give us any blood or placental samples. If you do not wish to participate in this study, you will receive the normal hospital treatment.

Reimbursements You will not be paid to take part in the study.

Confidentiality: Efforts will be made to keep personal information confidential. Your HIV status will be made known to the investigators of the research but we will keep this confidential. Your name will not be published in any research data or papers. However, absolute confidentiality cannot be guaranteed. Personal information may be disclosed if required by law.

Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as the Research Ethics Committee. If results are published, may lead to individual / cohort identification.

Contact details of researcher/s – If you want more information or are unhappy about anything you may contact Ms. Firoza Haffejee on 083 2918796

Contact details– for reporting of complaints/ problems:

BREC Administrator or Chair

Biomedical Research Ethics Committee

Private Bag X54001, Durban, 4000

Telephone: +27 (0) 31 260 4769 / 260 1074

Fax: +27 (0) 31 260 2384

Administrator: Ms. P Ngwenya email: ngwenyap@ukzn.ac.za

Chair: Prof. D.R. Wassenaar email: c/o ngwenyap@ukzn.ac.za

IsiZulu Translation of Information Document

INCWADI YESAZISO

Isihloko Sesifundo: Indima ye Liptin kwi HIV kanye ne Pre – eclampsia

Isibingelelo: Sawubona Ms Ngiwumcwaningi kwi midwife igama lami ngingu..... Ngiyabonga ukunginikeza isikhathi sokukhuluma nawe.

Isingeniso:

Ngenza ucwaningo kwi high blood pressure ekukhulelweni. Ucwaningo luyinqubo efundisa ukuphendula imibuzo. Abanye abantu besifazane bathola ihigh blood pressure ngesikhathi bekhulelwe. Kulesisifundo sifuna ukufunda kabanzi ngehigh blood pressure ekukhulelweni.

Ngiqinisekile ukuthi uma ingane isiyakheka ngaphakathi esibeledweni, ithola imisoco nomoya esicucwini (umva kokuzala) oxhumana nesibeledho. Umva kokuzala ubizwa ngomzanyana. Abantu abafunda kwilendima bakholelwa ukuthi into engaphakathi kumzanyana iyona imbangela ye high blood pressure. Sifuna ukubona ukuthi ingabe amahomoni abizwa nge leptin kanye ne insulin ayayiphazamisa yini ihigh blood pressure ekukhulelweni. Sifuna nokubona ukuthi isisindo kanye nempilo yezingane iyaphazamiseka yini uma umama kade ene ihigh blood pressure ngesikhathi ekhulelwe

Uma ingane isizelwe , umzanyana naye uyaphuma . Ukufunda kabanzi nge high blood pressure ekukhulelweni, sidinga isicubu senyama yomzanyana. Futhi elinye lamatube egazi. Sithanda ukuxilonga imininingwane yakho yasesibhedlela kanye neyomntwana wakho.

Isimemo sokuzibandakanya: Siyakumema ukuthi uzibandakanye kulesisifundo socwaningo. Sidinga imvume yakho yokuthatha isicubu esincane somzanyana uma usubelethe sidinga negazi lakho ukuyolihlola.

Sifuna futhi nokubona ukuthi I high blood pressure ngesikhathi sokukhulelwa iyakuphazamisa yini ukukhula komntwana esibeledweni. Enye yemininingwane eshadini lengane izosebenziswa. Yingakho sicela imvume yokhuthola futhi sifunde imininingwane yakho yasesibhedlela noma emtholampilo.

Yini ezokwenzeka kulolucwaningo: Sizozoqqa amasampula kwabesimame abawu 90 abane blood pressure ejwayelekile kanye nabawu 180 abane blood pressure ephezulu. Wonke lamasampula azoqoqwa ezibhedlela kwabesifazane base South Africa futhi kufundwe ngawo esikoleni sezempilo. Asidingi lutho kuwe ngaphandle kokuthatha isicubu somzanyana kanye negazi kanye neminye yemininingwane yomntwana wakho eshadini lasesibhedlela.

Isicucu somzanyana sizolebelwa ngaphandle kokubhala igama lakho kusona bese kuqhutshekwa ngokwenqubo yokulucwaninga. Emva kocwaningo, isampula lizogcinwa kwi wax medical school. Izinsalela zomzanyana ezingasetshenziselwanga ukufunda zisoshiswa esibhedlela.

Ubungozi: Isampula elizothathwa kumzanyana obuvele uzolahlwa ngakho akunabungozi ukuthathwa kwalo. Ukuthathwa kweTube eyodwa yegazi kuwena ngeke kube nabungozi, uzozwa nje kuphela ubuhlungu obuncane kungena inalithi. Lapho okuzongena khona inalithi kuzo hlanzwa ngezibulali magciwane ukunciphisa ubungozi bokusuleleka ngamagciwane. Uma kwenzekile kwasuleleka amagciwane ayokwelashwa ngama antibodies.

Inzuzo yokuba kulesisifundo: Angeke uzuze lutho oluzoza kuwe ngalesisifundo kodwa uzobe udlala indima ebalulekile kakhulu esifundweni sezempilo esizoshicilelwa futhi siphinde sisetshenziselwe iziqu ze PhD.

Ukuzibandakanya yesifundo uyavolontiya: Sicela wazi ukuthi ukuba yingxenywe yesifundo akuphoqiwe nakancane. Angeke uphoqwe ukuthi usinike igazi noma isicubu somzanyana wakho. Uma ungafisi ukuba yingxenywe kulesisifundo uzothola ukuphatheka kwasesibhedlela okujwayelekile.

Inkokhelo: Angeke ukhokhelwe ukuba yingxenywe yalesisifundo.

Ubumfihlo: Kuzokwenziwa konke okusemandleni ukugcina imininingwane yakho iyimfihlo. Ukuthi une HIV kuzokwaziswa kuba cwaningi kodwa kuyogcinwa kuyi mfihlo. Igama lakho ngeke laziswe kumbe Libhalwe kunoma nayi liphi uhlu noma iphepha locwaningo. Kodwa ubumfihlo ngeke buqinisekwe. Imininingwane yakho ingavezwa uma idingwa ngokusemthethweni. Izinhlangotho ezihlola amakhophi ocwaningo lamabhuku ukuze aqinisekise ukubayikho imininingwane ifaka amagatsha afana no Research Ethics Committee. Uma imiphumela isishicilelwe, ingaholela kumuntu ngamunye/ cohort identification.

Imininingwane yabacwaningi - Uma ufuna ulwazi kabanzi noma kukhona ongajabulile ngakho ungathintana no Ms. Firoza Haffeejee on 083 2918796

Imininingwane ye BREC Administrator – ukubika izikhalazo / izinkinga:

BREC Administrator or Chair
Biomedical Research Ethics Committee
Private Bag X54001, Durban, 4000

Telephone: +27 (0) 31 260 4769

Fax: +27 (0) 31 260 2384

Administrator: Ms. P Ngwenya email: ngwenyap@ukzn.ac.za

Chair: Prof. D.R. Wassenaar email: c/o ngwenyap@ukzn.ac.za

**Appendix VI
CONSENT DOCUMENT**

Consent to Participate in Research

You have been asked to participate in a research study towards a PhD degree.

You have been informed about the study by

Where applicable: You have been informed about any available medical treatment if injury occurs as a result of study-related procedures;

You may contact **Firoza Haffejee** at **083 2918796** any time if you have questions about the research or if you are injured as a result of the research.

You may contact the **Biomedical Research Office** at the Nelson R Mandela School of Medicine if you have questions about your rights as a research subject.

**BREC Administrator or Chair
Biomedical Research Ethics Committee
Private Bag X54001, Durban, 4000**

Telephone: +27 (0) 31 260 4769

Fax: +27 (0) 31 260 2384

Administrator: Ms. P Ngwenya email: ngwenyap@ukzn.ac.za

Chair: Prof. D.R. Wassenaar email: c/o ngwenyap@ukzn.ac.za

Your participation in this research is voluntary, and you will not be penalized or lose benefits if you refuse to participate or decide to stop.

If you agree to participate, you will be given a signed copy of this document and the participant information sheet which is a written summary of the research.

The research study, including the above information, has been described to me orally. I understand what my involvement in the study means and I voluntarily agree to participate.

Signature of Participant

Date

Signature of Witness
(Where applicable)

Date

Signature of Translator
(Where applicable)

Date

IsiZulu Translation of Consent Document

INCWADI YEMVUME

Imvume yo kubayingxenywe Yocwaningo

Ngiyaqonda ukuthi ngiceliwe ukuba ngizibandakanye kulolucwaningo lweziqo zePhD

Ngazisiwe kabanzi ngesifundo nge.....

Lapho bekudingekile: Wazisiwe ngokutholakala kwenkokhelo noma ukwelashelwa ukulimala okuzovela ngenxa yenqubo yesifundo;

Ungathintana no **Firoza Hafejee** ku **0832918796** noma zikhathi zonke uma unemibuzo ngocwaningo noma ulimele ngenxa yocwaningo.

Ungathintana ne **Biomedical Research Office** e Nelson R Mandela School of Medicine uma unemibuzo ngamalungelo akho ngokuba isihloko sesifundo.

BREC Administrator or Chair

Biomedical Research Ethics Committee

Private Bag X54001, Durban, 4000

Telephone: +27 (0) 31 260 4769

Fax: +27 (0) 31 260 2384

Administrator: Ms. P Ngwenya email: ngwenyap@ukzn.ac.za

Chair: Prof. D.R. Wassenaar email: c/o ngwenyap@ukzn.ac.za

Ukuzibandakanya kwakho kulolucwaningo akuphoqelekile futhi ngeke wajeziswa uma noma ungatholi inzuzo uma unqaba ukuzibandakanya noma ukhetha ukuyeka.

Uma uvuma ukuba yingxenywe, uzonikwa ikhophi elisayiniwe elizobe lichaza kimina. Ngiyaqonda ukuzibandakanya kwami kulesisifundo kuchaza ini futhi ngizivumele mina ukuba yingxenywe yaso.

Isiginisha yozibandakanyayo

Usuku

Isiginisha kafazi
(uma kunesidingo)

Usuku

Isiginisha yomhumushi
(uma kunesidingo)

Usuku

**APPENDIX VII
Data Sheet**

**THE ROLE OF LEPTIN IN HIV-ASSOCIATED
PRE-ECLAMPSIA**

Study no:

IP number

- Category (tick):**
- Pre-eclamptic HIV +ve CD4 < 200: 1
 - Pre-eclamptic HIV +ve CD4 > 200: 2
 - Pre-eclamptic HIV -ve CD4 : 3
 - Normotensive HIV +ve CD4 < 200: 4
 - Normotensive HIV +ve CD4 > 200: 5
 - Normotensive HIV -ve: 6
 - ☺ **No exclusion criteria present** (check against list)

Please place hospital sticker here

General hospital information

Admission date		PMMH no.	
----------------	--	----------	--

Demographics

Age			
Area of Residence (tick)	Rural	Urban	
Smoke (y/n)		No. of cigarettes/day	

HIV Status	+ve	-ve
CD4 counts		
Anti Retroviral therapy	yes	no
HAART		
PMCTC		

MATERNAL TREATMENT

Type of Treatment	Yes	No
Magnesium sulphate		
Aldomet		
Monoohydralazine		
Nifedipine		
Dihydralazine (nepresol)		
Labetalol		
Others		

Clinical Data

Parity	P: G:	Weeks gestation on admission		
Reason for previous pregnancy loss (If any)				
Highest BP	Systolic:	Diastolic:		
Maternal weight		Maternal height		
Midarm circumference		Triceps skin fold thickness		
Oedema (tick)	ankle	Up to knee	Up to groin Generalised (facial)	
(or attach copy of results)	proteinuria	Dipstick		
		Lab 24hr protein		
		Creatinine clearance		
	Full blood count	Red cell count		White cell count
		Haemoglobin		Neutrophils
		Haematocrit		Lymphocytes
		Mean cell volume		Monocytes
		Mean cell Hb		Eosinophils
		Platelets		Basophils
	Urea and electrolyte	Sodium		Urea
		Potassium		Creatinine
		Chloride		Anion gap
		CO2		
	Liver function tests	Total protein		Alkaline phos
		Albumin		AST
		Globulin		ALT
Alb : Glob			LDH	
Total bilirubin				

Antenatal Fetal Investigations

Type (tick)	Note any abnormalities
Sonar	
Doppler	
Electronic fetal HR	

Birth details

Weeks of gestation at time of birth			
Date of birth		Time of birth	
Indication for delivery (tick one)	Maternal interest	Fetal Distress	Combination of Maternal and fetal interest.
		CTG abnormal	
		MSL	
		IUGR	
	Explain above if relevant	Explain above if Relevant	Explain above if relevant
	Diagnosis: Eclampsia, severe abruptio infection		
Method of Delivery (tick one)	Normal vaginal		Caesarean
	Spontaneous		Elective
	Induced		Emergency
Complications in labour.	Eclampsia –related (tick)	Severe pre-eclampsia	Imminent eclampsia
	Abruptio-placentae		
	Other (explain)		

Baby details at birth

APGAR	1 min		5 min	
Baby (tick)	Live		Stillborn	
	Perinatal death (1 st 7days)		Neonatal death (up to 28 days)	
Baby weight (kgs)				

Placental details

shape	Normal	abnormal		
Weight (grams)				
Diameter (cm)				
Thickness (cm)	Less than 2cm	2-3cm	More than 4	
Colour	Maternal surface	Dark Maroon	Pale	
	Fetal surface	Dark	Pale	
Infarcts (maternal surface)	Amount of infarcted tissue	clear	mild	severe
	Colour of infarcts (if present)	Pale grey	Very dark	Both
Clots (maternal surface) tick	None	few	many	
Umbilical cord	Point of attachment	central	peripheral	
	Length	Less than 30 cm	30-90 cm	Greater than 90 cm
	No of vessels	3	2	
	Oedema	present	absent	

FOLLOW UP DATA PRIOR TO DISCHARGE FROM HOSPITAL

Date: _____ Inpatient / Outpatient visit: _____

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

Any other observations/clinical data/information of relevance for mother:
(Maternal complications / morbidity)

Baby weight: _____ Maternal BP: _____

Feeding choice	formula	Breast	flash heating	not fed	TPN
Cranial scan					

Morbidities in early NN period

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

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

Any other observations/clinical data/information of relevance for child:
(Neonatal complications / morbidity)

FOLLOW UP DATA AFTER DISCHARGE FROM HOSPITAL

Date: _____ Inpatient / Outpatient visit: _____

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

Baby weight: _____ Maternal BP: _____

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

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

Late Morbidities

Neurological impairment	
BPD	
ROP	
Nutritional	

Outcomes

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

APPENDIX VII

PEER REVIEW PUBLICATION (DOHET APPROVED)

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Role: Principle Investigator

Serum leptin levels are reversed in preeclamptic pregnancies complicated by human immunodeficiency virus infection

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Keywords

Preeclampsia, leptin, HIV, pregnancy

Synopsis

Leptin levels are lowered in HIV infected preeclamptics and therefore may not have a direct role in the aetiology of this pregnancy disorder.

Number of words 2 37

Abstract

Objective: To investigate whether leptin levels differ in HIV positive (+) and HIV negative (-) women with preeclampsia.

Design: Participants were recruited in the ante natal period and grouped as follows: normotensive HIV⁻ (n=30), normotensive HIV⁺ (n=60), preeclamptic HIV⁻ (n=30) and preeclamptic HIV⁺ (n=60). Anthropometric data were recorded and serum was analyzed for leptin using ELISA.

Results: In HIV⁻ groups, leptin levels were non-significantly elevated in the preeclamptic compared to the normotensive group (28.3±2.2 vs 24.6±1.0 ng/ml, $p=0.42$). In contrast, in HIV⁺ groups leptin was significantly lower in the preeclamptic compared to the normotensive group (10.0±1.1 vs 14.4±1.3 ng/ml, $p=0.03$). Leptin levels in HIV⁺ pregnancies were significantly lower than in HIV⁻ pregnancies in both preeclampsia (10.0±1.1 vs 28.3±2.2 ng/ml; $p<0.01$) and normotensive pregnancies (14.4±1.3 vs 24.6±1.0 ng/ml; $p<0.01$). In the HIV⁺ preeclamptic group, leptin was lower in HAART participants compared to those on PMTCT (8.56±1.4 vs 12.03±1.5 ng/ml; $p=0.04$). Leptin levels correlated with triceps skin-fold thickness ($r=0.35$, $p<0.01$).

Conclusion: This is the first study demonstrating decreased leptin levels HIV infected preeclampsia. Leptin may not have a direct role in the pathophysiology of this pregnancy disorder.

1. Introduction

Preeclampsia, a condition unique to human pregnancy is associated with significant maternal and perinatal morbidity and mortality.¹ The main cause of maternal deaths in South Africa is non-pregnancy related infections associated with HIV/AIDS, followed by obstetric haemorrhage and hypertension. The majority of maternal deaths occur in public sector hospitals, where 97.8% of maternal deaths due to hypertensive disorders of pregnancy occur.¹

The exact etiology of preeclampsia is not known, however, current views suggest a two stage disorder involving placental maladaptation arising as a result of immune dysfunction and genetic factors. Placental maladaptation leads to cellular hypoxia and the release of substances such as trophoblastic debris and necrotic cells eventually resulting in multi organ endothelial damage with clinical signs of hypertension, proteinuria, intrauterine growth restriction and thrombocytopenia. Exaggerated sterile inflammatory responses may also be implicated in its pathogenesis.²

Leptin, a satiety hormone produced primarily by adipocytes is involved in the regulation of body weight and in the development of inflammation.³ During pregnancy, leptin is also produced by the placenta and it has been reported that the excessive sterile maternal inflammatory response associated with preeclampsia is correlated with elevated leptin levels.⁴ It is also known that leptin enhances inflammation by promoting phagocytosis, chemotaxis and proliferation of circulating monocytes through the release of cytokines such as TNF- α and IL-6.⁵ Elevated levels of these cytokines lead to endothelial dysfunction, which is the pathological feature of preeclampsia.⁶

Since adipose tissue is the major contributor to serum leptin and its circulating levels are proportional to the amount of body fat³, low levels are present in conditions

such as HIV infection which is associated with lipoatrophy.⁷ Protease inhibitors, a component of highly active antiretroviral therapy (HAART), contribute to fat redistribution by a relative increase in abdominal fat, and peripheral lipoatrophy.⁸ However, loss of body fat also occurs in HIV positive (+) individuals not on HAART and in those who may not be benefiting from their medication.⁹ Importantly, low leptin levels impair immune function, thereby exacerbating the already suppressed immunity of HIV infection.⁷

Whilst the increase in serum leptin levels of HIV negative (-) preeclamptics may be translated as a possible role for leptin in the etiology of preeclampsia¹⁰, its association with HIV infection requires investigation. This study attempts to answer the following question: do leptin levels differ in HIV⁺ and HIV⁻ women with preeclampsia?

2. Materials and methods

2.1. Patients

Institutional ethical and regulatory permission was obtained and participants were recruited during the antenatal period from the maternity unit of a regional hospital in Durban, South Africa between September 2009 and December 2010. Pre-eclampsia was defined as a systolic blood pressure of at least 140 mmHg and /or a diastolic pressure of at least 90 mmHg with proteinuria of at least 300mg protein in a 24 hour urine sample or \geq 1+ on a urine dipstick. Participants were categorized as preeclamptic (n = 90) or normotensive (n = 90) with a further categorization according to HIV status.

The study groups included: normotensive HIV negative (NHIV⁻; n = 30), normotensive HIV positive (NHIV⁺; n = 60), preeclamptic HIV negative (PEHIV⁻; n = 30) and preeclamptic HIV positive (PEHIV⁺; n = 60).

Women with any medical or acute obstetric condition, and those on treatment with aspirin, warfarin, non-steroidal anti-inflammatory drugs, antibiotics, lipid lowering or anti-hypertensive drugs were excluded from the study. None of the participants were smokers.

In general, treatment of HIV infection depended on the level of the CD4 cell count; those with counts less than or equal to 200 cells/ μ l received highly active antiretroviral therapy (HAART) whilst those with counts greater than 200 cells/ μ l received zidovudine from the 28th week of pregnancy and a single dose of nevarapine in labor for the prevention of mother to child transmission of HIV (PMTCT).

Basic demographic data were obtained from all participants who were over the age of 18 years. The following anthropometric data were obtained: total body weight (kg), height (m), mid-arm circumference and triceps skin fold thickness (cm). The mid arm circumference was taken at the midpoint between the olecranon and the acromion process of the right arm. The subcutaneous tissue and skin of the midpoint of the posterior part of the right arm was measured with a skin fold caliper yielding the triceps skin fold thickness. The mid-arm muscle circumference was calculated by using the following formula: mid-arm muscle circumference = mid-arm circumference – (3.14 X triceps skin fold thickness).[13] Obesity was defined as a BMI of at least 30kg/m². [14]

2.2. *Determination of leptin levels*

Venous blood specimens were obtained ante-natally, centrifuged at 3 500rpm for 10 minutes and the serum stored at -70°C until assessed. Serum leptin levels were determined using a commercially available Quantikine Human Leptin enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, USA). The assay utilizes a quantitative sandwich enzyme immunoassay technique and has a sensitivity limit of 7.8 pg/ml. The

optical density was read at 450nm on a Bio-Rad ELISA plate reader (model 3350). All tests were performed in triplicate.

2.3. *Statistical Analysis*

SPSS for windows (version 20) was used for the statistical analysis. A square root transformation was used to normalize non-parametric data. Two-way analysis of variance (ANOVA) was used to test the significance between the groups. Where relevant the Bonferroni post hoc test was applied. Correlation between serum leptin and the anthropometric parameters was performed by the Pearson's or Spearman's rank correlation coefficient test, as appropriate. Values are presented as mean \pm SEM, except where stated otherwise. A p value < 0.05 was considered statistically significant.

3. **Results**

One hundred and eighty participants were enrolled. The clinical details of the participants are shown in Table 1. The overall mean age was 23.5 ± 0.37 years. The mean age of the HIV⁺ groups was significantly higher than that of the HIV⁻ groups ($p < 0.001$, Table 1). The mean gestational age and placental weight were similar amongst all study groups (Table 1). The placental weights ranged from 300 – 750g. Six placentae were below 400g, of which four were from mothers who delivered prior to 36 weeks. Neonatal weights were significantly lower in babies born of pre-eclamptic pregnancies compared to those born of normotensive pregnancies ($p = 0.036$). HIV status had no effect on neonatal weight. The Spearman's correlation coefficient showed a significant correlation between placental weight and gestational age ($r = 0.215$, $p = 0.004$).

All anthropometric measurements were lower in the HIV⁺ cohorts versus the HIV⁻ cohorts (Table 1). There was a weak correlation between the BMI vs mid-arm circumference ($r = 0.39$; $p < 0.01$), triceps skin-fold thickness ($r = 0.26$; $p < 0.01$) and mid-arm muscle circumference ($r = 0.40$; $p < 0.01$). A strong relationship was demonstrated between triceps skin-fold thickness and mid-arm muscle circumference ($r = 0.63$; $p < 0.01$).

Of the HIV⁺ participants, 58% ($n = 35$) of the preeclamptic and 45% ($n = 27$) of the normotensives were on HAART, while the remainder received prophylaxis for prevention of mother to child transmission of HIV (PMTCT).

A two-way ANOVA found a main effect on leptin with HIV status, $F(1,176) = 97.07$, $p < 0.001$, indicating a significance with regards to HIV status. This effect showed that leptin in the HIV⁺ group (mean = 12.21 ng/ml, CI 95%: 10.56 - 13.86) was lower than in the HIV⁻ group (mean = 26.46 ng/ml, CI 95%: 24.13 - 28.80). There was no overall main effect of pregnancy type (normotensive vs pre-eclamptic), $F(1,176) = 0.054$, $p = 0.816$, indicating no statistical significance. There was however an interaction between HIV status and pregnancy type present, $F(1,176) = 7.85$, $p = 0.006$. Bonferroni post hoc analysis indicated that in the HIV⁻ groups, serum leptin levels were elevated in pre-eclampsia compared to normotensive pregnancies, this difference was however, not statistically significant (mean = 28.32, CI 95%: 25.08 - 31.56 vs 24.6, CI 95%: 21.37 - 27.84 ng/ml; $p = 0.42$; Table 1). In the HIV⁺ groups, serum leptin was significantly lower in pre-eclampsia (mean = 10.02, CI 95%: 7.73 - 12.31 ng/ml) compared to normotensive pregnancy (mean = 14.41, CI 95%: 12.11 - 16.70 ng/ml, $p = 0.03$).

A negative correlation between leptin and maternal age existed ($r = -0.30$, $p < 0.001$). There was no relationship between serum leptin and BMI ($r = 0.15$; $p = 0.51$),

however a significant correlation existed between leptin and the other anthropometric measurements: triceps skin-fold thickness ($r = 0.37$; $p < 0.001$), midarm circumference ($r = 0.26$, $p = 0.001$) and midarm muscle circumference ($r = 0.18$, $p = 0.02$). There was no correlation between leptin levels vs gestational age ($p = 0.7$), neonatal weight ($p = 0.1$), systolic blood pressure ($p = 0.1$), diastolic blood pressure ($p = 0.1$) and CD4 cell counts of HIV⁺ participants ($p = 0.22$).

Within the HIV⁺ cohort, leptin levels were significantly lower in the preeclamptic group on HAART compared to the normotensive group receiving treatment only for PMTCT (8.56 ± 1.4 vs 14.45 ± 1.6 ng/ml, $p = 0.04$). The triceps skinfold thickness in the preeclamptic HAART group was significantly lower than the normotensive PMTCT group (0.65 ± 0.03 vs 1.32 ± 0.10 cm; $p < 0.01$). In the normotensive groups, leptin levels were similar between participants on HAART and PMTCT (14.36 ± 2.0 vs 14.45 ± 1.6 ng/ml; $p = 1.0$, Fig. 1). In the preeclamptic groups, leptin was lower in HAART participants compared to those on PMTCT (8.56 ± 1.4 vs 12.03 ± 1.5 ng/ml; $p = 0.04$, Fig. 1). The preeclamptic participants in HAART had lower leptin levels than the normotensive participants on HAART (8.56 ± 1.4 vs 14.36 ± 2.0 ng/ml, $p = 0.01$).

4. Discussion

This study demonstrates raised serum leptin levels, albeit non-significantly, in HIV⁻ preeclampsics compared with normotensive pregnant women and confirms the findings of existing reports.¹⁰ Leptin levels in the HIV⁻ negative preeclampsics in our study are similar to those reported by Kafulafula *et al.*, (2002) (28.3 ± 2.2 vs 26.66 ng/ml).¹⁰ The latter study enrolled Black African women within the same geographical region as our study and also reported a non-significant increase in leptin levels in preeclampsia. Raised leptin levels have been associated with placental hypo-perfusion, hypoxia and abnormal trophoblast

invasion that characterises the pathophysiology of pre-eclampsia.¹³ Leptin also promotes the release of cytokines such as TNF α and IL6,⁴ which may result in the exaggerated immune response associated with preeclampsia.⁵

An interesting finding of our study is that leptin levels are significantly decreased in HIV⁺ preeclamptic pregnancies compared to normotensive pregnancies ($p = 0.03$). The low immune response of HIV infection is counteracted by the hyper-immune response of preeclampsia. Previous studies have linked low leptin levels to decreased inflammatory responses.⁵ However, in the present study, there was no relationship between leptin and severity of HIV disease.

Our finding of lower BMI and triceps skinfold thickness in the HIV⁺ participants suggests a lower leptin production by adipose tissue. Previous studies in non-pregnant individuals indicate that a correlation exists between serum leptin levels and BMI.¹⁴ In the present study, correlation coefficient tests however, do not indicate a correlation between leptin levels and BMI. A possible explanation for this lack of correlation is that pregnant BMI reflects total body weight inclusive of the weight of the fetus, placenta, amniotic fluid and enlarged uterus as well as water retention. Nevertheless, the lower triceps skin fold thickness in the HIV⁺ group indicates decreased adiposity which could account for the lower leptin levels.³ This correlation signifies that adipose tissue is the main source of leptin in the body.

The low leptin levels and low triceps skinfold thickness in the preeclamptic HIV⁺ HAART cohort may be attributed to peripheral lipoatrophy. Lipodystrophy, comprising of an increase in abdominal fat, and peripheral lipoatrophy, has been shown in patients

receiving HAART.⁸ These changes in fat distribution have previously been linked to low leptin levels.¹⁵ However, leptin levels were also low in the participants who were not on HAART but were receiving prophylactic treatment for the prevention of maternal to child transfer of HIV (PMTCT). Poganelli *et al.* (1999) reported low leptin levels (6.3 ng/ml) in HIV⁺ non-pregnant patients not receiving HAART. The leptin levels of their study participants increased non-significantly to 10.5 ng/ml after 24 months on HAART.¹⁶ The leptin level of 8.56 ng/ml obtained in the preeclamptic HIV⁺ HAART cohort in our study is within the range of the previous study. This value is however lower than that obtained in the normotensive group, a reversal of the trend shown in studies of HIV⁻ pregnant women.¹⁰ Adipose tissue loss and leptin deficiency may contribute to the metabolic abnormalities associated with lipodystrophy.²¹

The lower mid-arm muscle circumference of HIV⁻ participants observed in this study indicates that the loss of body weight in HIV infection would be due to both lipoatrophy as well as lean tissue loss. Previous studies have indicated that weight loss in HIV infection reflects depletion of both lean and fat tissue. The loss of fat and lean tissue is related to the severity of the HIV infection and also to initial body composition changes before weight loss.¹⁷

Leptin plays a role in fetal growth and development.¹⁸ Despite the low maternal leptin levels in HIV⁺ mothers, the lack of correlation between birth weight and HIV status may be linked to the sequestration of placental leptin into the fetal circulation allowing for normal fetal growth. However, further investigation into placental and umbilical cord blood leptin is warranted.

In the present study, there was no correlation between leptin levels and placental weight. This is in contrast to the negative correlation reported previously between maternal serum leptin levels and placental weight.¹⁹ The latter study attributes this correlation to the involvement of leptin in regulating placental weight. The present study indicates a correlation between placental weight and gestational age. This corroborates previous findings of lower placental weight at smaller gestational ages.²⁰

In conclusion, this is the first study demonstrating decreased leptin levels in HIV infected preeclamptic women and leptin may therefore not have a direct role in the aetiology of this pregnancy disorder in HIV infected women.

Conflict of interest

We declare no conflict of interest.

Acknowledgements

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APPENDIX VIII

PEER REVIEW PUBLICATION (DOHET APPROVED)

To be submitted to: *Molecular Human Reproduction*

Role: Principle Investigator

Running Title: Leptin and insulin levels are decreased in HIV associated pre-eclampsia, compared to HIV positive normotensive pregnancies. This signifies that neither leptin nor insulin are involved in the aetiology of this syndrome when complicated by HIV infection.

A comparison of leptin and insulin in HIV positive and negative pregnancies with and without pre-eclampsia

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ABSTRACT

This study investigated leptin and insulin levels in HIV compromised normotensive and pre-eclamptic pregnancies. Participants were recruited antenatally and grouped as follows: normotensive HIV⁻ (n=30), normotensive HIV⁺ (n=60), pre-eclamptic HIV⁻ (n=30) and pre-eclamptic HIV⁺ (n=60). Anthropometric data were recorded and serum was analyzed for leptin and insulin by ELISA. Two-way ANOVA indicated that leptin levels were lower in the HIV positive groups (12.21ng/ml) compared to the HIV negative groups (24.46ng/ml, $p<0.001$). In the HIV positive groups, leptin levels were lower in pre-eclampsia (10.02ng/ml) compared to normotensive pregnancies (14.41ng/ml, $p=0.03$). Similarly, insulin levels were lower in HIV positive (4.15mU/l) compared to HIV negative pregnancies (7.57 mU/l, $p<0.001$). Insulin levels were also lower in pre-eclampsia compared to normotensive pregnancies, irrespective of HIV status (4.41mU/l vs 6.16mU/l, $p=0.02$). Whilst therapy with HAART indicated lower leptin levels in pre-eclampsia ($p=0.04$), treatment regimen had no effect on leptin levels in normotensive participants. Insulin levels were however lower in HAART treated participants in both the normotensive ($p=0.03$) and pre-eclamptic groups ($p=0.04$). Both leptin and insulin were not correlated with BMI. However, there was a correlation between leptin levels and triceps skin-fold thickness ($r=0.35$; $p<0.01$) and also between insulin levels and triceps skinfold thickness ($r=0.57$, $p<0.001$). The elevation of leptin in pre-eclampsia without HIV infection implicates it's role in the aetiology of this syndrome. However, this study demonstrates that both leptin and insulin levels are decreased in HIV associated pre-eclampsia signifying that neither leptin nor insulin are involved in the aetiology of this syndrome in HIV infection.

KEYWORDS: insulin/HIV/leptin/pre-eclampsia/pregnancy

INTRODUCTION

The most recent report of the National Committee on Confidential Enquiries into maternal deaths in South Africa has shown that the main cause of maternal deaths in this country is infections associated with HIV/AIDS, followed by obstetric haemorrhage and hypertension (Saving mother's Report: 2008-2010, 2012). Pre-eclampsia, a pregnancy specific condition associated with hypertension and proteinuria, accounts for 83% of the latter deaths (Cnossen *et al.*, 2006; Magnussen *et al.*, 2007; Saving mother's Report: 2008-2010, 2012). The aetiology of pre-eclampsia remains unknown, however genetic predisposition, maternal vascular disease, excessive maternal inflammatory responses and placental dysfunction are implicated (Cnossen *et al.*, 2006). Other predisposing factors may include metabolic abnormalities (Seely and Solomon, 2003). Previous studies have demonstrated an elevation of two protein hormones, insulin and leptin, in pre-eclampsia, implying a dysregulation of metabolism in affected individuals (Laivuori *et al.*, 2000; Seely and Solomon, 2003; Carpenter, 2007). The constraints of the latter reports are that the data does not report the HIV status of the participants.

Leptin is an adipose tissue hormone which is also produced by the placenta (Trayhurn *et al.*, 1998; Margetic *et al.*, 2002). It has a role in fetal development (Hoggard *et al.*, 2001; Souren *et al.*, 2008) but its primary role is in the regulation of body weight by activating hypothalamic centres involved in the control of energy intake and expenditure (Margetic *et al.*, 2002; Guzik *et al.*, 2007). Increased leptin is released into the circulation when body fat stores are high (Margetic *et al.*, 2002; Bluher and Mantzoros, 2007).

The pancreatic hormone, insulin, is involved in glucose homeostasis and 24 hour insulin levels reflect body energy stores (Niswender and Schwartz, 2003). As both hormones act on the same neurons of the hypothalamus, elevated levels of either hormone

stimulate an increased secretion of the other (Margetic *et al.*, 2002). In addition, when leptin levels are high, it binds to insulin receptors, thus high leptin levels are associated with insulin resistance (Margetic *et al.*, 2002).

During pregnancy, insulin is required for the placental production of leptin. Increased insulin may increase renal sodium reabsorption of water as well as stimulate the sympathetic nervous system, thereby elevating blood pressure (Seely and Solomon, 2003). Moreover, insulin resistance may lead to endothelial dysfunction which is a cardinal characteristic of pre-eclampsia (Seely and Solomon, 2003).

Pre-eclampsia is associated with a pro-inflammatory maternal response (Postovit *et al.*, 2001). Leptin has been previously implicated in enhancing pro-inflammatory responses, whilst low levels of this hormone have been reported in immune compromised conditions such as childhood diseases and starvation (La Cava and Matarese, 2004; Matarese *et al.*, 2005; Lago *et al.*, 2007). Extrapolation of the latter studies to pregnancies complicated by HIV infection, would translate to a lowering of leptin levels in these pregnancies. However, since pre-eclampsia is associated with an increased immune response (Postovit *et al.*, 2001), the effect of a combination of HIV and pre-eclampsia remains an enigma.

The relationship between pre-eclampsia and HIV infection is controversial, poorly documented and requires investigation (Wimalasundera *et al.*, 2002; Frank *et al.*, 2004; Mattar *et al.*, 2004; Suy *et al.*, 2006; Conde-Agudelo *et al.*, 2008). This study is novel in that it attempts to elucidate the relationship between leptin and insulin in HIV compromised pre-eclampsia.

METHODS

Institutional ethical approval (BF155/08) and written, informed consent from participants were obtained. Pregnant women were recruited between September 2009 and December 2010 from the Obstetric Unit of a regional hospital in KwaZulu-Natal, South Africa. Pre-eclampsia was defined as a sustained systolic blood pressure of at least 140 mm Hg and/or a diastolic pressure of at least 90 mmHg with proteinuria of at least 300 mg protein in a 24 hour urine sample or $\geq 1+$ on a urine dipstick (Magnussen *et al.*, 2007).

We enrolled 180 women, over the age of 18 years with term pregnancies. The participants were divided into 4 groups viz. normotensive HIV negative (n = 30), normotensive HIV positive (n = 60), pre-eclamptic HIV negative (n = 30) and pre-eclamptic HIV positive (n = 60). Women with diabetes, chronic hypertensive disorders and those who smoked were excluded.

Basic demographic profile was obtained from all participants. The following anthropometric data were obtained: total body weight (kg), height (m), mid-arm circumference and triceps skin fold thickness (cm). The mid arm circumference was taken at the midpoint between the olecranon and the acromion process of the right arm. The subcutaneous tissue and skin of the midpoint of the posterior part of the right arm was measured with a skin fold caliper yielding the triceps skin fold thickness. The mid-arm muscle circumference was calculated by using the following formula: mid-arm muscle circumference = mid-arm circumference – (3.14 X triceps skin fold thickness; Huang *et al.*, 2001). Obesity was defined as a BMI of at least 30kg/m² (Kafulafula and Moodley, 2001).

Venous blood was obtained in the antenatal period, centrifuged at 3 500 rpm for 10 minutes and the serum was stored at -70°C until required. Serum leptin concentrations

were determined using a commercially available Quantikine Human Leptin ELISA test kit (R&D Systems, USA). The assay utilizes a quantitative test with a sensitivity limit of 7.8 pg/ml. Serum insulin concentrations were determined by a human insulin ELISA test kit (DRG Diagnostics, USA). The sensitivity limit of this test kit was 0.07 mU/l. All tests were carried out in triplicate. The optical density for both tests was read at 450 nm on a Bio-Rad 3350 ELISA plate reader. Results were extrapolated from standard curves generated for both hormones.

SPSS for windows (version 20) was used for the statistical analysis. Non-parametric data were corrected by logarithmic transformations and two-way ANOVA, followed by the Bonferroni post hoc test were subsequently used to test the significance between the groups. Correlation between serum leptin, insulin, CD 4 T lymphocyte counts and the anthropometric parameters was performed by the Spearman's rank correlation coefficient test. A p value < 0.05 was considered statistically significant.

RESULTS

The demographic data of participants are shown in Table 1. The overall mean age was 23.5 ± 0.37 years. The mean age of the HIV positive groups differed from that of the HIV negative groups ($p < 0.001$, Table 1).

Of the 120 HIV positive participants, 35 pre-eclamptic and 27 normotensives were on highly active antiretroviral therapy (HAART), while the remainder received prophylaxis for prevention of mother to child transmission of HIV (PMTCT).

The BMI and triceps skinfold thickness were significantly different across the study groups ($p = 0.01$, Table 1). These measurements were lower in the HIV positive groups.

There was an overall weak correlation between the BMI and triceps skin-fold thickness ($r = 0.26$; $p < 0.01$).

Leptin

A two-way ANOVA found a main effect of HIV status on serum leptin levels [$F(1,176) = 97.07$, $p < 0.001$]. This effect showed that serum leptin levels in the HIV positive group (mean = 12.21 ng/ml, CI 95%: 10.56 - 13.86) were lower than in the HIV negative group (mean = 26.46 ng/ml, CI 95%: 24.13 - 28.80). There was no overall main effect of pregnancy type (normotensive vs pre-eclamptic [$F(1,176) = 0.054$, $p = 0.816$] on serum leptin levels. There was, however, an interaction between HIV status and pregnancy type on serum leptin levels [$F(1,176) = 7.85$, $p = 0.006$]. This effect showed that within the HIV positive groups, serum leptin levels in pre-eclampsia (mean = 10.02 ng/ml, CI 95%: 7.69 - 12.35) were significantly lower than that of normotensive pregnancies (mean = 14.41 ng/ml, CI 95%: 12.08 - 16.74).

Bonferroni post hoc analysis indicated that in the HIV negative groups, serum leptin levels were elevated in pre-eclampsia compared to normotensive pregnancies, this difference was however, not statistically significant (mean = 28.32 ng/ml, CI 95%: 25.08 - 31.56 vs 24.6, CI 95%: 21.37 - 27.84; $p = 0.42$). Conversely, in the HIV positive groups, serum leptin was significantly lower in pre-eclampsia (mean = 10.02 ng/ml, CI 95%: 7.73 - 12.31) compared to normotensive pregnancy (mean = 14.41 ng/ml, CI 95%: 12.11 - 16.70, $p = 0.03$).

In normotensive pregnancies, serum leptin levels were significantly lower in the HIV positive group (mean = 10.02 ng/ml, CI 95%: 7.73 - 12.31) compared to the HIV negative group (mean = 14.41 ng/ml, CI 95%: 12.11 - 16.70 vs 24.61 ng/ml; $p < 0.01$). Similarly, in pre-eclamptic pregnancies, leptin levels were significantly decreased in the

HIV positive group (mean 10.02 ng/ml, CI 95%: 7.73 – 12.31) compared to the HIV negative group (mean = 28.32 ng/ml, CI 95%: 25.08 - 31.56; $p < 0.01$).

In the HIV positive groups normotensive group, leptin levels were similar in the different treatment regimen, ie. HAART vs treatment for the prevention of maternal to child transfer (PMTCT) 14.36 ± 2.0 vs 14.45 ± 1.6 ng/ml, $p = 1.0$, Fig. 1a). However in pre-eclampsia leptin was significantly lower in HAART participants compared to those on PMTCT therapy (8.56 ± 1.4 vs 12.03 ± 1.5 ng/ml, $p = 0.04$, Fig. 1b)

Insulin

A two-way ANOVA found a main effect of HIV status on serum insulin levels [$F(1,176) = 17.18$, $p < 0.001$]. This effect showed that insulin levels in the HIV positive group was lower (mean = 4.15 mU/l, CI 95%: 0.14 - 1.53) than in the HIV negative group (mean = 7.57 mU/l, CI 95%: 3.10 – 10.55). There was also a main effect of type of pregnancy (normotensive vs pre-eclamptic) on serum insulin levels [$F(1,176) = 5.329$, $p = 0.022$]. This effect showed that serum insulin levels in pre-eclampsia were lower (mean = 4.41 mU/l, CI 95%: 0.00 – 5.67) than that in normotensive pregnancies (mean = 6.16 mU/l, CI 95%: 1.27 – 7.84). Finally, there was no interaction between HIV status and type of pregnancy [$F(1,149) = 0.004$, $p = 0.950$].

The Bonferroni post hoc test indicated that serum insulin levels were lower in the HIV positive normotensive group (mean = 4.97 mU/l, CI 95%: 3.45 - 6.0) compared to the HIV negative normotensive group (mean = 8.55 mU/l, CI 95%: 6.39 - 10.70, $p = 0.009$). Similarly in pre-eclampsia serum insulin levels were lower in the HIV positive (mean = 3.33 mU/l, CI 95%: 1.80 - 4.85) compared the HIV negative groups (mean = 6.59 mU/l, CI 95%: 4.43 - 8.74, $p = 0.032$).

In the HIV positive normotensive groups, serum insulin levels were significantly lower in HAART ($3.16 \text{ mU/l} \pm 0.85$) compared to those on PMTCT therapy ($6.29 \text{ mU/l} \pm 1.13$, $p = 0.03$, Fig. 2a). Similarly in the HIV positive pre-eclamptic groups, serum insulin levels were significantly lower in HAART ($1.28 \pm 0.30 \text{ mU/l}$) participants compared to those on PMTCT therapy ($4.55 \pm 1.55 \text{ mU/l}$; $p = 0.04$, Fig 2b).

There was a significant overall correlation between leptin and insulin levels ($r = 0.27$, $p < 0.001$). There was also no relationship between leptin and CD4 cell counts, however, a weak but significant correlation between insulin and the CD4 cell count in the normotensive HIV positive group ($r = 0.33$, $p = 0.009$) was observed.

There was no relationship between leptin and BMI ($r = 0.15$; $p = 0.51$), however, a correlation with triceps skin-fold thickness was observed ($r = 0.35$; $p < 0.01$). Similarly a relationship between insulin levels and triceps skinfold thickness ($r = 0.57$, $p < 0.001$) but not with BMI was demonstrated ($r = 0.01$, $p = 0.89$). A significant negative correlation between leptin levels and maternal age ($r = -0.20$; $p < 0.001$) as well as insulin levels and maternal age ($r = -0.36$; $p < 0.001$) was noted.

There was a significant negative correlation between age and CD4 cell counts in the HIV positive normotensive group ($r = -0.39$; $p = 0.002$).

DISCUSSION

During pregnancy, circulating leptin levels are produced by both adipose tissue and the placenta (Malik *et al.*, 2005; Haugen *et al.*, 2006) suggesting a role for leptin in gestation (Hoggard *et al.*, 2001; Mise *et al.*, 2007). The elevation of leptin in pre-eclampsia may be directly implicated in the aetiology of this syndrome since delivery of the placenta

results in the resolution of the condition (Laivuori *et al.*, 2000; Seely and Solomon, 2003; Carpenter, 2007).

There is a paucity of data on the relationship between leptin and insulin in HIV compromised normotensive and pre-eclamptic pregnancies. In the present study, leptin levels were raised in HIV negative pre-eclampsia compared to HIV negative normotensive pregnancies. Although this difference was not statistically significant ($p = 0.42$), this trend confirms previous reports (Kafulafula *et al.*, 2002). We report leptin levels in a similar range as that of the previous study which also enrolled Black South African women from the same geographic region as our study, also report a non-significant rise in leptin levels in pre-eclampsia was also reported in the previous South African study (Kafulafula *et al.*, 2002). A plausible explanation may be a reduction in the range of leptin levels between normotensive and pre-eclamptic pregnancies in Black South African population. It must be noted that other studies that reported raised leptin levels in pre-eclampsia were conducted on Caucasian populations and that the factors that contribute to pre-eclampsia are an extreme end of a range of factors that are present in normal pregnancies as well (Laivuori *et al.*, 2000; Redman and Sargent, 2003; Laivuori *et al.*, 2006).

In contrast, leptin levels were significantly lowered in the immuno-compromised pre-eclamptic pregnancies ($p = 0.03$). This shift implies that whilst leptin may play a role in the aetiology of HIV negative pre-eclampsia, the same does not necessarily hold true in the presence of an HIV infection.

The data on the incidence of pre-eclampsia in HIV positive women is conflicting. Some studies report increased incidence of pre-eclampsia development (Suy *et al.*, 2006), whilst others report a lower occurrence of pre-eclampsia in HIV associated pregnancies (Wimalasundera *et al.*, 2002; Mattar *et al.*, 2004). Other studies also report no reduction in the incidence of pre-eclampsia (Frank *et al.*, 2004). Additionally, studies have attributed

this conflicting data to HAART treatment, indicating that pre-eclampsia was lower in untreated HIV infection but increased in HIV positive women on HAART (Wimalasundera *et al.*, 2002; Suy *et al.*, 2006; Conde-Agudelo *et al.*, 2008). HIV infection is associated with immune suppression, whilst pre-eclampsia is linked to an exaggerated immune response thus an amelioration of the immune hyper-reactivity will occur in HIV associated pre-eclampsia. The latter argument has also been put forward to explain the lower incidence of pre-eclampsia in untreated HIV infected pregnancies (Turner and Summers, 1999; Ibrahim *et al.*, 2004; Redman and Sargent, 2005).

Wimalasundera *et al.* (2002) proposed that HAART restores the immune response of HIV positive women, thus triggering the process that results in pre-eclampsia. Leptin is a pro-inflammatory hormone and stimulates the TH-1 inflammatory response by promoting the release of cytokines that cause inflammation (La Cava and Matarese, 2004). We could therefore expect leptin levels to be decreased in untreated HIV but raised in HIV infected women on HAART. The converse was observed in our pre-eclamptic participants where the leptin levels were lower in those on HAART ($p = 0.04$). We also demonstrate that in normotensive pregnancies, leptin levels do not differ in the participants on HAART compared to those on treatment for PMTCT ($p = 1.0$). This indicates that the mechanism for the improvement of the immune response of HIV positive women on HAART occurs independently of leptin.

In contrast to previous reports (Laivuori *et al.*, 2000; Seely and Solomon, 2003; Suy *et al.*, 2006; Carpenter, 2007), this study demonstrates that insulin levels are lower in pre-eclampsia with or without HIV infection (Table 1). Elevated leptin and insulin levels are associated with an increase in inflammatory responses which in turn is associated with vascular dysfunction (Redman *et al.*, 1999; Carpenter, 2007). The low levels of both these hormones in HIV positive pre-eclampsia implicates an underlying vascular dysfunction

that may occur independently of insulin resistance. Kaaja *et al.* (1996) proposed that in pre-eclampsia there is a state of insulin resistance which was possibly present prior to pregnancy. Insulin resistance and concomitant changes in lipid and lipoprotein on endothelial cell function favour vasoconstriction (Kaaja *et al.*, 1999; Suy *et al.*, 2006). The present study shows that insulin levels are lower in both HIV positive and negative pre-eclamptic pregnancies suggesting that insulin resistance is not the underlying mechanism for the development of pre-eclampsia in our study population. Furthermore, in the present study, the HIV positive participants on HAART had lower insulin levels than those not on HAART. Although this difference did not reach statistical significance, it indicates that HAART does not cause insulin resistance as reported by Suy *et al.* (2006).

The literature on the relationship between insulin resistance and pre-eclampsia is controversial and conducted on different ethnic/race groups. It is probable that the different insulin levels reflect their genetic profiles (Sowers *et al.*, 1995; Kaaja *et al.*, 1999; Parretti *et al.*, 2006). A study on Caucasian populations indicates both low glucose and insulin in pre-eclampsia (Caruso *et al.*, 1999), whilst that performed on African American women showed decreased insulin levels in the postpartum elevation of blood pressure indicating that blood pressure elevation in pre-eclampsia is not associated with insulin resistance (Jacobson *et al.*, 1994).

The low insulin levels observed in the present study reflect data obtained from a Black South African population attending an urban regional health referral centre. Since our cohort is derived from a low socio-economic background (both urban and rural), this raises the possibility that nutrition may be inadequate thereby influencing leptin and insulin levels. Nutritionally poor diets have previously been reported amongst low-income population groups in South Africa (Bourne *et al.*, 2002). A further explanation for the low insulin and leptin levels is a possible link to decreased incidence of metabolic syndrome in

HIV infection, which has previously been reported to be lower than in the general population (Samaras *et al.*, 2007). Metabolic syndrome includes disturbances in insulin and in lipid metabolism (Grundy *et al.*, 2004). Since metabolic syndrome, is associated with an increase in leptin and insulin (Samaras *et al.*, 2007), the lower rates of metabolic syndrome in HIV infection could also account for the lower insulin and leptin in our HIV positive cohorts. In addition, decreased exocrine pancreatic function has been reported in HIV infection (Price *et al.*, 2005). Whilst lowered pancreatic endocrine functions remain unreported, autopsy records indicate infection of the pancreas with beta cell destruction (Brown, 2011).

This study also shows a correlation between insulin and CD4 cell counts ($p = 0.009$). Since there is a lower incidence of metabolic syndrome with HIV infection (Samaras *et al.*, 2007), as disease progression occurs, the CD4 cell count decreases and there may be less lipid and insulin disturbances. We also demonstrate a positive relationship of triceps skinfold thickness with both leptin and insulin. Leptin is correlated with absolute fat mass (Prolo *et al.*, 1998), thus the decreased adiposity in HIV infection, could account for the lower leptin levels.

It should be noted that in the present study, we measured random insulin levels and not insulin resistance or sensitivity, which quantifies the ability of insulin to lower blood glucose concentration by stimulating the uptake of glucose thereby suppressing it's production. The latter is measured either by the glucose clamp or the euglycemic hyperinsulinaemic clamp techniques (Pacini and Mari, 2003). Both these techniques require the measurement of fasting glucose levels and require additional blood samples. Furthermore, the tests take between two and three hours to perform (Pacini and Mari, 2003). The current study participants were recruited at presentation to the hospital maternity unit, after the onset of labour. It was thus not feasible to perform the glucose clamp or the euglycemic

hyper-insulinaemic clamp tests on them, neither was it feasible to determine fasting insulin and leptin levels. We therefore determined random serum insulin and leptin levels. In addition to the experimental complexity of both the clamp techniques, a further disadvantage of these methods is that they do not provide information on the effects of insulin on glucose uptake in the basal condition and this is physiologically important (Pacini and Mari, 2003). Previous studies report that insulin resistance is accompanied by high insulin levels (Joffe *et al.*, 1992; Saltiel, 2001). The low insulin levels obtained in the present study thus indicate the absence of insulin resistance. Furthermore HIV has previously been reported to cause wasting which in turn results in ameliorating insulin resistance (Panz and Joffe, 1999).

In conclusion, this novel study demonstrates that both leptin and insulin levels are decreased in HIV associated pre-eclampsia signifying that neither leptin nor insulin resistance are involved in the aetiology of this syndrome in HIV infection.

Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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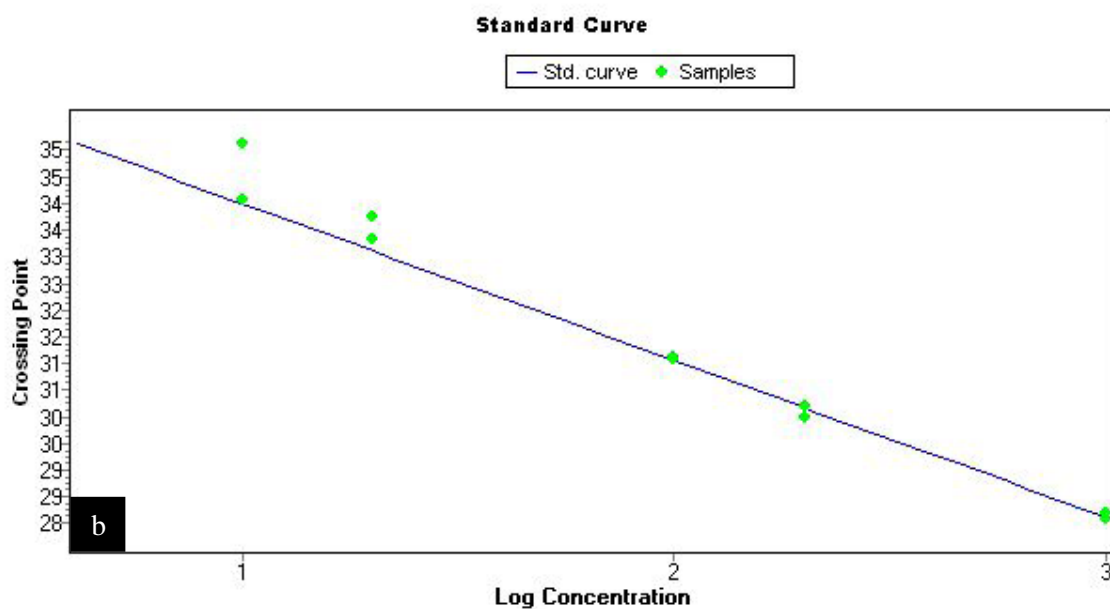
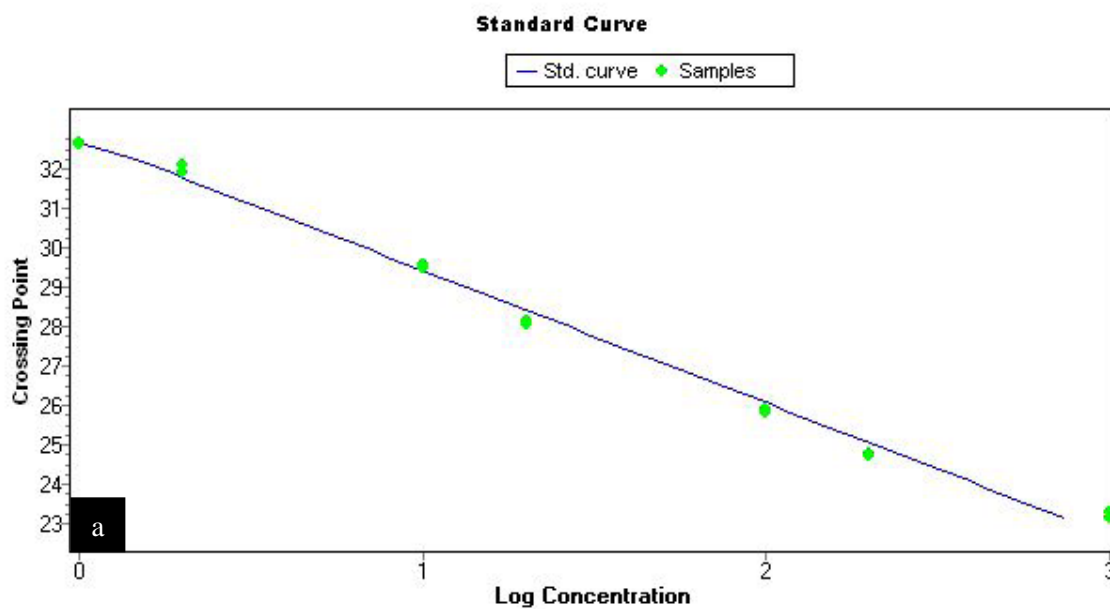
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APPENDIX IX

RT PCR STANDARD CURVES FOR (a) GAPDH AND (b) LEPTIN



APPENDIX X

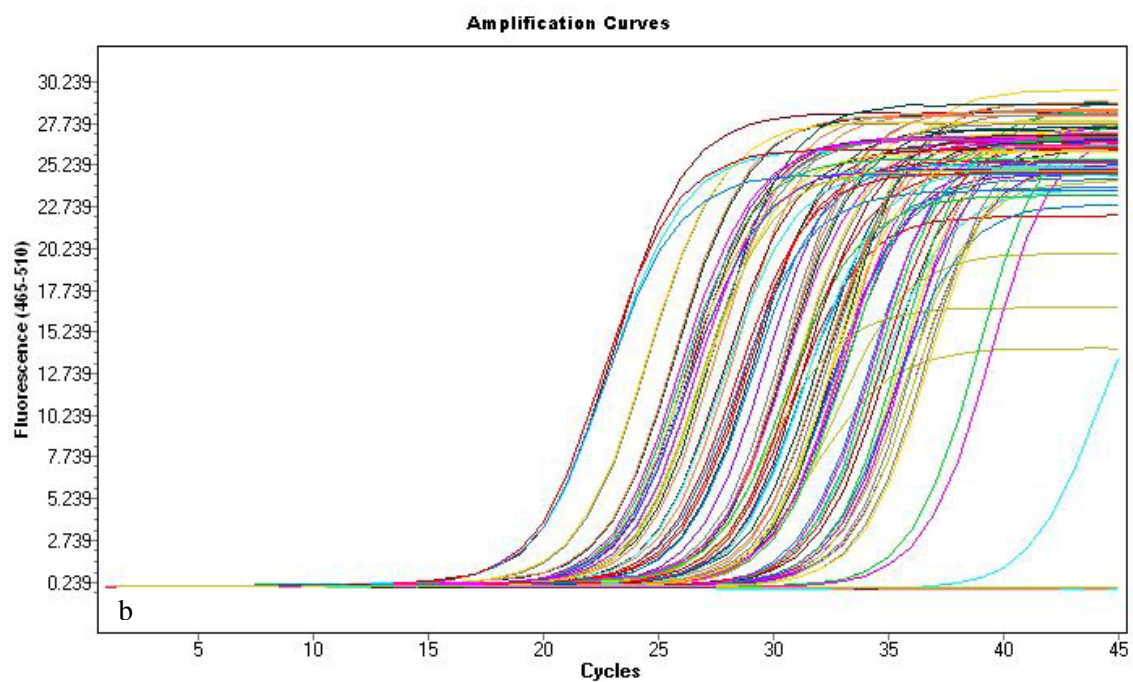
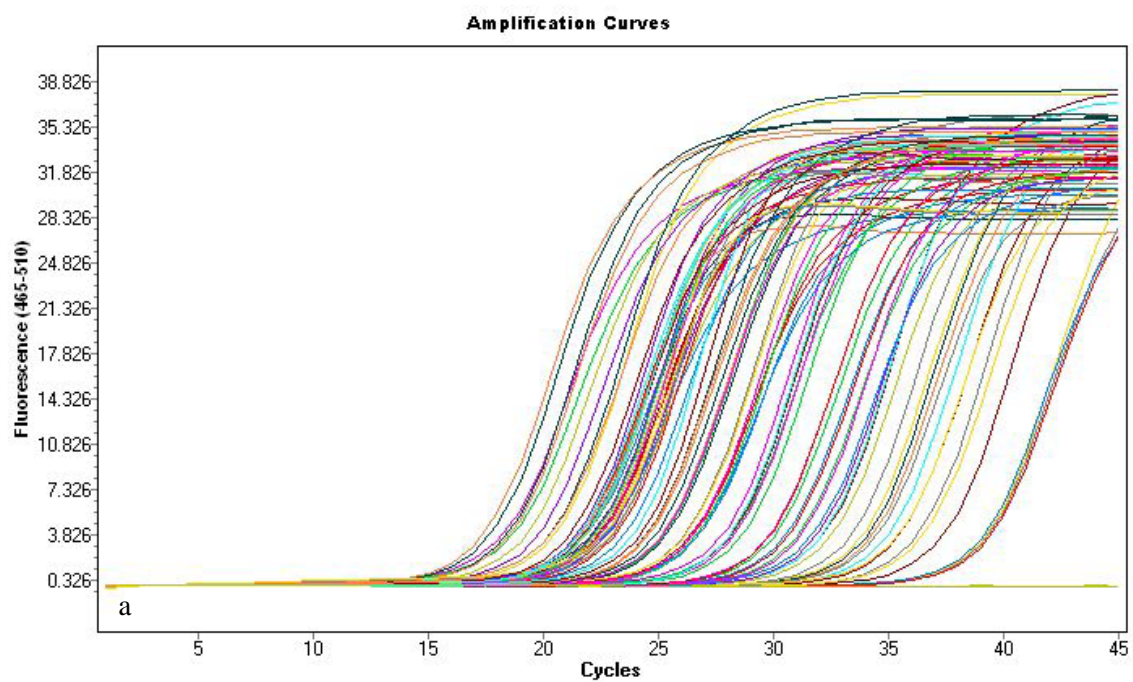
PROCEDURE FOR IMMUNOHISTOCHEMISTRY

PROCESS	CHEMICAL	TIME
Dewax	Xylene	2 X 5 min
Rehydrate	100% ethanol	2 X
	95% ethanol	1 X
	70% ethanol	1 X
	Running tap water	5 min
	Distilled water	5 min
		} 5 min
Rinse	Phosphate buffered saline (PBS)	2 X 5 min on slide shaker
Antigen retrieval treatment	Citrate buffer solution (Dako) diluted 1:10 – Microwave at Medium High (LG; 1 200W) to boiling point for 10 minutes Cool sections to 60°C	10 min
Rinse	PBS + Tween 20	3 X 5 min on slide shaker
	PBS	1 X 5 min on slide shaker
	Aspirate excess liquid	
Hydrophobic barrier to localize staining	Circle specimen with pap pen	
Blocking	3 drops 3% hydrogen peroxide	5 min
Rinse	Distilled water	Few dips
	PBS + Tween 20	5 min
	3 drops 10% bovine serum albumin (BSA in 1X PBS)	30 min
Rinse	PBS + Tween 20	3 X 5 min on slide shaker
	PBS	1 X 5 min on slide shaker
	150 µl primary Leptin antibody diluted 1:25 in 2% BSA (BSA in 1X PBS)	Overnight at 4°C
Rinse	PBS + Tween 20	3 X 5 min on slide shaker
	PBS	1 X 5 min on slide shaker
	Aspirate excess liquid	

PROCESS	CHEMICAL	TIME
Link to secondary antibody	3 drops secondary link to cover specimen (Yellow)	30 min
Rinse	PBS + Tween 20	3X 5 min on slide shaker
	PBS	1X 5 min on slide shaker
	Aspirate excess liquid	
Streptavidin peroxidase	3 drops of Streptavidin peroxidase to cover specimen (Red)	30 min
Rinse	PBS + Tween 20	3X 5 min on slide shaker
	PBS	1X 5 min on slide shaker
	Aspirate excess liquid	
Visualisation	3 drops substrate-chromogen solution (made up of 1 ml buffered substrate + 1 drop DAB chromogen)	3 min
Wash	Tap water	Dip
	Running tap water	5 min
Counterstain	Mayer's haematoxylin	3 min
Rinse	Running tap water	5 min
Dehydrate	70% ethanol	1X 5 min
	80% ethanol	1X
	95% ethanol	1X
	100% ethanol	2X
	Xylene	2X
		} 5 min
Mount	DPX Mountant	

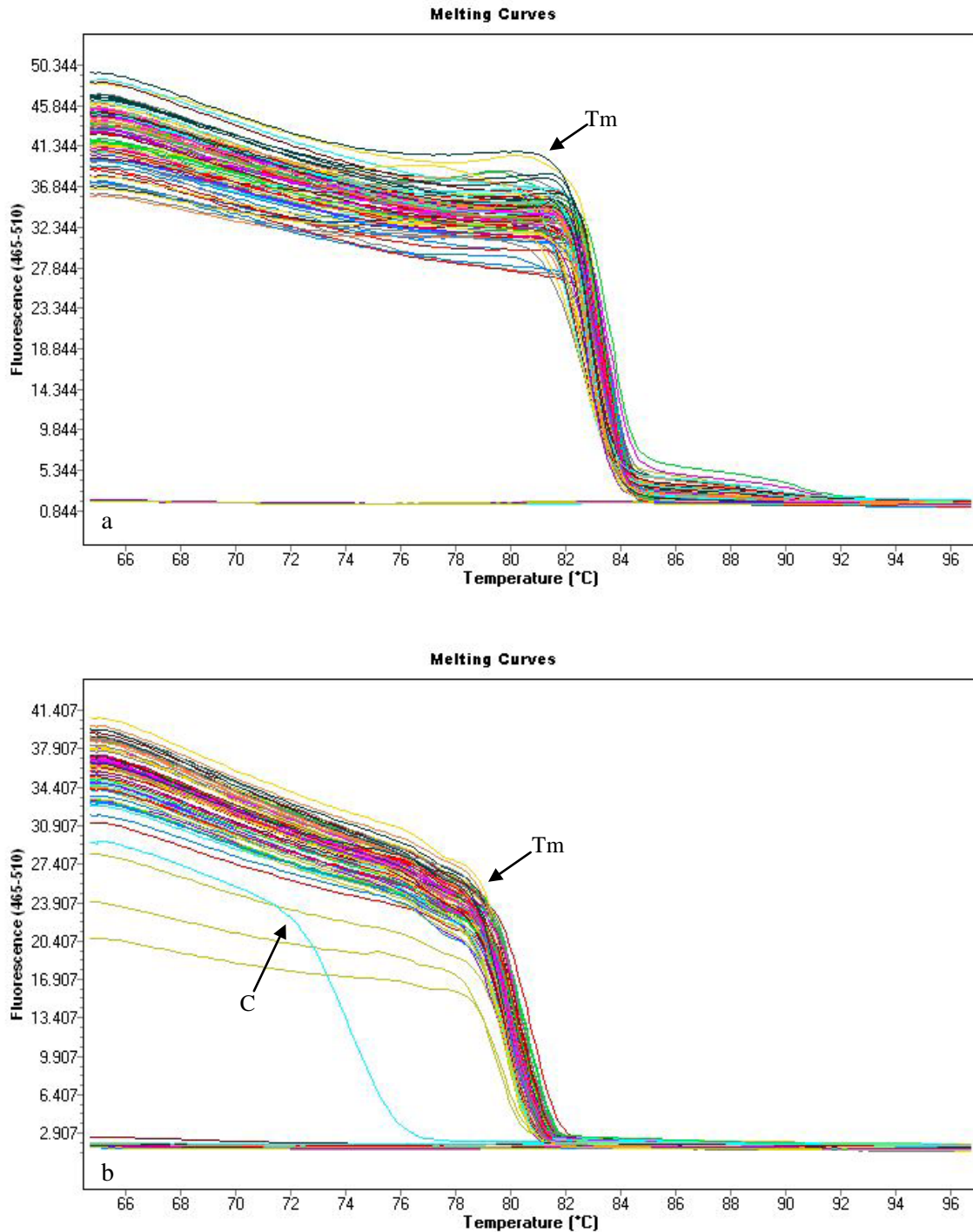
APPENDIX XI

Graphical representation of amplification curves for (a) GAPDH and (b) leptin showing gene amplification between cycles 15 - 40.



APPENDIX XII

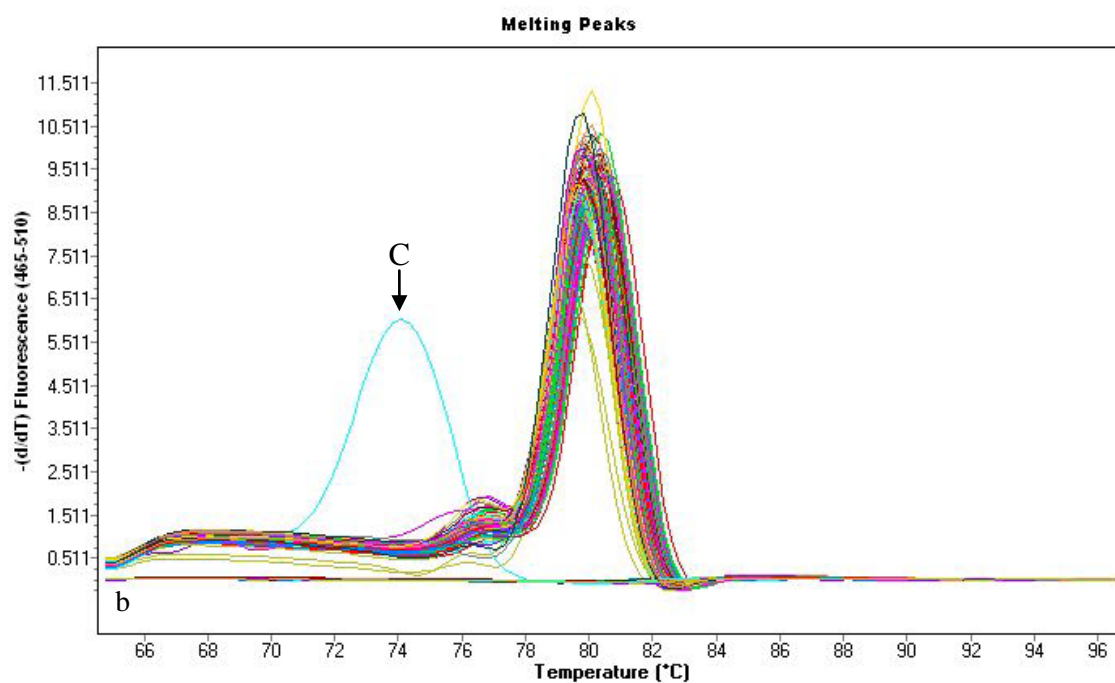
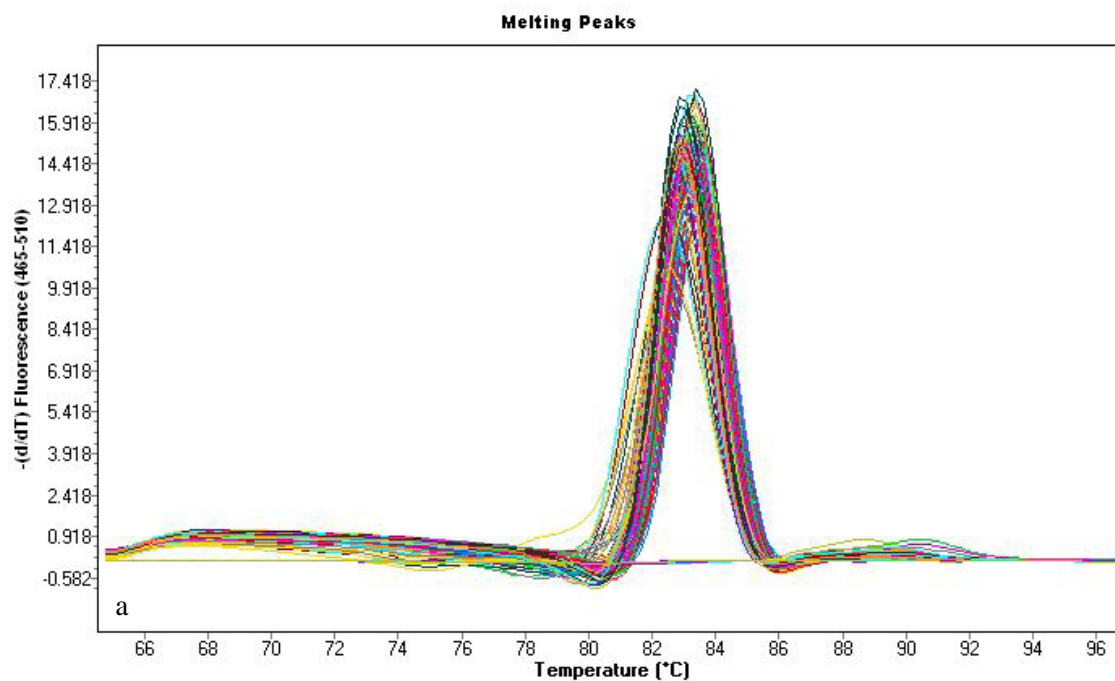
Graphical representation of melting curve analysis of (a) GAPDH and (b) leptin



Amplification reaction demonstrating the gradual reduction in fluorescence with temperature increase. The rapid fall off at 83°C and 80°C (T_m) indicates the presence of GAPDH and leptin in (a) and (b), respectively. At point C, an unspecified product had a lower melting temperature.

APPENDIX XIII

Graphical representation of melting peaks for (a) GAPDH and (b) leptin.



The melting of the DNA product is easily identified as a sharp peak centred at the T_m of the product. The unspecified product with a lower T_m is shown at point C.