THE ROLE OF SOLUBLE FMS-LIKE TYROSINE-KINASE-1, VASCULAR ENDOTHELIAL GROWTH FACTOR AND PLACENTAL GROWTH FACTOR IN HIV ASSOCIATED PRE-ECLAMPTIC PREGNANCIES: A SOUTH AFRICAN PERSPECTIVE

by

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submitted in partial fulfillment of the requirements for the degree of

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PREFACE

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

The research described in this dissertation was carried out in the Optics & Imaging Centre, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa under the supervision of Professor T. Naicker.

_____	_____
Nalini Govender	**Professor Thajasvarie Naicker**
(9304501)	**(Supervisor)**

DECLARATION

I, Nalini Govender declare that:

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DEDICATION

To my son Joshua Govender,

and my parents, for instilling in me the desire to progress

To God, without whose love, grace, wisdom, knowledge and mercy none of my achievements would have been possible

"Do not be anxious about anything, but in everything, by prayer and petition, with thanksgiving, present your requests to God. Whatever you have learned or received or heard from me, or seen in me -- put it into practice. And the God of peace will be with you."

Philippians 4:6

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VI Maternal imbalance between pro- and anti-angiogenic factors in HIV infected preeclampsia

VII Soluble fms-like tyrosine kinase-1 and soluble endoglin in preeclampsia associated with HIV infection.

LIST OF ABBREVIATIONS

KZN	KwaZulu-Natal
USA	United States of America
VEGF	vascular endothelial growth factor
PlGF	placental growth factor
VEGFR-1	vascular endothelial growth factor receptor 1
VEGFR-2	vascular endothelial growth factor receptor 2
sFlt-1	soluble fms-like tyrosine kinase receptor 1
Eng	endoglin
sEng	soluble endoglin
TGF-β_1	transforming growth factor-beta 1
TβRI	transforming growth factor-beta type I receptor
TβRII	transforming growth factor-beta type I receptor
ALK1	activin receptor like kinase receptor I
ALK5	activin receptor like kinase receptor II
Ang 1	angiopoeitin 1
Ang 2	angiopoeitin 2
tie-1	tyrosine kinase receptor 1
tie-2	tyrosine kinase receptor 2
HIV	human immunodeficiency virus
N-	HIV negative normotensive pregnant women
N+	HIV positive normotensive pregnant women
P-	HIV negative pre-eclamptic pregnant women
P+	HIV positive pre-eclamptic pregnant women
ng	nanograms
Fig	figure

AU	arbitrary units
pg	picograms
ns	non significant
µm	micrometer
µg	micrograms
cm	centimeter
µl	microliters
ml	milliliters
mM	millimolar
nm	nanometers
dH_2O	distilled water
mmHg	millimetres mercury
BP	blood pressure
wks	weeks
g	grams
°C	degrees celcius
kD	kilo dalton
min	minute
sec	seconds
rpm	revs per minute
RT	room temperature
hrs	hours
PBS	phosphate buffered saline
BSA	bovine serum albumin
DPX	dibutylphthalate
DAB	diamino-benzidine

ANOVA	analyses of variance
r	correlation coefficient
SD	standard deviation
RT-PCR	Real time polymerase chain reactions
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
DNA	deoxyribonucleic acid
DEPC	diethyl pyrocarbonate
RT	reverse transcription mastermix
ELISA	enzyme-linked immunosorbent assays
IHC	immunohistochemistry
IEM	immunoelectron microscopy
H&E	hematoxylin and eosin
ST	syncytiotrophoblast
CT	cytotrophoblast
mv	microvilli
M	mitochondria
BM	thickened basement membrane
Mc	mesenchymal cells
C	fetal capillaries
L	lumen
EC	endothelial cells
RBC	red blood cells
P	platelets
N	nuclei
n	nucleolemma

ER	endoplasmic reticulum
IgG	immunoglobulin G
EVT	extravillous trophoblast cells
rF	Rohr fibrinoids
F	intravillous and perivillous fibrin
f	fibrin
L	Langhans cells
AV	anchoring villi
SL	spongy layer
SB	syncytial bridges
SK	syncytial knots
CI	confidence interval

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ABSTRACT

Introduction and aims

South Africa is the epicenter of the HIV/AIDS pandemic. Hypertensive disorders of pregnancy (15.7%) are the second cause of maternal deaths of which pre-eclampsia represents 83%. Normal pregnancy requires a balance between pro- and anti-angiogenic factors to necessitate effective vasculogenesis, angiogenesis and placental development, however, pre-eclampsia is characterised by an excess anti-angiogenic state. The hypoxic placenta releases excess anti-angiogenic factors into the maternal circulation causing endothelial dysfunction. However, there is no data to verify if HIV infection affects pre-eclampsia, or if the angiogenic imbalance is affected. Contradictory data exists on the association between HIV infection and pre-eclampsia. In an attempt to assess the role of HIV infection in pre-eclampsia, this study examined the immunolocalisation of sFlt-1, sEng, PlGF and VEGF in placentae of HIV negative and positive normotensive and pre-eclamptic pregnancies at term using immunohistochemistry (IHC) and immunoelectron microscopy (IEM). Additionally, we estimated the placental expression of sFlt-1, sEng, PlGF and VEGF to verify if the HIV negative differed from the HIV positive cohorts. We further evaluated the maternal serum to determine if variations existed in the circulating levels of these factors in HIV negative and positive normotensive and pre-eclamptic pregnancies

Methods

Following institutional ethical approval and informed consent, placental biopsies and maternal serum were collected post-delivery. For IHC and IEM, 130 and 25 placentae were evaluated, respectively. Following conventional immunohistochemical processing, 5µm sections were immunostained & immunoexpression of the various antibodies were evaluated with the Zeiss Axioscope A1 interfaced with an AxioVision Image analysis software package (version 4.8.3) in

combination with the auto-measurement module (Carl Zeiss, Germany). Post-conventional immunoelectron processing, ultra-thin sections were immunolabelled. Sections were post-fixed, contrast enhanced with uranyl acetate and Reynolds lead citrate and viewed on a Jeol 1011 Transmission Electron Microscope. Additionally, the placental expressions of these factors were assessed using RT-PCR. In an attempt to confirm if maternal circulating levels of these factors differed, we quantitatively evaluated these factors in serum from HIV negative normotensives, HIV negative pre-eclamptics, HIV positive normotensives, and HIV positive pre-eclamptics using ELISA techniques.

Results and Discussion

The expression of sFlt-1, sEng, PlGF and VEGF was confirmed using immunohistochemistry, RT-PCR and ELISAs. Irrespective of the HIV status, sFlt-1 and sEng was elevated with the concomitant reduction in PlGF in pre-eclamptic compared to normotensive pregnancies. The levels of VEGF were however undetectable across all study groups. It is plausible that this lack of effect of HIV status on the factors under study may be attributed to the treatment regimen as HAART is known to restore the immune response of HIV positive preeclamptic women. However, a concise anti-retroviral treatment history in our study was unavailable.

Additionally, this study is novel in that it ultrastructurally immunolocalises sFlt-1, sEng, PlGF and VEGF within the placenta. This immunoelectron localisation data corresponds to our immunohistochemical data. Our study further demonstrates strong immunoreactivity of both placental sFlt-1 and sEng in pre-eclampsia with concurrent elevations in the maternal circulation. A qualitative increase in the occurrence of syncytial knots in the pre-eclamptics compared to the normotensive pregnancies was noted. These observations support the detachment of anti-

angiogenic rich microparticles from syncytial knots and their subsequent deportation and elevation in the maternal circulation. Moreover, their consequent antagonistic effects on VEGF, PlGF and TGF-β, disrupts the vascular endothelial maintenance.

The strong immunoreactivity of sFlt-1, sEng, PlGF and VEGF was observed in villous endothelial cells. Moreover, a strong sFlt-1 and sEng but a weak PlGF and VEGF immunoreactivity was noted in syncytio- and cytotrophoblasts. This immunoexpression within trophoblasts is suggestive of their autocrine mode of action on normal trophoblast functions including invasion, differentiation and production. It is plausible that the angiogenic imbalance observed in our study, will impact on placental function, by modifying trophoblast activity thereby contributing to abnormal placentation.

Conclusion

Our study supports the hypothesis that pre-eclampsia is characterized by an imbalance between pro- and anti-angiogenic factors. Whether the pregnancy is complicated by immune insufficiencies or not, does not affect the role of the anti-angiogenic factors in pre-eclampsia development. Nevertheless, the neutralising effect of HIV infection on the immune system may be insufficient in the development of pre-eclampsia. To our knowledge, the quantification of serum pro-/anti-angiogenic factors in HIV-associated pre-eclampsia is novel. In conclusion, our data reinforces the hypothesis that increased concentrations of sFlt-1 and sEng are involved in the pathogenesis of pre-eclampsia and indicates their possible use as discriminatory factors between diseases.

CHAPTERS 1 and 2

Introduction and Methods

1.1 Context

Human immunodeficiency virus (HIV) is a 'retrovirus' consisting of ribonucleic acid (RNA) and reverse transcriptase, which allow HIV to convert its RNA to DNA and then integrate, and invade, a cell's own genetic material (Arnold *et al.*, 1992). HIV is a transmissible virus that causes acquired immunodeficiency syndrome (AIDS) in humans. Two types of HIV have been documented namely, HIV-1, the main cause of AIDS in Europe, North and South America and most parts of Africa; and HIV-2, a less virulent form which has a longer latency phase and is mainly recognised in West Africa (Arnold *et al.*, 1992). The disease extends across genders, all age groups, geographic areas, and ethnic/race groups (UNAIDS, 2011).

The recent World AIDS Day Report, UNAIDS (2011) have shown that approximately 34 million people are living with HIV, indicative of a 17% increase since 2001. Sub-Saharan Africa remains the epicentre of this global pandemic with an estimate of 5.6 million people living with HIV (UNAIDS, 2011). Additionally, in 2010, an estimated 70% of all new global infections were found to occur in sub-Saharan Africa. According to global estimates by the World Health Organization, 585 000 females die per annum due to pregnancy related complications (UNAIDS, 2011). The latest South African National Confidential Enquiries into Maternal Deaths indicates that HIV/AIDS contributes to approximately 41% of all maternal deaths (Saving Mothers Report, 2012). Geographic variations of the epidemic in South Africa reflect a provincial difference with KwaZulu-Natal showing the highest prevalence rate of 39.1% (Saving Mothers Report, 2012).

Hypertensive disorders (14%) and haemorrhage (14.1%) are the next commonest direct cause of maternal death in pregnancy (Saving Mothers Report, 2012). More specifically, pre-eclampsia, a pregnancy-related disease characterized by hypertension and proteinuria after 20 weeks of gestation, complicates almost 3-5% of all pregnancies in high-income countries (Powe *et al.*, 2011). The incidence of pre-eclampsia, however, varies considerably in different parts of the world and ranges from 0.05% in developed countries to 27% in developing countries (Duley, 1992). In South Africa, a low-to-middle income country, pre-eclampsia accounts for 83% of maternal deaths due to hypertension, whilst at King Edward VIII Hospital, KwaZulu-Natal (KZN), 16% of patients admitted to the Obstetric Unit are complicated by hypertension (Moodley, 2008). This maternal disorder is also related with intra-uterine growth restriction or death, preterm birth, ante- and post-partum haemorrhages (Noris *et al*., 2005).

The HIV epidemic has devastating consequences on women of reproductive age 15 -24 years (Moodley and Moodley, 2005). Globally, more than half of the HIV infected adults (57%) are young females aged between 15 to 24 years (Kim and Watts, 2005). Young women are three-fold more likely to be infected than young men (Kim and Watts, 2005, de Groot *et al.*, 2003, Petropoulou *et al.*, 2006). In South Africa, HIV prevalence in the 20-24 year age group are 28.0%, whilst in older age groups (30–34, 35–39; 40+ years), the percentages are 37.0%; 29.6% and 21.3% respectively (Saving Mothers Report, 2012). In view of this increased vulnerability, it is also a major obstetric dilemma, since two-thirds of HIV infected women of reproductive age reside in sub-Saharan Africa (Coovadia and Coutsoudis, 2000). Thus, in light of the high prevalence rates of HIV/AIDS and pre-eclampsia in South Africa, it is important to adequately clarify the interaction of these two diseases.

The human placenta undergoes major vascularisation during fetal development (Agarwal and Karumanchi, 2011, Cerdeira and Karumanchi, 2012). Additionally, human placentation involves vasculogenesis, angiogenesis and pseudovasculogenesis, or physiological remodelling of the maternal spiral arteries (Brosens *et al.*, 2011). This physiological remodeling involves both decidua and trophoblast associated stages, whereby placental trophoblasts undergo an epithelial-endothelial phenotype conversion (Brosens *et al.*, 2011, Cerdeira and Karumanchi, 2012). Fetal development requires a sufficient nutritional supply, thus an unstable uterine blood supply is related with increased perinatal morbidity and mortality (Zygmunt *et al.*, 2003).

Placental vascularisation thus requires a subtle balance between pro- and anti-angiogenic factors. However, in pre-eclampsia, elevated levels of several anti-angiogenic factors, such as soluble Flt-1 and soluble endoglin, are produced by the placenta compared to normotensive pregnancies. These angiogenic imbalances perhaps activate abnormal placental vascularization and pre-eclampsia development. Pregnancy in itself reflects a modest maternal inflammatory response, whilst pre-eclampsia is identified as an excessive inflammatory response (Redman *et al.*, 1999). Thus, when combined with the immune insufficiency stimulated by HIV infection, it is possible that the immune hyper-reactivity is perhaps prevented thereby inhibiting pre-eclampsia development. It is also possible that HIV infection may influence angiogenesis in pre-eclampsia development. Moreover, a combination of the immune activation in pre-eclampsia and the immunosuppression during HIV infection may impact on pro- and anti-angiogenic factors.

1.2 Placental vasculogenesis and angiogenesis

Placental vasculogensesis, an essential embryonic requirement, is initiated by the differentiation of mesenchymal cells into endothelial progenitor cells (Smith, 2001). It is defined as the formation of new blood vessels and interconnecting capillaries through endothelial differentiation and migration, in the absence of pre-existing vascular structures (Felmeden *et al.*, 2003, Charnock-Jones *et al.*, 2004, Cines *et al.*, 1998, Zygmunt *et al.*, 2003).

Embryonic and post-embryonic angiogenesis or neovascularisation, however, is defined as being the constant growth or sprouting of new blood capillaries from pre-existing vessels (Wu *et al.*, 2010, Charnock-Jones *et al.*, 2004). During placental angiogenesis, the vasculogenic formation of endothelial tube segments are transformed into an organised vasculature through branching and non-branching angiogenesis (Cerdeira and Karumanchi, 2012). Placental development therefore requires both vasculogenesis and angiogenesis for efficient gaseous exchange and transport of nutrients and wastes. During pregnancy, placental angiogenesis extends from the maternal circulatory system through the trophoblastic cellular region of the villi and the growing fetal circulation and finally to the developing fetus (Kaufmann *et al.*, 2004, Charnock-Jones *et al.*, 2004).

The vascular system is made up primarily of vascular endothelial cells that are responsible for the regulation of vascular integrity and the thrombotic homeostatic balance plus its role in cellular transfer (Charnock-Jones *et al.*, 2004). In adult females, this system is able to acclimatise within defined physiological conditions, especially within the ovaries, the endometrial layer of the uterus and the placenta. Vascular growth is therefore essential for the

development of placental villi, allowing for peripheral gas and nutrient-exchange (Kingdom *et al.*, 2000). These processes are under the influence of regulatory molecules such as pro- and anti-angiogenic factors.

In addition, oxygen regulates the development of the maternal villi and the fetoplacental vasculature (Charnock-Jones *et al.*, 2004). The oxygen is released by the different trophoblast placental cells, including the vascular endothelial cells, pericytes, pro-angiogenic factors, their respective receptors and anti-angiogenic factors. Changes in blood flow therefore affect oxygen and nutrient level with concomitant alteration of the metabolism of endothelial cells and the development and maturation of the vascular tree. This growth and progression of the fetoplacental unit is subject to the effects of pro-angiogenic factors and their specific receptors.

1.2.1 Physiological effects of vasculogenesis and angiogenesis

A balance between pro-and anti-angiogenic factors are essential to ensure normal placental development and function. An angiogenic imbalance in either direction will thus result in various pathologies such as pre-eclampsia and IUGR, hypertension, diabetes and nephropathy, thereby compromising the wellbeing of women (Wu *et al.*, 2010).

1.2.2 Mechanism of vasculogenesis

Vasculogenesis during early gestation occurs via the differentiation and migration of endothelial progenitor cells into endothelial cells that line the vascular network (Charnock-Jones *et al.*, 2004, Agarwal and Karumanchi, 2011). Transformation of mesenchymal

precursor cells into haemangioblastic endothelial precursor cells (**Fig. 1.1**) enhances their differentiation into new placental blood vessels (Cines *et al.*, 1998).

The haemangioblastic cells/primitive yolk sac initiates vasculogenesis via the:

- differentiation of endothelial precursor cells which allows for the generation and proliferation of endothelial cells by the interaction between vascular endothelial growth factor (VEGF) and its receptors [tyrosine receptor kinases VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1)]. This promotes contact between endothelial cells and tubal growth.
- transition from vasculogenesis to angiogenesis via the differentiation of mesenchymal cells into endothelial cells mediated by angiogenic promoters such as angiopoeitin 1 and 2 and their tyrosine kinase receptors, tie-1 and -2. This creates an environment conducive for the maintenance of the vascular network and angiogenesis (Felmeden *et al.*, 2003, Cines *et al.*, 1998, Zygmunt *et al.*, 2003).

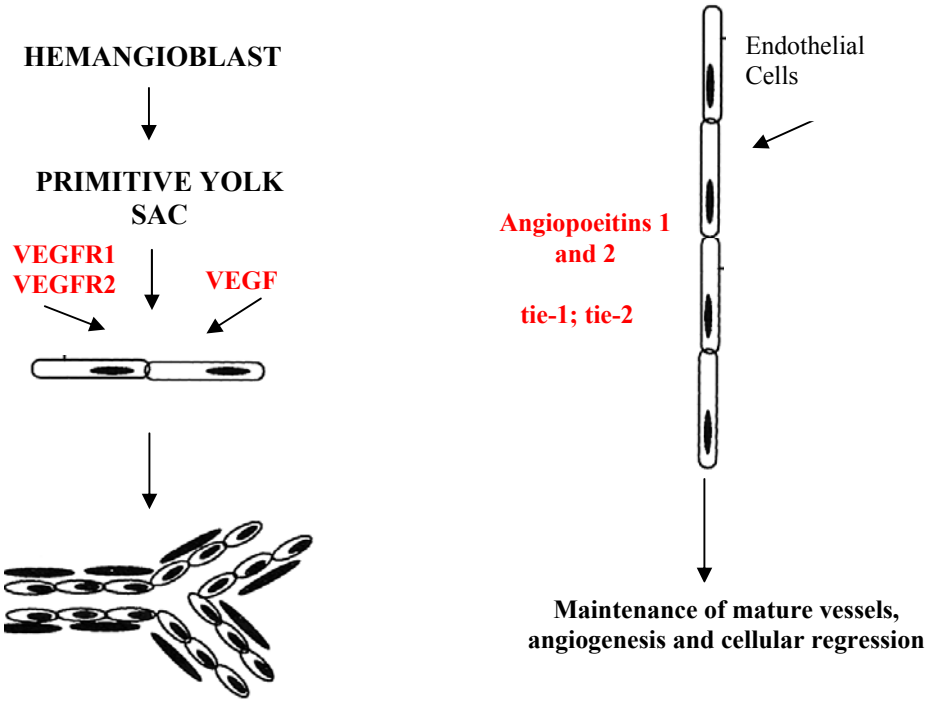


Figure 1.1: Formation of blood vessels via vasculogenesis (Adapted from Cines *et al*., 1998)

1.2.3 Mechanism of angiogenesis

Following vasculogenesis, placental angiogenesis occurs during embryonic development. This process occurs (**Fig. 1.2**) in four distinct pathways (Charnock-Jones *et al.*, 2004, Wu *et al.*, 2010) viz.,

- non-sprouting angiogenesis or proliferative elongation, which allow for the extension of existing endothelial tubes;
- non-sprouting angiogenesis or intercalative elongation which allow for the extension of existing endothelial tubes by incorporating circulating endothelial/progenitor cells;
- non-sprouting angiogenesis or intussusceptions, which allow for the longitudinal or oblique lumen partitioning of existing endothelial tubes via the formation of endothelial pillars;
- sprouting angiogenesis which allow for the formation of new side branches by lateral outgrowths (Charnock-Jones *et al.*, 2004).

Consequently, fetal growth can only be sustained if there is co-ordinated growth of the fetal villous tree. Hence the vascular development that occurs within the immature intermediate villi is characterised by branching angiogenesis whilst non-branching angiogenesis occurs in the mature intermediate villi to produce the terminal villi (Kingdom *et al.*, 2000). These progressions enable gas and nutrient exchange between the growing fetus and mother. Additionally, the branching angiogenesis occurring within intermediate villi undergoes major expansion thereby contributing to the production of stem villi (Kingdom *et al.*, 2000).

Angiogenesis guarantees to be an effective therapeutic target as a result of the development of numerous pathologies arising from either excessive or inadequate angiogenesis (Wu *et al.*, 2010). Treatment regimes would consider pro-angiogenic therapy aspiring to increase angiogenic promoters or decrease angiogenic inhibitors whilst anti-angiogenic therapy would decrease angiogenic promoters and increase the inhibitors. Thus, treatment targets the receptors of the VEGF family whereby VEGF promotes pro-angiogenic signals through VEGFR-2. However, the recently identified soluble fms-like tyrosine kinase-1 (sFlt-1/sVEGFR1), the soluble form of VEGFR-1 receptor, prevents angiogenic signalling (Maynard *et al.*, 2003).

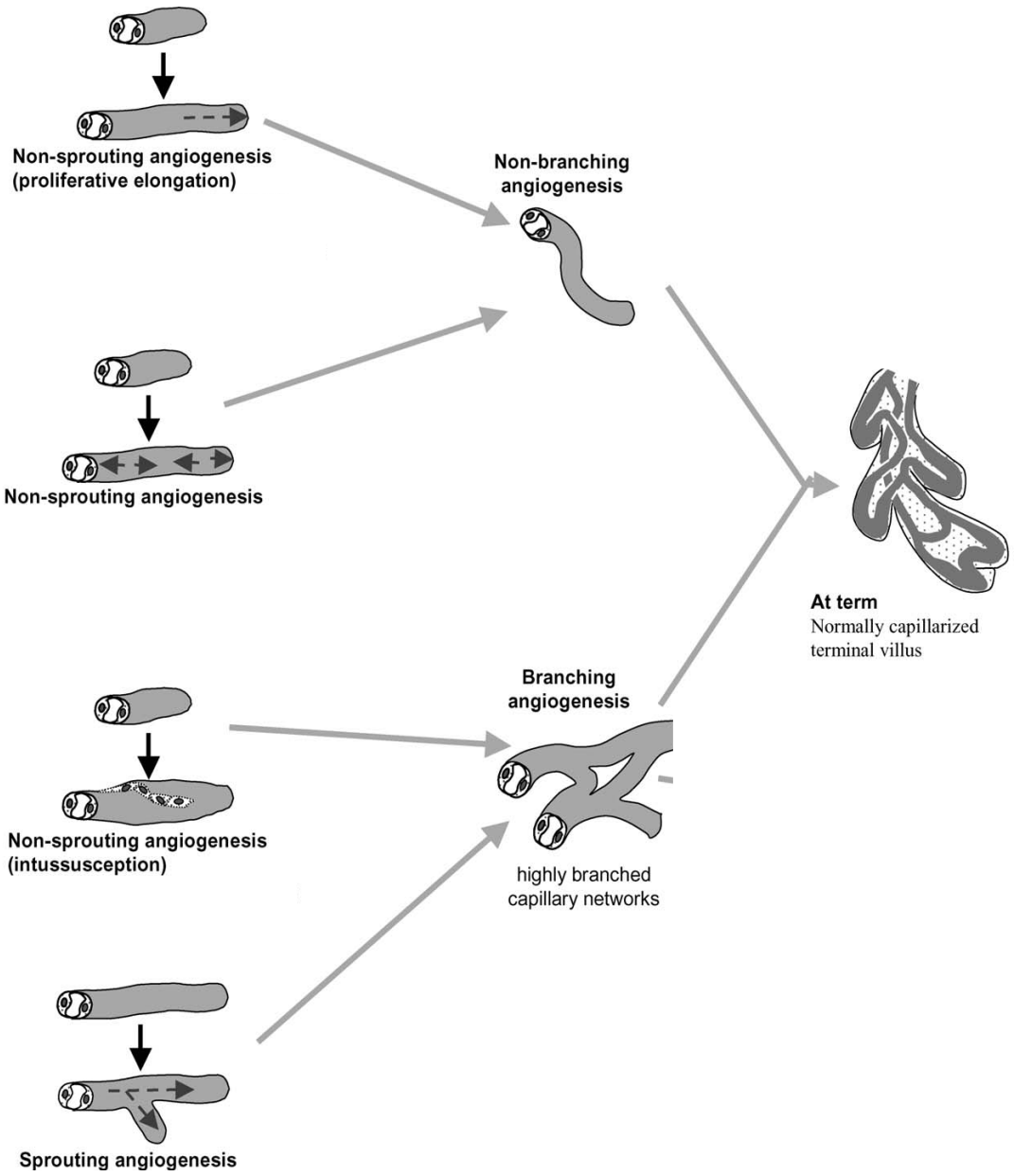


Figure 1.2: Angiogenic processes and patterns of villous formation (Adapted from Charnock-Jones *et al.*, 2004)

1.3 Remodelling of maternal vasculature

Both vasculogenesis and angiogenesis contribute extensively to the normal progression of pregnancy, with a significant role in the development of the placenta. Importantly, their influence on the conversion of the spiral arteries from low resistance blood vessels to flaccid, higher calibre blood conduits impacts on the increased oxygen and nutrient demands required for maintaining the growing fetus throughout pregnancy (Karumanchi *et al.*, 2005).

1.3.1 Early development of the human placenta

Consequently, placental development requires remodelling of maternal spiral arteries and decidualisation (Aplin, 2000, Red-Horse *et al.*, 2004). Cell differentiation continues via two main pathways, ie., the villous/extravillous and invasive pathway of trophoblast migration, giving rise to the placenta (Forbes and Westwood, 2010). Furthermore, various factors such as hormones, transcription and growth factors, cytokines and varying oxygen levels, contribute to the differentiation of cytotrophoblasts to syncytiotrophoblasts or extravillous trophoblast cells, thereby enabling spiral arterial invasion and the subsequent formation of a conducive fetal oxygenation and nutritional environment (Lunghi *et al.*, 2007).

Hypoxia via the alteration of gene expression induces the proliferation of cytotrophoblast but prevents the differentiation of extravillous trophoblast and syncytiotrophoblast. In contrast, growth factors such as VEGF induces the differentiation of cytotrophoblasts into syncytiotrophoblast and endovascular extravillous trophoblasts (Fukushima *et al.*, 2005). However, the angiogenic effect of these factors are prevented by the anti-angiogenic sFlt-1, which act by neutralising the effects of VEGF (Fukushima *et al.*, 2005). In contrast, the

differentiation of the invasive cytotrophoblasts are inhibited by transforming growth factor-β (TGF-β) and its receptor, endoglin (Caniggia *et al.*, 1997).

The feto-maternal exchange area is thus characterised by these three main cell types (**Fig. 1.3**), viz.,

- villous cytotrophoblast cells which provide a proliferative pool of cells during gestation;
- syncytiotrophoblast cells that are exposed to the maternal interface, serves as a protective fetal barrier, and also functions as the feto-maternal interface;
- and the extravillous trophoblast cells that invade the maternal endometrium forming connecting villi. This leads to the formation of the placenta (Red-Horse *et al.*, 2004, Vitiello and Patrizio, 2007, Norwitz *et al.*, 2001, Forbes and Westwood, 2010).

Beneath the cytotrophoblast layer is the villous stromal tissue which consists of various cell types including fibroblasts, placental macrophages or Hofbauer cells and endothelial cells (Forbes and Westwood, 2010).

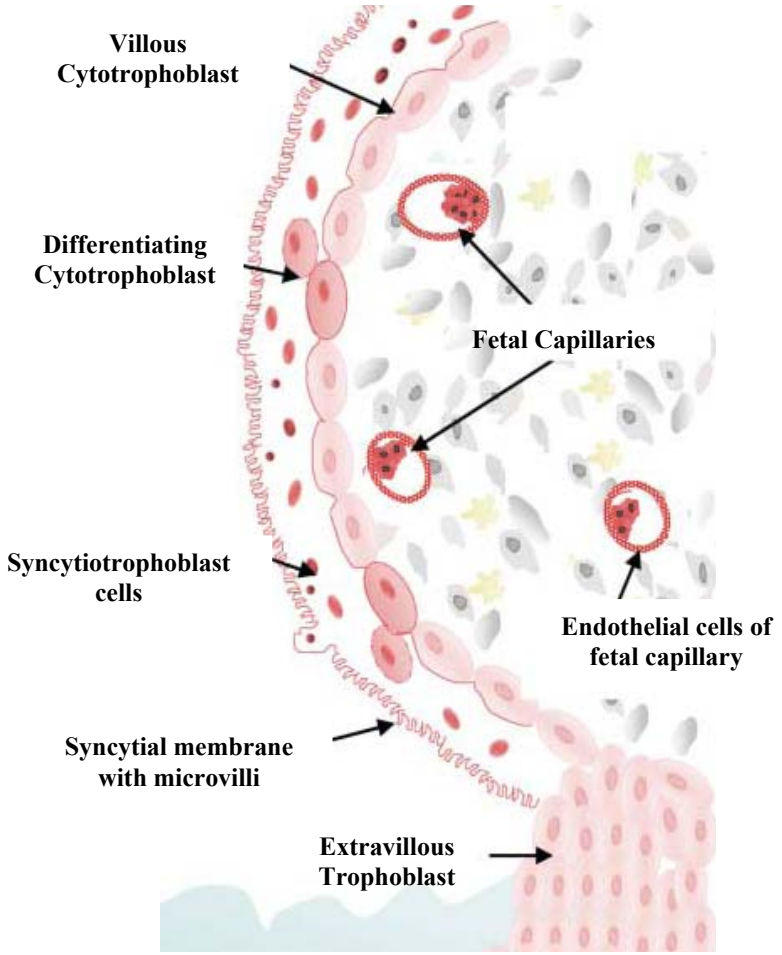


Figure 1.3: Illustration of the human placental cell types during first trimester.

The proliferation and differentiation of the cytotrophoblasts result in the formation of invasive extravillous trophoblasts and the syncytiotrophoblast (Adapted from Forbes and Westwood, 2010).

Whilst the formation of the feto-maternal interface is regulated by several physiological elements, oxygen tension is a key regulator for the differentiation and proliferation of trophoblasts (Red-Horse *et al.*, 2004). During early development, trophoblast cells derived from the basal plate and tips of the anchoring villi infiltrate into the decidua and inner myometrium in a timed sequence (Red-Horse *et al.*, 2004, Pijnenborg *et al.*, 2006).

During the early gestational period, placental growth occurs within a fairly hypoxic setting that essentially enhances the proliferation of cytotrophoblasts along the invasive pathway (**Fig. 1.4A**). As placental development continues, these cytoptrophoblasts continue to invade the uterine arteries of the decidua (**Fig. 1.4B**). This remodelling of the arterial wall results in complete plugging of spiral arteries thus creating a low oxygen micro-environment conducive to the proliferation and development of cytotrophoblast cells (**Fig. 1.4A-B**) (Aplin, 2000, Pijnenborg *et al.*, 2006, Red-Horse *et al.*, 2004). Vascular plugging also offers potential protection of the growing fetus from increased arterial pressure (Aplin, 2000, Pijnenborg *et al.*, 2006).

Towards the end of the first trimester (10-12 weeks), vascular flow is initiated within intervillous spaces. With endovascular invasion, the cytotrophoblasts migrates along the luminal regions of the spiral arterioles and adopt a vascular endothelial phenotype (Aplin, 2000, Pijnenborg *et al.*, 2006). Also, increased oxygen levels enhance cytotrophoblastic differentiation (**Figs. 1.4C; 1.5**). These endovascular trophoblasts invade the arterial walls, with the replacement of vascular smooth muscle and elastic tissue with a fibrinoid type material (Aplin, 2000). Thus, remodelling of the uterine arterioles during normal pregnancy requires

infiltration of interstitial trophoblastic cells into decidual stroma until it reaches the myometrium, followed by migration and luminal arterial invasion of endovascular trophoblasts (Red-Horse *et al.*, 2004, Maynard *et al.*, 2005, Pijnenborg *et al.*, 2006, Wang *et al.*, 2009).

This arterial transformation by the trophoblasts, called a "*vascular mimicry*" or pseudovasculogenesis, results in the diameter of the spiral arteries increasing 4- to 6- fold relative to that in non-pregnant women. Hence these low-resistance high-capacitance vessels supply the placenta with a maximal blood flow which allows for an enhanced supply of nutrients to the growing fetus independent of the influence of maternal vasomotor control. Failed or inadequate endovascular invasion results in miscarriages, whilst shallow invasion up to the decidua has been related to pre-eclampsia and intra-uterine growth restriction.

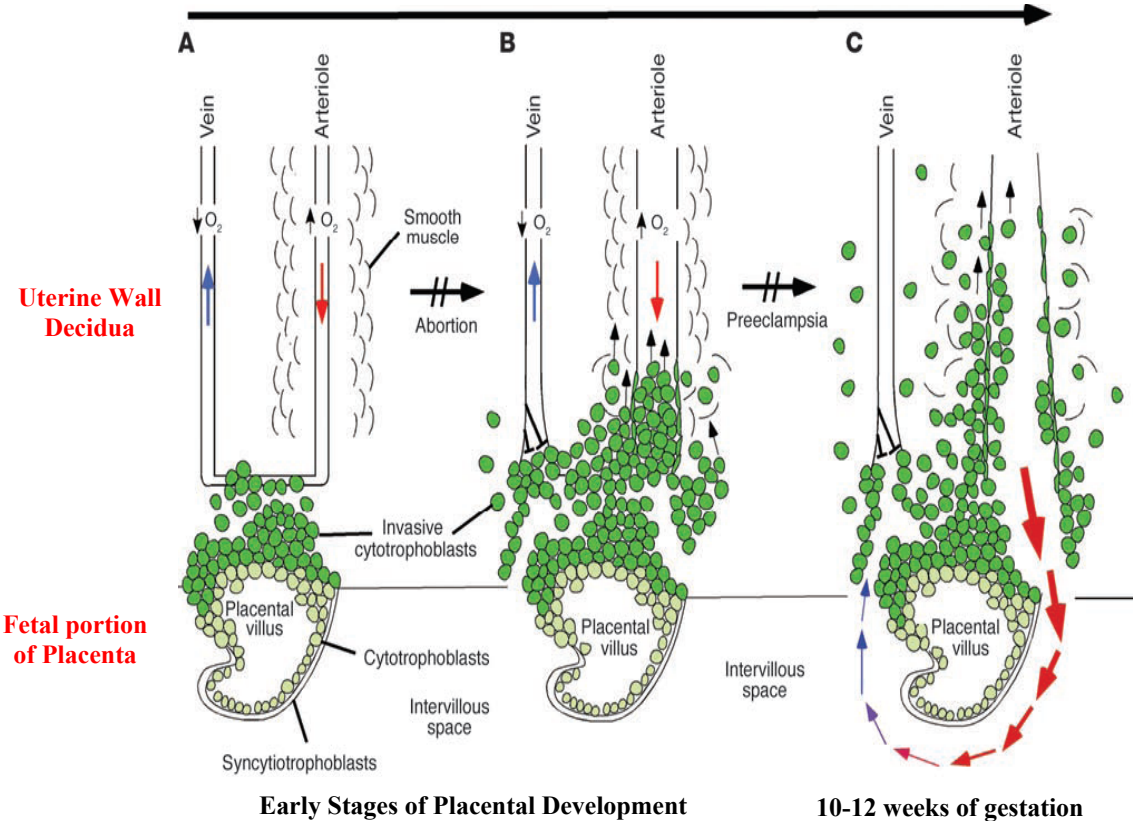


Figure 1.4: Uterine invasion of cytotrophoblasts

The blunt arrows (⊣) indicate that the cytotrophoblasts migrate further up arteries than veins (Adapted from Red-horse *et al.*, 2004).

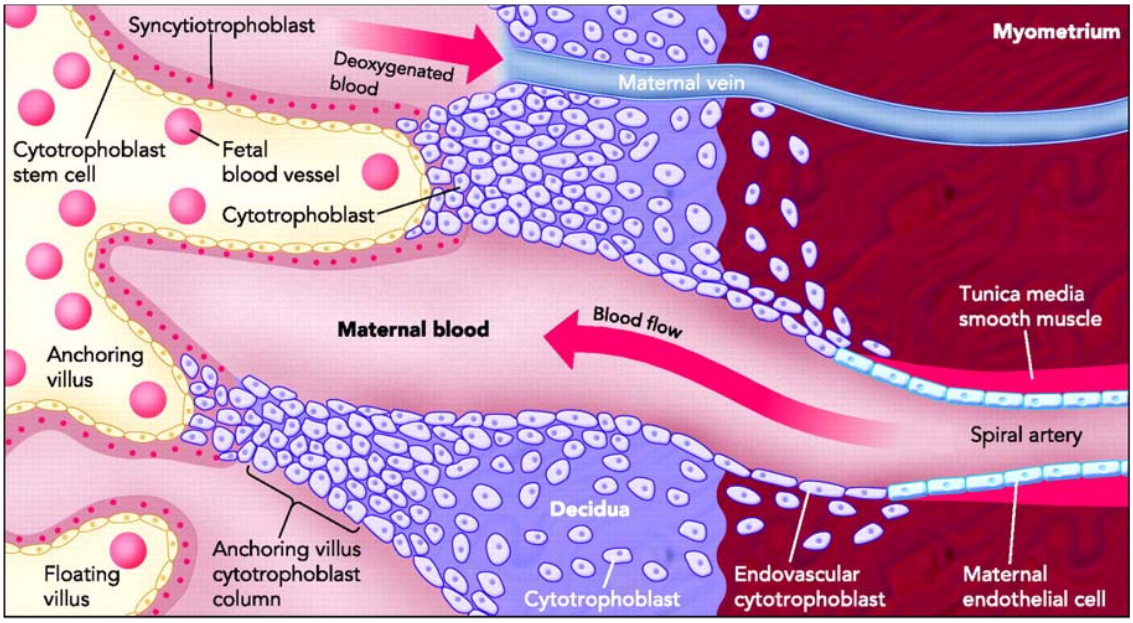


Figure 1.5: Normal placental development (with permission from Wang *et al*., 2009)

Remodelling of maternal spiral arteries by invasive cytotrophoblasts through the differentiation of cytotrophoblast from an epithelial phenotype to an endothelial phenotype resulting in the conversion of from small-calibre resistance to high-calibre capacitance vessels.

1.3.2 Histological features of term placenta

The human placenta is a haemochorial villous structure, produced through a series of sequential events that enables the maternal-feto exchange between mother and fetus (Gude *et al.*, 2004). It consists of the maternal region (maternal plate) that is produced from the endometrium and the fetal region (chorionic plate) that is derived from the chorionic sac (Gude *et al.*, 2004). Consequently, the functional unit of the placenta is the chorionic plate, which is separated from the maternal plate by the intervillous space. This intervillous space consists of several villous types that are lined by syncytiotrophoblasts. The villi are highly branched with major fetal vascular networks (Gude *et al.*, 2004). These villi include the stem villi or conducting vessels, the intermediate and terminal villi, or exchange vessels (**Fig. 1.6**).

According to Bernischke *et al*., (2006), the histological features of these villi include:

i. Stem villi which forms the central branches of the villous trees comprising dense fibrous stomal tissue (Bernischke *et al.*, 2006). Larger stem villi contain mainly arteries and veins, whilst peripheral regions contain capillaries or sinusoids with distinct muscular walls. They have a thick trophoblastic cover and are devoid of epithelial plates. Beneath the superficial syncytiotrophoblasts are the cytotrophoblast cells. At term, this trophoblast layer degenerates and is replaced with perivillous fibrinoid. These villi provide mechanical support to the villous tree.

ii. Immature intermediate villi are present in fewer numbers at term. They consist of a consistently thick trophoblastic layer also devoid of epithelial plates. They are characterised by a reticular stromal region with several pale staining stromal channels that consists of Hofbauer cells or macrophages. These cells are rounded and vacuolated

in appearance. The stroma lack large arteries and veins and contain few fetal capillaries. These villi serve as proliferation centres of the villous trees.

iii. Mature intermediate villi are slender, rounded branches of stem villi that consist of a thin trophoblastic cover (Bernischke *et al.*, 2006). Stromal region is made up of loose connective tissue with fixed cellular types but fewer fibers. Fetal vessels include mostly capillaries. These villi give rise to terminal villi.

iv. Terminal villi are branches of the mature intermediate villi (**Fig. 1.6**). They are characterised by thin trophoblastic covers with several epithelial plates, extremely coiled fetal vessels and dilated sinusoids. These fetal vessels bulge against the trophoblasts layer, thereby transforming into a thin vasculosyncytial membrane that lack nuclei. These villi form the feto-maternal exchange area.

Additionally, terminal together with the other villous types are characterised by syncytial knotting (Bernischke *et al.*, 2006). These knots appear as polyploid outgrowths of trophoblast tissue at the surface of villi and as syncytial bridges that unite adjacent villi. Placental septa also project into the intervillous space and contain interspersed anchoring villi with extravillous trophoblasts cells from the septal surfaces.

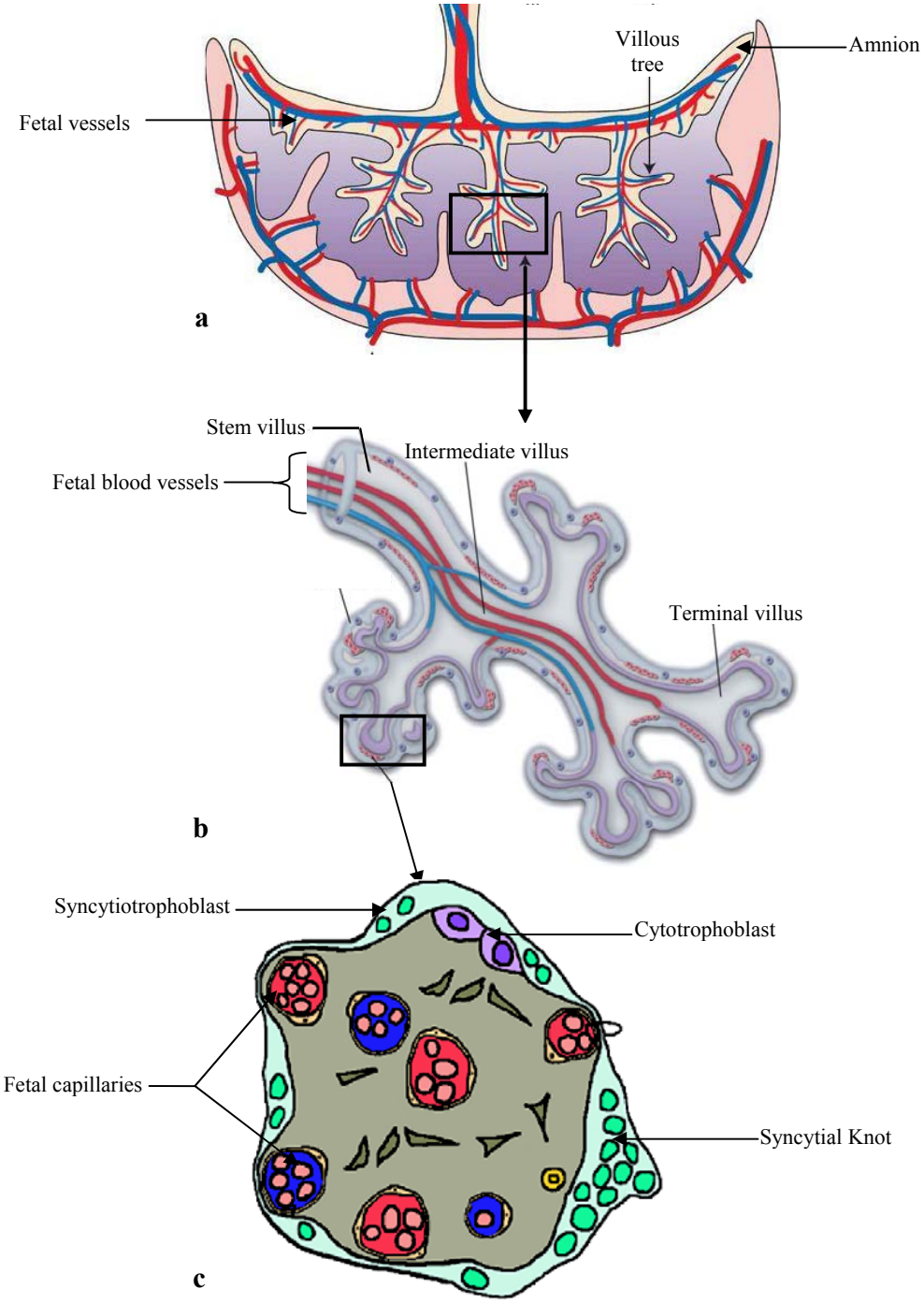


Figure 1.6: Illustration of term human placenta depicting (a) structure of villous tree (b) fetal placental circulation and (c) terminal villus (Adapted from Gude *et al.*, 2004 and Cerdeira and Karumanchi, 2012).

1.4 Regulation of angiogenesis

Angiogenesis is regulated by various angiogenic regulators including cytokines, hormones and growth factors; hypoxia and hypoglycemia; shear stress and stretch; components of extracellular matrix (laminin, fibronectin) and their receptors; matrix metalloproteinases (MMPs) and their tissue inhibitors; other proteases (urokinase-type-uPA and tissue-type plasminogen activator, tPA); fibrin; inflammatory cells and pericytes (Hoeben *et al.*, 2004, Felmeden *et al.*, 2003). The growth factors that regulate angiogenesis (**Fig. 1.7**) include basic fibroblast growth factor (FGF), vascular endothelial growth factor, tumour necrosis factor-alpha (TNF-α) and placental growth factor (PlGF) (Felmeden *et al.*, 2003, Cines *et al.*, 1998, Yla-Herttuala *et al.*, 2007, Klagsbrun and Moses, 1999, Charnock-Jones *et al.*, 2004).

Angiogenic progression involves binding of angiopoietins 1 and 2 with the tyrosine kinase receptor tie-2 (Hansson *et al.*, 2006), an interaction that maintains both vasculogenesis and angiogenesis (Charnock-Jones *et al.*, 2004). The maintenance of vessels, expansion of new vessels, and degeneration of formed vessels promotes the activation of endothelial cells (Charnock-Jones *et al.*, 2004, Cines *et al.*, 1998). Once this is achieved, degradation of endothelial basement membranes of existing vessels follows, together with the relocation, production and differentiation of endothelial cells. This is followed by reconstitution of the basement membrane and the final maturation and stabilisation of blood vessels (Cines *et al.*, 1998, Felmeden *et al.*, 2003, Yla-Herttuala *et al.*, 2007). The resultant effect is the proliferation and functional maturation of the endothelium, including enhanced vessel permeability and proteolytic enzyme activity of the extracellular matrix by specific proteolytic

enzymes such as collagenases and plasminogen activators (Felmeden *et al.*, 2003, Cines *et al.*, 1998, Yla-Herttuala *et al.*, 2007, Charnock-Jones *et al.*, 2004).

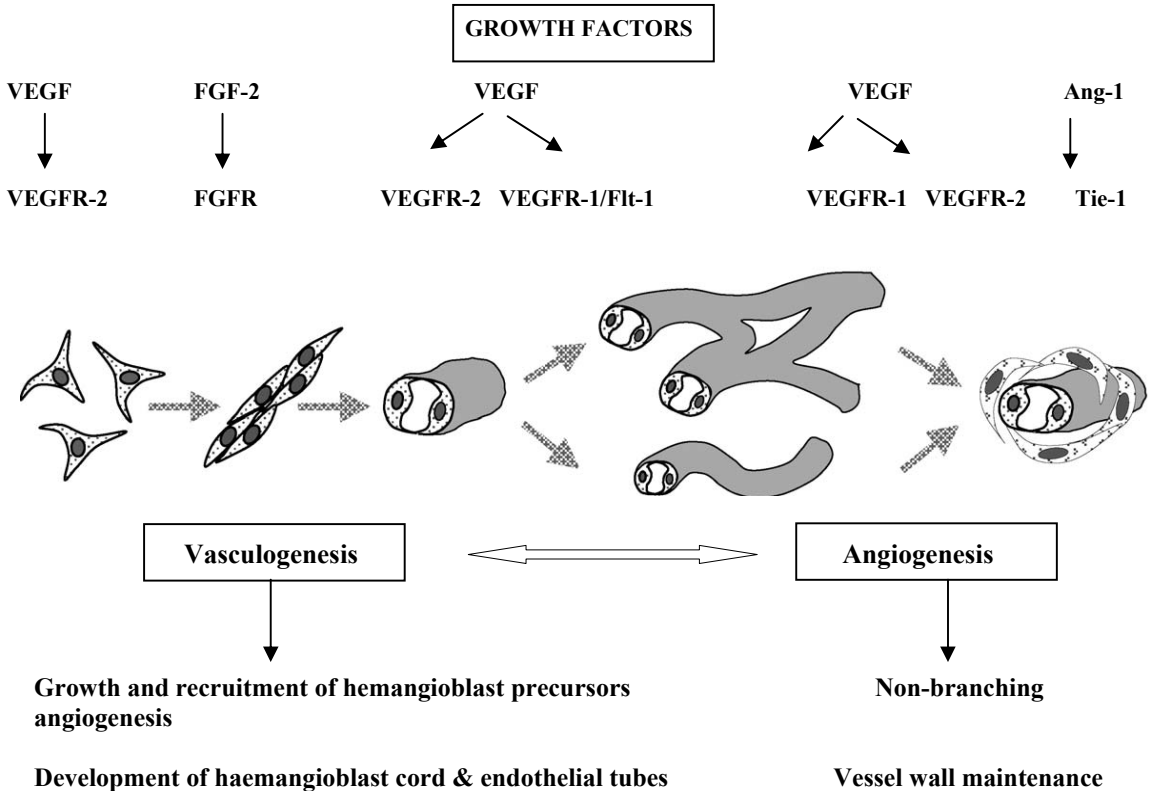


Figure 1.7: Role of growth factors and their receptors involved in vasculogenesis and angiogenesis

Ang-1 and tie-1 denotes angiopoietin-1 and angiopoietin receptor respectively; FGF-2 denotes (basic) fibroblast growth factor-2; FGFR is its receptor; VEGF denotes vascular endothelial growth factor; VEGFR-1 (Flt-1) and VEGFR-2 are its receptors (Adapted from Charnock-Jones et al., 2004).

1.5 Growth factors of placental angiogenesis

The placenta produces various angiogenic inhibitors and growth factors (Charnock-Jones *et al.*, 2004). Fetal vessels undergo several characteristic modifications during development, indicating that angiogenic growth factors together with their inhibitors may possibly play a role in this process. Angiogenic growth factors include FGF, angiopoetin proteins (Ang-1/-2), VEGF and PlGF (Kopcow and Karumanchi, 2007, Reynolds and Redmer, 2001, Zygmunt *et al.*, 2003, Charnock-Jones *et al.*, 2004). These growth factors are produced by different types of cells and have a significant role in organ development and growth of tumours, plus *in vivo* angiogenesis as outlined below.

1.5.1 Fibroblast Growth Factor (FGF) and their receptors

This family comprises approximately 18 members, including 2 major isoforms viz., FGF-1, a 16 kD peptide (acidic FGF), and FGF-2, a 18 kD peptide (basic FGF) (Felmeden *et al*., 2003, Zygmunt *et al*., 2003). These promoters are produced by vascular smooth muscle cells and function as powerful stimulators for migration of endothelial cells and in the formation of endothelial tubes (Klagsbrun and Moses, 1999). Both isoforms can induce endothelial expansion and alter endothelial functioning. In addition, these isoforms prevent apoptosis, induce development of fibroblasts and endothelial cells and have a role in wound healing as well as growth of tumours (Felmeden *et al*., 2003 and Zygmunt *et al*., 2003). Their biological function is mediated via low affinity receptors such as heparan sulfate proteoglycans (HSPG) and high affinity receptors characterised by FGF receptors 1-4 (FGFR1-4). The FGFR 1-4 receptors are tyrosine kinase receptors which have a role in signalling whilst the HSPG receptors allow for ligand sequestration and strength.

1.5.2 Angiopoietins and their receptors

The angiopoietins are essential during initial angiogenesis and vasculogenesis (Zygmunt *et al.*, 2003, Felmeden *et al.*, 2003). They have been identified as ligands for the tyrosine kinase tie-2 receptor, which are localised on endothelial, cytotrophoblast and hematopoietic cells. Angiopoietins have an established role in capillary modifications and the continued survival of endothelial cells.

There are currently four isoforms of angiopoietins (Klagsbrun and Moses, 1999, Felmeden *et al.*, 2003, Zygmunt *et al.*, 2003); viz.,

- ***Angiopoietin 1*** (Ang-1): ligand for tie-2 receptors, found in tissues next to blood vessels, expressed in cytotrophoblasts and syncytiotrophoblasts and heart myocardium;
- ***Angiopoietin 2*** (Ang-2): shares 60% homology to Ang-1, however does not induce tie-2 phosphorylation, may be an intrinsic antagonist of Ang-1 that prevents activation of Ang-1 and angiogenesis, located at tissue remodelling areas and mainly expressed in cytotrophoblasts;
- ***Angiopoietin 3*** (Ang-3): found in mouse and
- ***Angiopoietin 4*** (Ang-4): found in humans.

1.5.3 Vascular Endothelial Growth Factors (VEGF)

This group of homodimeric glycoproteins are essential for vasculogenesis, lymphangiogenesis and angiogenesis (Holmes *et al.*, 2007). Their initial recognition as strong vascular permeability factors contributes extensively to their primary angiogenic role in both physiological and pathological conditions (Wu *et al.*, 2010). This family consists of seven

members, viz., VEGF-A, PlGF, VEGF-B, VEGF-C, VEGF-D, and VEGF-E and snake venom VEGF (svVEGF), each sharing a similar VEGF homology domain (Holmes *et al.*, 2007, Yla-Herttuala *et al.*, 2007).

The scope of pro-angiogenic and anti-angiogenic treatment involves the VEGF system together with its receptors and ligands as primary targets of interest. Their clinical contribution to cardiovascular biology, tumour angiogenesis and retinal neovascularisation is widely documented (Ferrara, 2004, Ferrara and Davis-Smyth, 1997, Ferrara and Keyt, 1997, Felmeden *et al.*, 2003, Holmes *et al.*, 2007, Yla-Herttuala *et al.*, 2007).

1.5.3.1 Structure, gene organisation and isoforms of VEGF ligands

This family consists of seven members that share a similar VEGF homology domain, viz., VEGF-A, VEGF-B, VEGF-C, VEGF-D and PlGF (Holmes *et al.*, 2007, Yla-Herttuala *et al.*, 2007, Wu *et al.*, 2010). Structurally, these biologically active crystallized homodimers are covalently joined by two disulfide bonds, with a prominent cysteine-knot motif comprising an eight cysteine residue ring (**Fig. 1.8a-b**) (De Falco *et al.*, 2002, Iyer and Acharya, 2002, Iyer *et al.*, 2001). This motif consists of one interchain and three intrachain disulfide bridges that are symmetrically positioned opposite to each other (Ferrara and Davis-Smyth, 1997, Iyer and Acharya, 2002). This framework provides structural stability to these proteins and their receptor binding regions.

The biologic function of these disulphide linked dimeric glycoproteins are mediated via 3 VEGF tyrosine kinase signalling receptors (Felmeden *et al.*, 2003). Additionally, the PlGFs

share a 53 % identity with the VEGF gene. The structural and biological properties of VEGF have been extensively documented (Yla-Herttuala *et al.*, 2007, Hoeben *et al.*, 2004, Felmeden *et al.*, 2003, Holmes *et al.*, 2007).

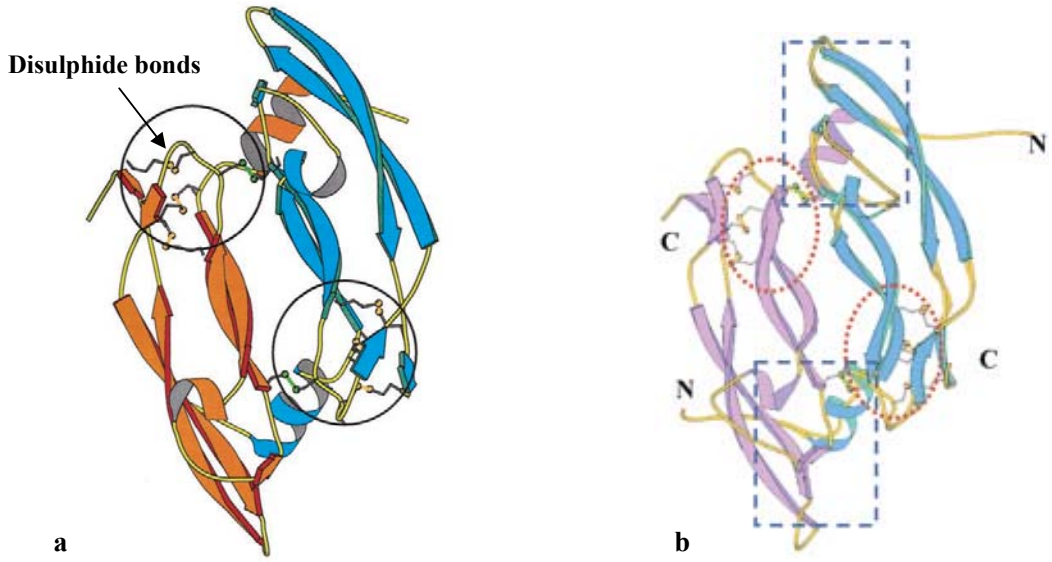


Figure 1.8: Structural model of human (a) VEGF and (b) PlGF

The cysteine-knot motif and the receptor-binding sites have been highlighted as circles whilst the receptor binding sites on PlGF are highlighted as rectangles (Adapted from Iyer *et al.*, (2001) and Iyer and Acharya (2002) respectively).

1.5.3.1.1 Vascular Endothelial Growth Factor A (VEGF-A)

The structural properties of the VEGF A gene is documented as an anti-parallel 34-42 kDa disulphide dimeric peptide structured as 8 exons divided by 7 introns in humans (Takahashi and Shibuya, 2005, Hoeben *et al.*, 2004, Roy *et al.*, 2006, Wu *et al.*, 2010). All members of the VEGF family are encoded by exons 1 to 5 with a chromosome location of 6p21.3. This gene consists of 9 homodimeric isoforms, viz., $VEGF_{121}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{162}$, $VEGF_{165}$, $VEGF_{165b}$, $VEGF_{183}$, $VEGF_{189}$ or $VEGF_{206}$ respectively (**Fig. 1.10**) (Takahashi and Shibuya, 2005, Wu *et al.*, 2010). The $VEGF_{165}$, isoform or VEGF A is documented as the main human VEGF isoform. The biological property of this isoform arises from its interaction with VEGFR-1, -2 and neuropilin-1 and -2. This isoform plays a significant role in angiogenesis and vasculogenesis both in health, disease (such as growth of tumours and wound healing) and therapeutic interventions.

An additional isoform of VEGF-A which contains 110 amino acids has been reported (Takahashi and Shibuya, 2005, Roy *et al.*, 2006, Hoeben *et al.*, 2004, Ferrara and Keyt, 1997, Ferrara, 2004, Felmeden *et al.*, 2003). This isoform is a product of a proteolytic cleavage. With the exception of $VEGF_{121}$ all isoforms can homo-dimerize, bind to heparan and contain a common 141 amino acid terminal that serves as a signal peptide permitting receptor recognition. The production of VEGFs occur within most maternal and fetal cells such as endothelial cells, placental tissue, uterine smooth muscle cells, macrophages and T cells (Ramesh and Shenoy, 2003, Roy *et al.*, 2006, Yla-Herttuala *et al.*, 2007). The expression of these factors is controlled by growth factors, oestrogen, tumour stimulators, hypoxia, nitric oxide and the thyroid stimulating hormone.

The function of VEGF includes a role in the development of blood vessels, anti-apoptotic characteristics, vasodilatory and hypotensive effects, micro-vascular permeability, coagulation effects, neuro-protective effects on hypoxic motor neurons, hematopoietic effects and the production and migration of vascular smooth muscle cells (Ramesh and Shenoy, 2003, Roy *et al.*, 2006, Yla-Herttuala *et al.*, 2007, Wu *et al.*, 2010). Its vasodilatory effects occur via the stimulation of endothelial nitric oxide synthase and the subsequent raised nitric oxide generation.

1.5.3.1.2 Placental Growth Factors (PlGF)

The PlGFs are expressed in the human placenta (especially villous syncytiotrophoblasts) during different gestational stages (Shore *et al.*, 1997, Vuorela *et al.*, 1997, Iyer and Acharya, 2002). They were also identified in reduced quantities within the thyroid gland, skeletal muscle, lungs and heart tissue (Takahashi and Shibuya, 2005, Shibuya and Claesson-Welsh, 2006, Roy *et al.*, 2006). The human PlGF gene comprises seven exons identified on chromosome 14q24 (Roy *et al.*, 2006, Torry *et al.*, 1999). The structure-based sequence alignment of PlGF-1 with VEGF is shown in **Fig. 1.9**. Initial reports suggest 3 isoforms of the PlGF gene occurring as a consequence of alternative splicing (Torry *et al.*, 1999, Iyer and Acharya, 2002), however, recent studies have classified 4 isoforms (**Fig. 1.10**) viz., PlGF-1 ($PlGF_{131}$), PlGF-2 ($PlGF_{152}$), PlGF-3 ($PlGF_{203}$) and PlGF-4 ($PlGF_{224}$) (De Falco *et al.*, 2002, Roy *et al.*, 2006, Takahashi and Shibuya, 2005).

Despite their size, these isoforms also vary in terms of binding affinities and their secretions. Biological activities of these growth factors are due to their receptor binding affinity with

VEGFR-1 and neuropilin 1 and 2 (Nrp-1 and Nrp-2) (De Falco *et al.*, 2002, Roy *et al.*, 2006, Takahashi and Shibuya, 2005). The structural differences that exist between these isoforms include the presence of 21 basic amino acid insertion sites at their carboxyl terminus for both PlGF-2 and PlGF-4 compared to PlGF-1 and PlGF-3. In addition, there are heparan binding domains that aid their binding to Nrp-1 and Nrp-2.

The therapeutic and functional role of PlGF within the cardiovascular system includes its effect in myocardial angiogenesis, macrophage chemotaxis via its regulation of vascular growth in atherosclerosis. Its role in pathologies is widely documented (De Falco *et al.*, 2002).

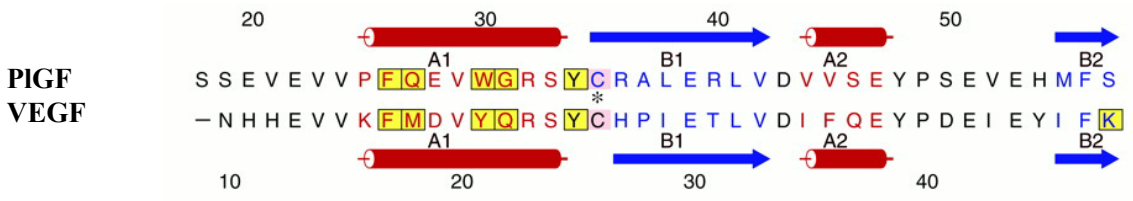


Figure 1.9: Sequence position between PlGF and VEGF

The amino acid residues are colour coded. *Blue* and *red* indicates the b-strands and helices of the genes, whilst pink denotes the cysteine residues and *yellow* denotes VEGF residues involved in Flt-1 (VEGFR-1) binding and the equivalent residues in PlGF-1(with permission from Iyer *et al.*, 2001).

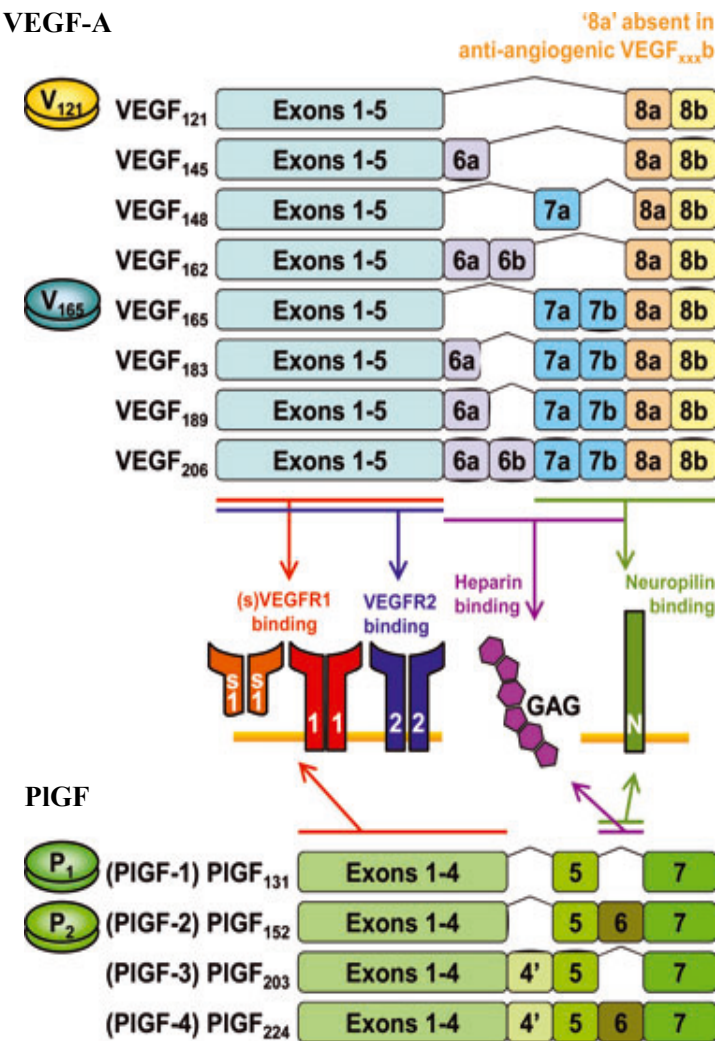


Figure 1.10: Gene structure of the human VEGF-A mRNA splice variants.

This gene consists of 8 exons that encode various structural motifs. Alternative splicing of the exon results in various isoforms of VEGF-A, PlGF and VEGF-B, with VEGF165 being the main human isoform (with permission from Wu *et al.*, 2010)

1.5.3.1.3 Vascular Endothelial Growth Factor B (VEGF-B)

All isoforms of VEGF-B have a molecular composition of 188 amino acids (**Fig. 1.11**) and are expressed within the heart, skeletal muscle and pancreas (Yla-Herttuala *et al.*, 2007, Takahashi and Shibuya, 2005). Alternative splicing of exon 6 of the human VEGF-B gene produces a 21 kDa soluble peptide VEGF-B_{167} and a 32 kDa VEGF-B_{186} that is both intra and extracellularly bound.

The VEGF-B_{167} isoform is expressed mainly in skeletal muscle, myocardium and brown adipose tissue. Both these peptides bind to VEGFR-1 and neuropilin 1, whilst VEGF-B_{167} additionally binds to heparan sulphate proteoglycans. The functions of VEGF-B include its inflammatory role in vascular remodelling and muscle angiogenesis (Takahashi and Shibuya, 2005, Roy *et al.*, 2006, Hoeben *et al.*, 2004, Ferrara and Keyt, 1997, Ferrara, 2004, Felmeden *et al.*, 2003, Wu *et al.*, 2010).

1.5.3.1.4 Vascular Endothelial Growth Factor C (VEGF- C)

This isoform is a 47 kDa glycoprotein consisting of 419 amino acids (**Fig. 1.11**) and is widely distributed within the placenta, heart, skeletal muscle, ovaries, small intestine, thyroid gland, platelets, and in some tumour growths (Takahashi and Shibuya, 2005, Roy *et al.*, 2006, Hoeben *et al.*, 2004, Ferrara and Keyt, 1997, Ferrara, 2004, Felmeden *et al.*, 2003, Yla-Herttuala *et al.*, 2007, Wu *et al.*, 2010). Biological activity of VEGF-C is due to its high affinity to both VEGFR-2 and -3. It also has a significant role in the development and protection of the lymphatic system.

1.5.3.1.5 Vascular Endothelial Growth Factor D and E (VEGF-D and -E)

VEGF-D is extensively expressed in endothelial cells, lung, skeletal muscle, colon, small intestine and heart (Roy *et al.*, 2006, Wu *et al.*, 2010). Their functional ability is due to their interaction with the receptors VEGFR-2 and -3. Additionally, they play a major role in the development of the endothelial cells as well as *in vitro* and *in vivo* angiogenic and lymphangiogenic functions (Roy *et al.*, 2006, Wu *et al.*, 2010). Both VEGF-D and E have the capacity to bind to VEGFR-2 and they contribute to the pathological angiogenesis that occur during viral infections (Yla-Herttuala *et al.*, 2007, Hoeben *et al.*, 2004, Felmeden *et al.*, 2003).

A summary of the VEGF ligands, its isoforms and biological roles are outlined in **Table 1.1**.

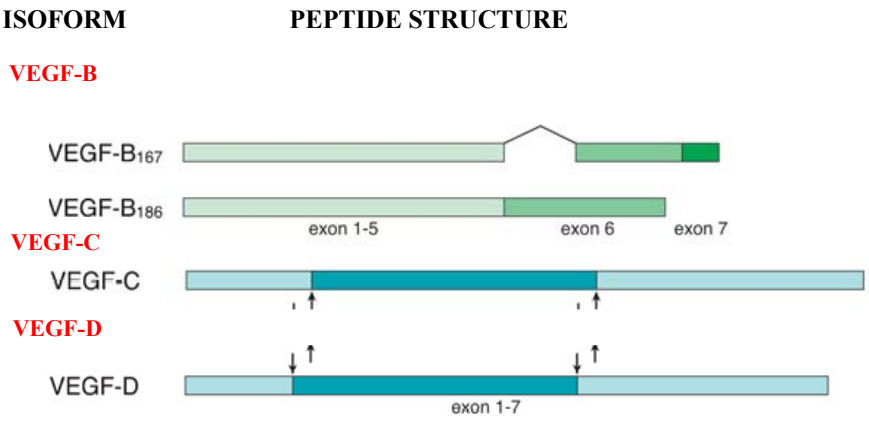


Figure 1.11: Isoforms of VEGF B-D

Isoforms of VEGF-B are generated by alternative exon splicing. Arrows denote positions of proteolytic cleavage that give rise to mature VEGF-C or VEGF-D (Adapted from Takahashi & Shibuya, 2005).

Table 1.1: Overview of the Characteristic Features of VEGF Isoforms (Adapted from Yla-Herttuala *et al*., 2007)

VEGF Ligands	Soluble VEGF Isoform	Solubility Effect	Biological Role
VEGF (VEGF-A)	VEGF-A_{121}, VEGF-A_{165} VEGF-A_{189}, VEGF-A_{206} ($VEGFA_{138/145/162/165b}$)	VEGF-A_{121} soluble, longer isoforms bind to heparan sulphates with increasing affinity	Vasculogenesis, angiogenesis, vascular homeostasis, vascular permeability, recruitment of bone marrow derived cells
VEGF-B	VEGF-B_{167}, VEGF-B_{186}	VEGF-B_{167} binds to heparan sulphates VEGF-B_{186} soluble	Angiogenesis and recruitment of bone marrow-derived cells
VEGF-C/-2	No, Unprocessed and proteolytically processed	Soluble	Growth of lymphatic & lymphangiogenesis, angiogenesis
VEGF-D	Yes	Soluble	lymphangiogenesis, angiogenesis
VEGF-E	-	Soluble	Angiogenesis
VEGF-F	-	Binds to heparan sulphates	Angiogenesis and vascular permeability
PlGF	$PlGF_{131}$ (PlGF-1),$PlGF_{152}$ (PlGF-2), $PlGF_{203}$ (PlGF-3); $PlGF_{224}$ (PlGF-4)	$PlGF_{131}$ and $PlGF_{203}$ soluble, $PlGF_{152}$ binds to heparan sulphates	Angiogenesis, monocyte migration, recruitment of bone marrow-derived cells

1.6 Receptors of the VEGF family

The biologic activity of VEGF ligands on the human endothelium is dependent upon their binding affinity with their respective receptors (Yla-Herttuala *et al.*, 2007, Hoeben *et al.*, 2004, Felmeden *et al.*, 2003, Veikkola *et al.*, 2000, Neufeld *et al.*, 1999, Lambrechts and Carmeliet, 2006). These ligands interact in an overlapping manner to three diverse but structurally related VEGF receptors. These receptors are classified either as membrane-bound signalling/tyrosine kinase receptors; high-affinity non-signalling co-receptors and matrix proteins; or as soluble receptors (Neufeld *et al.*, 1999, Desai *et al.*, 1999, Wu *et al.*, 2010).

1.6.1 Membrane-bound or tyrosine kinase receptors

To date, 3 full-length membrane-bound tyrosine kinase receptors have been recognised, viz., VEGFR-1 or fms-like tyrosine kinase (Flt-1), VEGFR-2 or kinase insert domain-containing receptor (KDR) or fetal liver kinase 1 (Flk-1) and finally the vascular endothelial growth factor receptor 3 (VEGFR-3) or fms-like tyrosine kinase (Flt-4) (Wu *et al.*, 2010). These receptors are stimulated via homo- or hetero- dimerization and ligand induced trans-phosphorylation of intracellular tyrosine residues.

VEGFR-1 receptors are 180 kDa transmembrane high-affinity glycoproteins, whilst VEGFR-2 receptors are 200–230 kDa high-affinity receptors and VEGFR-3 are 195 kDa high-affinity receptors (Takahashi and Shibuya, 2005, Neufeld *et al.*, 1999, Wu *et al.*, 2010). In addition, the VEGFR-3 receptors are proteolytically split at the fifth extracellular immunoglobulin domain into 2 disulphide bridge linked isoforms, 120 kDa and 75 kDa.

VEGFR-1 and VEGFR-2 are highly expressed in trophoblast cells, monocytes, renal mesangial cells, hematopoietic stem cells, megakaryocytes, vascular and lymphatic endothelial cells and retinal progenitor cells, fetal endothelial cells of the heart, lung and kidney, but reduced in the liver and brain (Torry *et al.*, 1999, Wu *et al.*, 2010, Takahashi and Shibuya, 2005). In contrast, VEGFR-3 receptors are expressed on blood and lymphatic vessels. Additionally, VEGFR-1 receptors mediate both pro- and anti-angiogenic effects depending on the type of ligand and binds to both VEGF-A and PlGF. In contrast, VEGFR-2 is the key pro-angiogenic signaller for sprouting angiogenesis and the survival of endothelial cells. VEGFR-3 on the other hand, is crucial for the growth of lymphatic endothelial cells and has a role in adaptive immunity (Wu *et al.*, 2010). Consequently, an over-expression of a soluble form of VEGFR-3 prevents fetal lymphangiogenesis.

Receptor-binding affinity varies with the different receptors, ie., the affinity of VEGFR-1 is greater for VEGF and PlGF, whilst VEGFR-2 binds only to VEGF (Veikkola *et al.*, 2000, Takahashi and Shibuya, 2005, Wu *et al.*, 2010, Iyer and Acharya, 2002). Receptors VEGFR-1 is specific for VEGFA, VEGF-B and PlGF whilst VEGFR-2 has a greater affinity to VEGF-A. VEGF-E and VEGFR-3 is specific for VEGF-C and VEGF-D. Alternatively PlGF, which shares a similar structural homology to VEGF (**Fig. 1.12**), binds strongly to VEGFR-1/Flt-1.

The pro- and anti-angiogenic effects of VEGFR-1 are due to their location of binding regions in both VEGF and PlGF (Veikkola *et al.*, 2000, Takahashi and Shibuya, 2005). These binding regions are located within the second immunoglobulin-like area of VEGFR-1 (Veikkola *et al.*, 2000, Takahashi and Shibuya, 2005, Wu *et al.*, 2010). Anti-angiogenic effects emanate from

the binding of VEGFR-1 to VEGF-A as a consequence of VEGF-A sequestration. This reduces the accessibility of VEGF-A to stimulation. Alternatively, the pro-angiogenic effects of VEGFR-1 with PlGF may be two-fold. It may be due to either direct intracellular interaction with VEGFR-1 via the dislocation of VEGF-A, thereby interacting with and signalling via VEGFR-2, or by intracellular crosstalk between the PlGF-stimulated VEGFR-1 and the VEGF-stimulated VEGFR-2 (Wu *et al.*, 2010).

VEGFR-1 averts the movement of human umbilical vein endothelial cells in response to VEGF-A, indicative of its key role in endothelial cell migration (Veikkola *et al.*, 2000, Takahashi and Shibuya, 2005, Wu *et al.*, 2010). It also works together with PlGF and VEGF-B, to induce development of a vascular network that leads to an *in vivo* recruitment of endothelial precursor cells. In contrast, VEGFR-2 and -3 are important during mitogenesis, angiogenesis and lymphangiogenesis (Takahashi and Shibuya, 2005, Yla-Herttuala *et al.*, 2007). It also promotes the permeability of VEGF-A. The expression of VEGFR-1 and VEGFR-2 is greatly stimulated by hypoxia (Veikkola *et al.*, 2000, Neufeld *et al.*, 1999).

1.6.2 Non-signalling co-receptors and matrix proteins

Extracellular heparan-associated proteins necessitate interaction with the longer VEGF-A (>$VEGF_{121}$) isoform (Wu *et al.*, 2010). Likewise, the glycosaminoglycan chains of HSPGs, such as perlecan, agrin and collagen XVIII comprise a rich source of interstitial binding sites for VEGF. Additionally, HSPGs located on the plasmalemma enable surface VEGF-VEGFR binding.

On the other hand, the non-signalling co-receptors viz., neuropilin 1 and -2 (Nrp-1/-2) were initially identified as semaphorin receptors involved in neuronal regulation; however they were later identified as isoform specific receptors for VEGF-A (Takahashi and Shibuya, 2005, Neufeld *et al.*, 1999, Wu *et al.*, 2010). The Nrp-1 co-receptors are 130–140 kDa cell-surface glycoproteins, whilst the Nrp-2, which shares 44% identity to Nrp-1, also shares a sequence homology with Nrp-1. Structurally, neuropilins have short intracellular loops (**Figure 1.12**), suggestive of their inability to serve as independent biological signal transducers.

The role of neuropilins in angiogenesis is evident by the formation of complexes via their interaction with VEGF receptors (Wu *et al.*, 2010). Neuropilins and HSPGs also bind to VEGF but do not seem to induce biological responses in the absence of the tyrosine-kinase receptors, thereby implying that each receptor has the ability to bind to different VEGF isoforms and initiate different functions (Yla-Herttuala *et al.*, 2007). The binding specificity thus varies for both co-receptors. Neuropilin-1 has a high affinity to $VEGF_{165}$, VEGF-B and PlGF-2, whilst Nrp-2 is specific for $VEGF_{145}$, $VEGF_{165}$, PlGF-2 and VEGF-C.

The specificity of Nrp-1 co-receptors for $VEGF_{165}$ and their binding capacity is regulated by amino acids located at the carboxyl-terminal chain of the exon 7-encoded peptide of $VEGF_{165}$ (**Fig. 1.12**) (Takahashi and Shibuya, 2005, Neufeld *et al.*, 1999, Wu *et al.*, 2010). In addition, Nrp-1 co-receptors act synergistically in two ways to produce pro-angiogenic effects. Firstly, it reduces the inhibitory signalling of VEGFR-1 with $VEGF_{165}$ by allowing less substrate to be accessible; and secondly, it is unable to directly interact with VEGFR-2, although it can produce the VEGFR-2-$VEGF_{165}$-Nrp-1 complex via non-overlapping binding sites of both VEGFR-2 and Nrp-1 on the $VEGF_{165}$ bridge. Pro-angiogenic signalling of VEGFR-2 via $VEGF_{165}$ isoform is thereby improved (Takahashi and Shibuya, 2005, Neufeld *et al.*, 1999, Wu *et al.*, 2010).

Characteristic features and biologic functions of all these receptors are summarized in **Table 1.2** and **Fig. 1.12.**

Table 1.2: Summary of Functions of VEGF receptors (Adapted from Felmeden *et al*., 2003)

VEGF Receptor	Ligand/ Protein	Biological Function
VEGFR-1 (Flt-1)	VEGF-A_{121} VEGF-A_{165} VEGF-B PlGF-1 and -2	Enhances migration of cells, organisation of vascular network and gene expression of monocytes and macrophages
VEGFR-2 (KDR/Flk-1)	VEGF-A_{121} VEGF-A_{145} VEGF-A_{165} VEGF –C VEGF-D	Stimulates mitogenesis and differentiation of endothelial cells, cell migration and vascular permeability
VEGFR-3 ((Flt-4)	VEGF-A_{145} VEGF-A_{165} VEGF-A_{189} VEGF-A_{167} PlGF-2	Promote remodelling of primary capillary blood vessels, growth of the cardiovascular system and controls development and maintenance of lymphatic system
Neuropilin 1 (Nrp-1)	VEGF-A_{165} PlGF-2	Growth of the cardiovascular system
Neuropilin 2 (Nrp-2)	VEGF-A_{165}	Stimulates organization of peripheral nerve fibres and growth of vascular networks

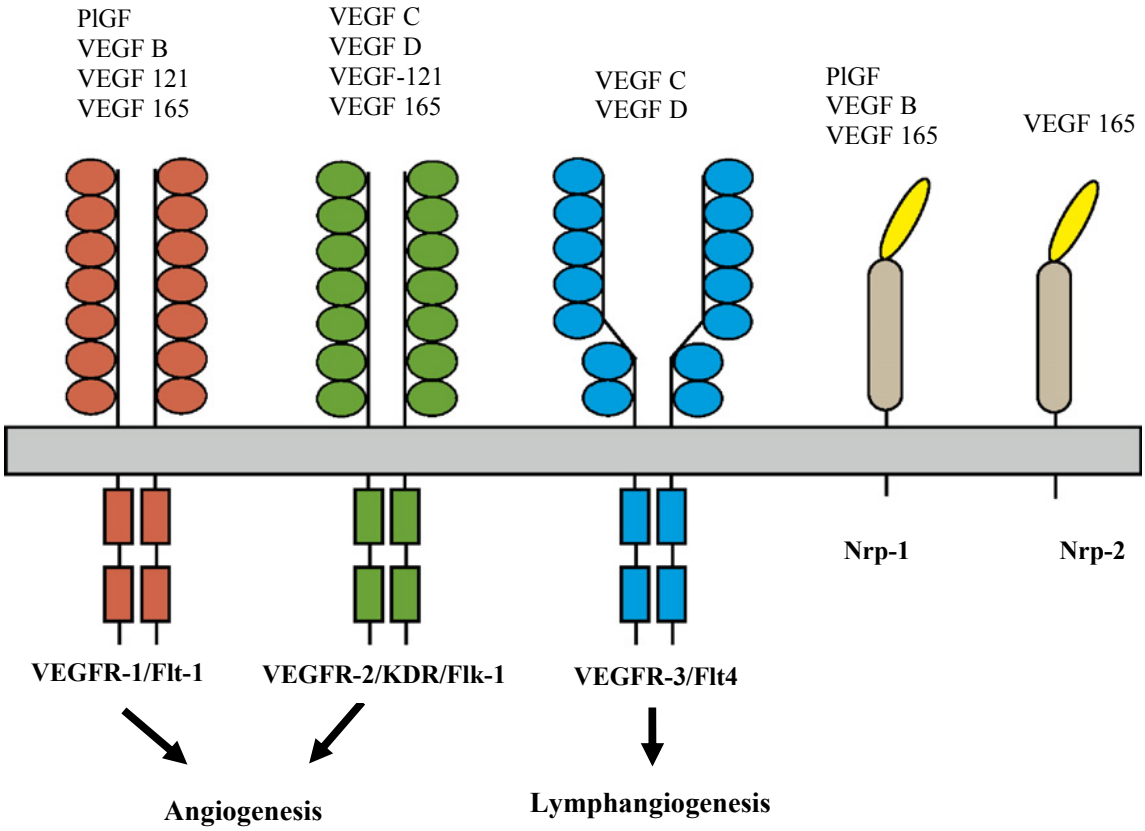


Figure 1.12: VEGF ligands and their receptors (Adapted from Lambrecht and Carmeliet, 2006).

1.6.3 Soluble receptors (soluble Fms like tyrosine kinase-1/sFlt-1)

Soluble VEGFR-1/Flt-1 is a truncated 110 kDa splice variant of the 180 kDa, full length membrane-bound VEGFR-1 (Takahashi and Shibuya, 2005, Hagmann *et al.*, 2012, Maynard *et al.*, 2005). This truncated form of soluble VEGFR-1 is secreted mainly by syncytiotrophoblasts. It contains an extracellular ligand binding domain that lacks a transmembrane and intracellular signalling domain (**Fig. 1.13**) (Takahashi and Shibuya, 2005, Hagmann *et al.*, 2012, Maynard *et al.*, 2005). The incidence of this spliced variant as an anti-angiogenic protein in pre-eclampsia is well documented (Wu *et al.*, 2010, Karumanchi and Bdolah, 2004).

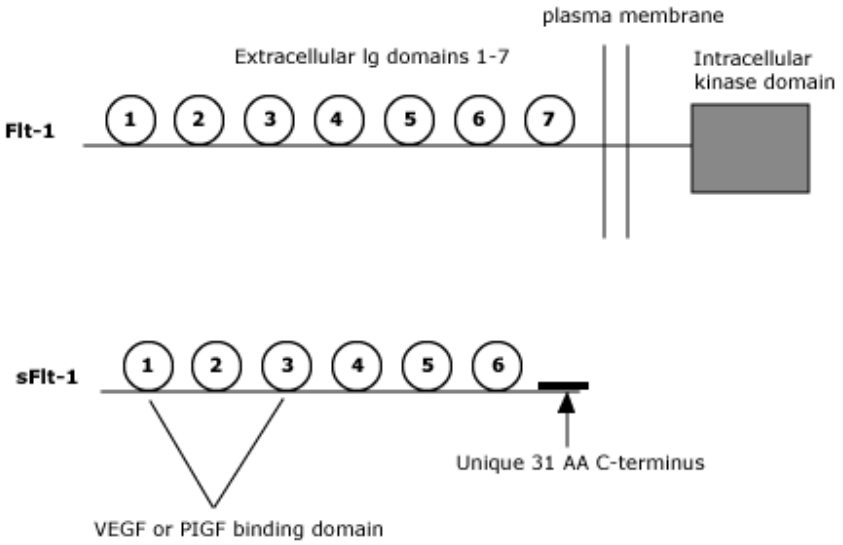


Figure 1.13: Structure of Flt-1 and sFlt-1/sVEGFR-1 (with permission from Maynard *et al.*, 2005)

Furthermore, it is known to bind with VEGF in both dimeric and monomeric forms *in vitro*. Both VEGFR-1 and sFlt-1 share the first 6 immunoglobulin-like loops on their N-terminus (**Fig. 1.14**); suggestive that sFlt-1 theoretically inherits VEGFR-1's affinity for VEGF and accessory molecules such as interstitial HSPGs and Nrp's plus VEGFR-1's dimerisation properties. These anti-angiogenic features characterise its antagonistic effects on VEGF plus its protein-specific role which is possibly due to VEGF-A sequestration and the formation of non-signalling heterodimers with VEGFR-2 (Wu *et al.*, 2010, Karumanchi and Bdolah, 2004).

Recent studies have demonstrated that the human placenta expresses a family of four different splice variants of sVEGFR-1/sFlt-1, namely sFlt1_v1, v2, v3 and v4 (Heydarian *et al.*, 2009, Silasi *et al.*, 2010). The expression of at least three of these proteins are elevated in pre-eclamptic placentae (Heydarian *et al.*, 2009). Another splice variant, sFlt-14 is specific for primates and is possibly the major VEGF-inhibiting factor with elevated expression within syncytial knots in pre-eclamptic placentae (Sela *et al.*, 2008). Further work is however, warranted to elucidate its precise role in pre-eclampsia.

The biological functions of VEGF and their receptors are illustrated in **Fig. 1.15**.

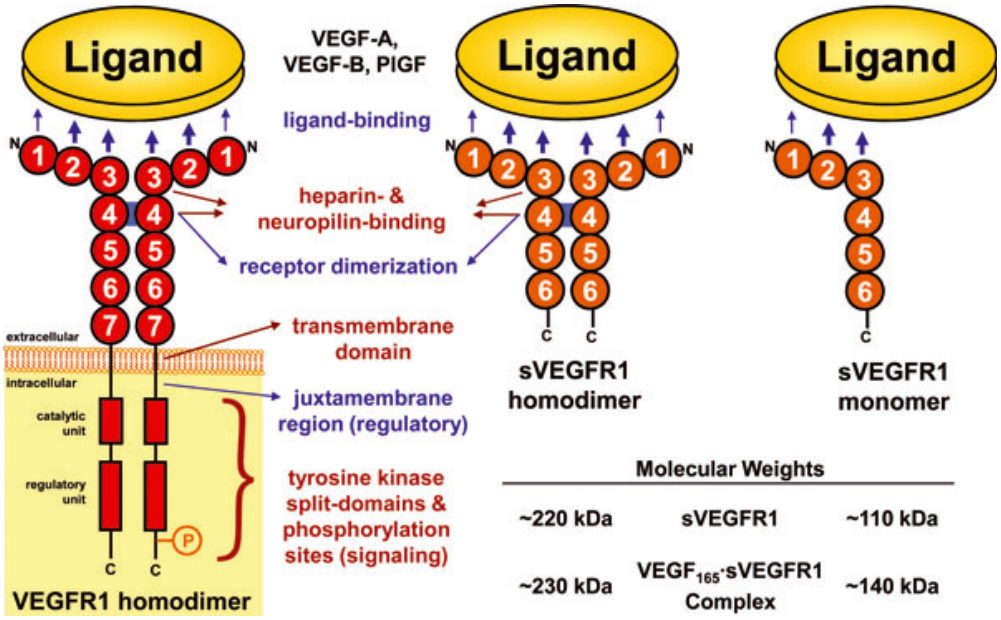


Figure 1.14: Protein structures of soluble VEGFR-1 versus membrane anchored VEGFR-1 (with permission from Wu *et al*., 2010)

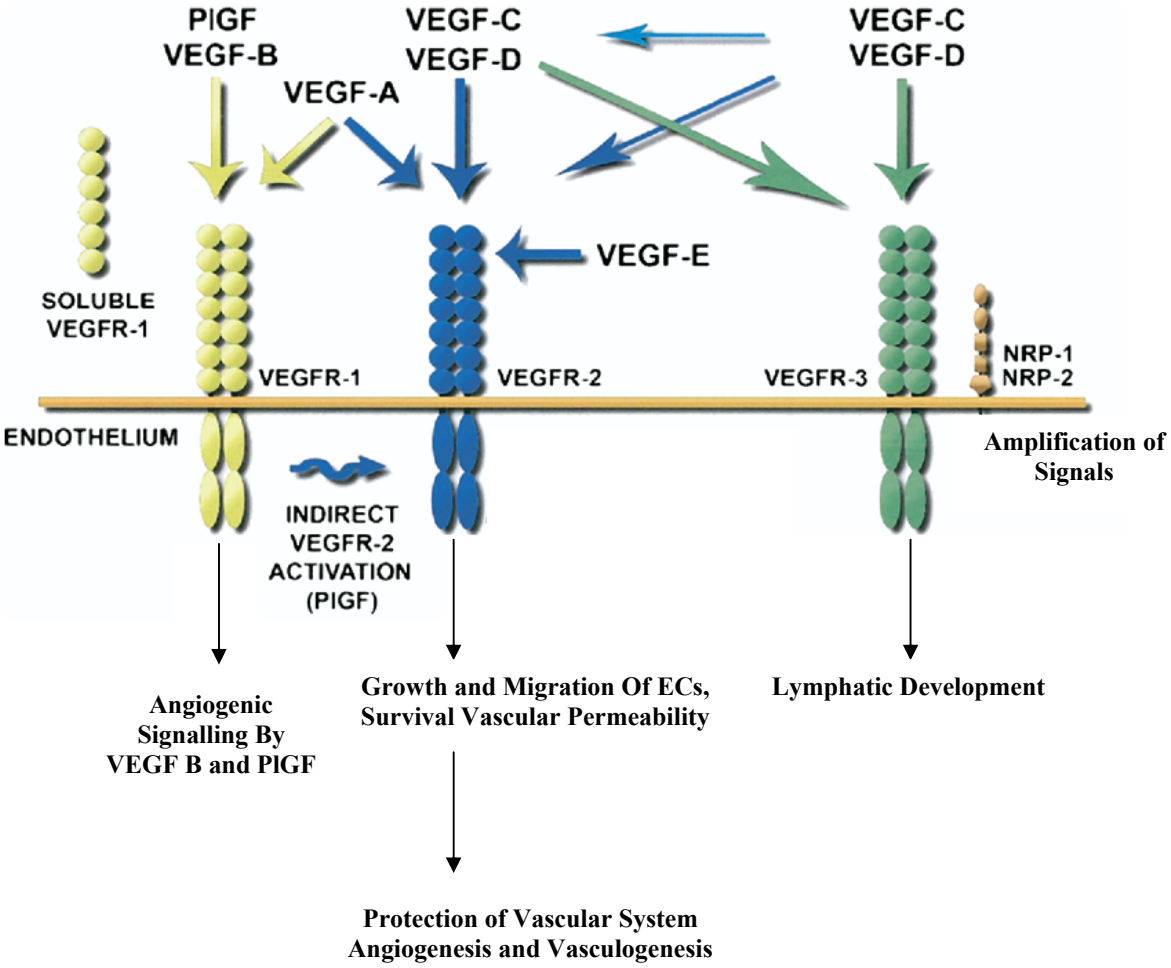


Figure 1.15: Biological Functioning of VEGF and its receptors

This figure illustrates the presence of 7 extracellular immunoglobulin homology domains, a single trans-membrane region, and a consensus tyrosine kinase domain interrupted by a kinase insert domain characteristic of VEGFR-1 and VEGFR-2. VEGFR-3 on the other hand, shows the 5th immunoglobulin domain which is cleaved into disulfide-linked subunits. Co-receptors, Nrp-1 and -2 lack VEGFR-2 kinase activity. (Adapted from Yla-Herttuala *et al.*, 2007).

1.7 Transforming growth factor-beta (TGF-β)

1.7.1 Expression and isoforms

The transforming growth factor superfamily encompasses a large set of multi-functional, pleiotrophic cytokines, including the TGF-βs, the bone morphogenetic proteins, the activins and inhibins, Mullerian inhibitory substance, and decapentaplegic peptides (Lebrin *et al.*, 2005, Ghosh *et al.*, 2005, Bertolino *et al.*, 2005, Massague, 1998). All these growth factors share a cluster of conserved cysteine residues that form a common cysteine knot structure (**Fig. 1.16**) held together by intra-molecular disulphide bonds (Iyer *et al.*, 2001, Massague, 1998).

The transforming growth factor-beta cytokines are derived from amino acid precursors that contain an N terminal, a pro-segment and a bioactive C-terminal (Goumans *et al.*, 2009, Hu *et al.*, 1998). The precursor cleavage site is a sequence of four basic amino acids immediately preceding the bioactive domain. After secretion, the cleaved pro-segment remains associated with the bioactive dimer forming a latent complex, which only becomes activated by specific proteases or thrombospondins. The biologically active 25kD homodimer is found within the placenta, in blood platelets, and the bovine kidney (Sporn and Roberts, 1992, Hu *et al.*, 1998).

There are three related dimeric isoforms of TGF-β , viz., TGF-β_1, TGF-β_2 and TGF-β_3 (Fonsatti *et al.*, 2001). Each isoform is expressed in a tissue-specific manner (**Table 1.3**) and is encoded by different genes, thereby having multiple cell surface receptors that mediate signal transduction (Sporn and Roberts, 1992, Lebrin *et al.*, 2005, Ten Dijke and Arthur, 2007, Ghosh *et al.*, 2005, Fonsatti *et al.*, 2001).

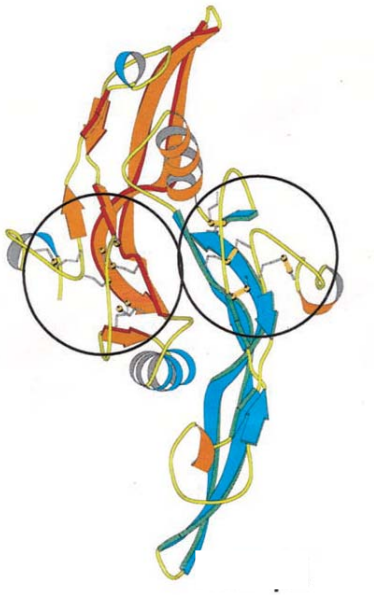


Figure 1.16: Structural model of TGF-ß

The cysteine-knot motif and the receptor-binding sites have been highlighted as circles (Adapted from Iyer *et al.*, 2001)

Table 1.3: Characterisation of TGF-ß Isoforms (Adapted from Ghosh *et al.*, 2005)

	TGF-$ß_1$	**TGF-$ß_2$**	**TGF-$ß_3$**
Gene Localisation	Chromosome 19q13	Chromosome 1q14	Chromosome 14q24
Cellular Expression	Endothelial cells Hematopoietic cells Smooth muscle cells Fibroblasts	Epithelial cells Neurons	Mesenchymal cells

1.7.2 Receptors of transforming growth factor-beta

Intracellular signalling of TGF-β proteins are regulated by high affinity cell surface receptors and Smad effectors (Gordon and Blobe, 2008, Sporn and Roberts, 1992, Bertolino *et al.*, 2005, Ten Dijke and Arthur, 2007). Three types of receptors which control the extracellular availability of TGF-β have been identified based on their molecular weight. These include Type I/activin receptor-like kinase receptor *(*ALK1), Type II/ALK5 (serine/threonine kinase receptors, including activin receptors) and Type III accessory receptors viz., betaglycans and endoglin (Ghosh *et al.*, 2005, Lebrin *et al.*, 2005, Gordon and Blobe, 2008).

The efficiency of TGF-β receptors are dependent on intracellular effectors /signalling mediators called Smads (Gordon and Blobe, 2008, Lebrin *et al.*, 2005, Ten Dijke and Arthur, 2007). These mediators also function as direct nuclear effectors for TGF-β type I receptors via phosphorylation.

1.7.2.1 Type I and II receptors

To date, seven transforming growth factor-beta type I (TβRI) and type II (TβRII) receptors have been identified within mammals (Bertolino *et al.*, 2005). These receptors are structurally related with small cysteine rich extracellular domains (**Fig. 1.17**), solitary transmembrane-spanning and intracellular components that largely include serine/threonine kinase domains (Ten Dijke and Arthur, 2007). In addition, the type I receptors operate downstream of type II receptors, thereby establishing the intracellular signalling specificity of the receptor compound.

Following the configuration of the ligand-stimulated heteromeric compound, the type II receptors then transphosphorylates and activates the type I receptors (Ten Dijke and Arthur, 2007, Bertolino *et al.*, 2005). This then enables signal transmission at the two C-terminal Ser residues by phosphorylating particular receptor-regulated (R-) Smad transcription molecules. Once stimulated, there is formation of heteromeric compounds by the R-Smads with the Co-Smads (Smad4 in mammals). This leads to transcriptional regulation of target genes due to their subsequent accretion within the nucleus (Ten Dijke and Arthur, 2007).

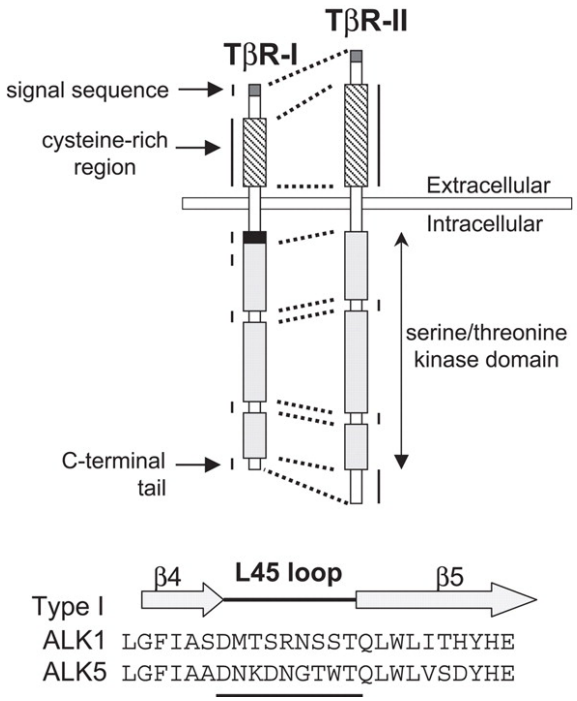


Figure 1.17: Transforming growth factor-beta type I (TβRI) and type II (TβRII) serine/ threonine kinase receptors (Adapted from Lebrin *et al*., 2005)

1.7.2.2 Type III accessory receptors

Both betaglycan and endoglin are transmembrane proteins with small intracellular domains.(Bertolino *et al.*, 2005, Lebrin *et al.*, 2005). These proteins are devoid of enzymatic motifs but include several serine and threonine residues (**Fig. 1.18**). In addition, betaglycans allow TGF-β to bind to the serine/threonine kinase receptors thus assisting in signal transduction.

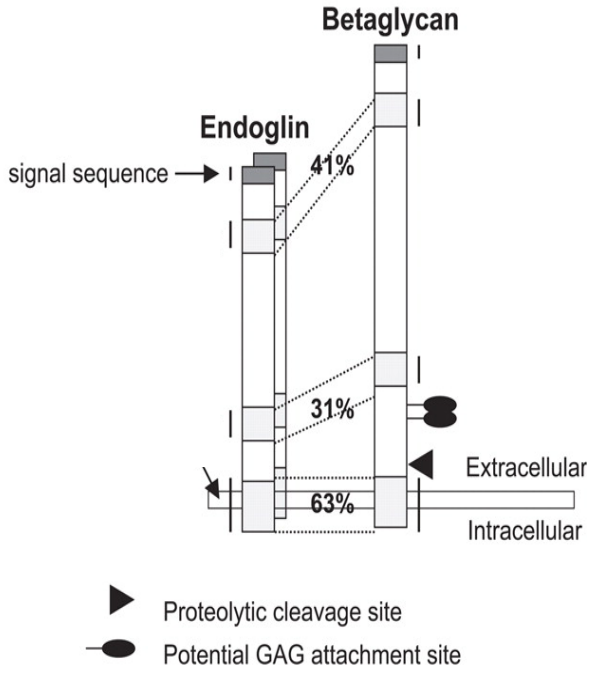


Figure 1.18: TGF- β Type III Accessory receptors (Adapted from Lebrin *et al.*, 2005)

1.7.2.2.1 Endoglin

Endoglin, or CD105 is a 180-kDa disulphide linked homodimeric type I membrane glycoprotein that comprises a long extracellular domain, a solitary hydrophobic transmembrane domain, plus a small cytoplasmic domain (Fonsatti *et al.*, 2001, López-Novoa and Bernabeu, 2010, Fonsatti *et al.*, 2003, Ten Dijke *et al.*, 2008). This extracellular domain comprises a zona pellucida (ZP) domain of 260 amino acids and an orphan domain (**Fig. 1.19**).

On the other hand, the cytoplasmic domain is phosphorylated at the serine/threonine residues, and consists of a consensus postsynaptic density 95/Drosophila disk large/zonula occludens-1 (PDZ)-binding motif at the carboxyl terminus (Fonsatti *et al.*, 2001, López-Novoa and Bernabeu, 2010, Fonsatti *et al.*, 2003). Additionally, the cytoplasmic domains are targeted by the TGF-βI and II receptors, whilst the PDZ region allows for the phosphorylation of distal threonine residues and the interaction of endoglin with PDZ domain-containing proteins. To date, 2 different alternatively spliced isoforms of endoglin have been identified (**Fig. 1.20**) viz., long (L)-endoglin and short (S)-endoglin (López-Novoa and Bernabeu, 2010, Fonsatti *et al.*, 2003, Fonsatti *et al.*, 2001).

Additionally, L-endoglin is more expressed than S-endoglin, and differs based on variations in the amino acid composition of their cytoplasmic tails (**Figure 1.20**). Expression of endoglin occurs mainly on proliferating endothelial cells (López-Novoa and Bernabeu, 2010, Lopez-Novoa, 2007). Angiogenesis is therefore one of the major functions of endoglin, thus an abnormal endoglin expression may lead to vascular disorders such as tumour angiogenesis, hereditary hemorrhagic telangectasia and pre-eclampsia (Ten Dijke *et al.*, 2008).

Endoglin is identified as a hypoxia-inducible and growth-related protein that regulates signal transduction of TGF-β by associating with TGF-β receptors I and II (Lebrin *et al.*, 2005, Lopez-Novoa, 2007, Luft, 2006). Receptor-ligand interaction occurs only when it is linked with TGF-βR-II (**Figure 1.21**). This enhances the interaction of both the extracellular and intracellular domains of endoglin with TGF-βR-II and ALK5, in which the cytoplasmic domain rich in serine and threonine residues are phosphorylated by ALK5 or TGF-βR-II (Lebrin *et al.*, 2005, Lopez-Novoa, 2007, Luft, 2006).

1.7.2.2.2 Soluble endoglin (sEng)

The recent identification of increased levels of placental-derived soluble endoglin in pre-eclampsia has indeed gained noteworthy significance (Venkatesha *et al.*, 2006). This soluble endoglin was found to work together with soluble Flt-1/VEGF-R1 by stimulating endothelial dysfunction (Agarwal and Karumanchi, 2011).

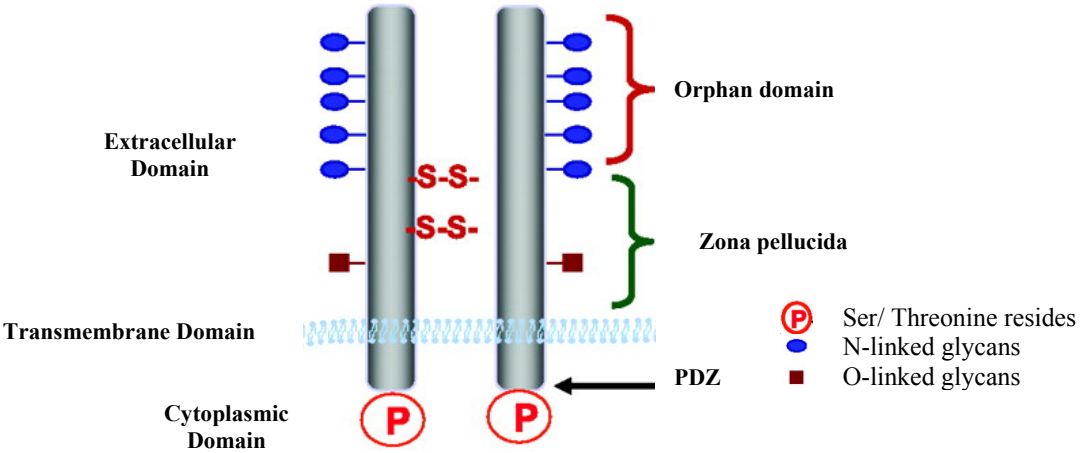


Figure 1.19: Structure of Endoglin

This receptor consists of a large extracellular domain that includes a Zona pellucida and an Orphan domain, which forms dimers. The analogous monomers are disulphide-linked that attach to N-linked glycans and O-linked glycans attach to the extracellular domain via consensus motifs. Cytoplasmic domains are phosphorylated (P) at Ser/Thr residues and include a consensus postsynaptic density 95/Drosophila disk large/zonula occludens-1 (PDZ) binding motif at the carboxyl terminus (Adapted from Lopez-Novoa and Bernabeu, 2010).

Figure 1.20: Endoglin isomers illustrating amino acid sequences from short (S) - and long (L)-endoglin cytoplasmic tails

Blue denotes variations in sequences between 2 isomers and the PDZ binding motif in L-endoglin is underlined (Adapted from Lopez-Novoa and Bernabeu, 2010).

1.7.2.3 Smad intracellular effectors

To date three types of Smads have been identified (**Table 1.4**), viz., Receptor Regulated (R-), the Common Mediator (Co) and the Inhibitory Smads (I-) (Lebrin *et al.*, 2005, Gordon and Blobe, 2008, Bertolino *et al.*, 2005). The R-Smads include Smad1, 2, 3, 5 and 8 which are phosphorylated by TGF-βR-I at the C-terminal serine residues. The Co Smad (Smad4) forms a complex with the activated R Smads which then propagates to the nucleus. Their subsequent interaction with transcription factors, co-activators or co-repressors within the nucleus aids in the regulation of transcription of target genes (Lebrin *et al.*, 2005, Gordon and Blobe, 2008). The I-Smads include Smad6 and 7, whose expressions are stimulated by TGF-β, whilst the activation of the R- Smads is inhibited by endothelial shear stress.

Table 1.4: Transforming Growth Factor-β Ligands and Respective Receptors Function (Adapted from Bertolino *et al.*, 2005)

Ligand	Type II Receptor (TβRII)s	Type I Receptors (TβRI)	R- Smads	Co-Smads
BMP	BMP R-II	ALK2, 3 or 6	Smad 1/5/8	Smad 4 → Transcription of target genes
	ActR-II/R-IIB			
TGF-β	TβRII	ALK1		
		ALK5	Smad 2/3	
Activin	ActR-II/B	ALK4		

1.7.3 Signal transduction of transforming growth factor-beta

Signalling of the TGF-β superfamily is produced by their specific cell surface type I and type II receptors (**Figure 1.21**) (Gordon and Blobe, 2008, Jones *et al.*, 2006, Goumans *et al.*, 2009, Lebrin *et al.*, 2005). The accessory receptors endoglin and betaglycans adjust this signal transduction via type I and type II receptors, whilst the soluble endoglin and betaglycans may possibly sequester these ligands, thereby preventing the binding of receptors (Ten Dijke *et al.*, 2008). In majority of the cells, signal transduction is produced by the interaction of TGF-β with TβRII and ALK5, whilst during endothelial signalling it occurs via ALK1 (Gordon and Blobe, 2008, Jones *et al.*, 2006, Goumans *et al.*, 2009, Lebrin *et al.*, 2005). Ligand-receptor interaction is mediated by the recruitment of a specific Type I receptor, resulting in the formation of a heteromeric complex consisting of two type I and two type II serine/threonine kinase receptors.

Signal transduction of the activins occurs via the activin receptor ActR-II and the type I receptor ALK4 (Gordon and Blobe, 2008, Jones *et al.*, 2006, Goumans *et al.*, 2009, Lebrin *et al.*, 2005). In contrast, the bone morhogenetic proteins (BMPs) function via the BMP receptor BMPR-II, ActR-IIA and ActR-IIB, and the type I receptors ALK1, 2, 3 and 6, whilst the biological functioning of the betaglycans and endoglin are mediated via the type II receptors TβRII/ALK5 and TβRII/ALK1 (Gordon and Blobe, 2008, Jones *et al.*, 2006, Goumans *et al.*, 2009, Lebrin *et al.*, 2005). Additionally, intracellular signal transduction occurs via two central Smad signalling pathways viz., ALK5 stimulates phosphorylation of Smad2 and Smad3, whilst ALK1, 2, 3 and 6 allows for phosphorylation of Smad1, 5 and 8. This results in the formation of heteromeric compounds that build-up within the nucleus. This contributes to gene

expression regulation by binding to DNA together with other DNA binding transcription factors (**Figure 1.21**) (Gordon and Blobe, 2008, Jones *et al.*, 2006, Goumans *et al.*, 2009, Lebrin *et al.*, 2005).

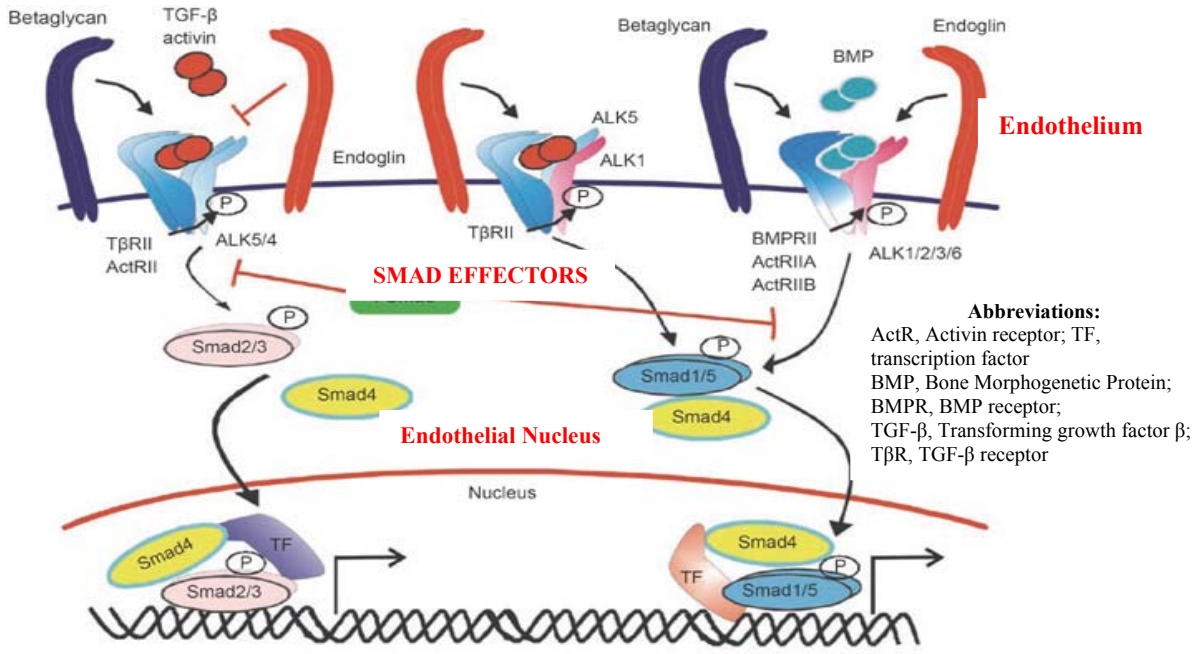


Figure 1.21: Endothelial signal transduction by TGF-β superfamily

Signalling of TGF-β occurs via the type I receptor ALK1, the activins via ActRII and ALK4. The BMPs function via BMPRII and ActRII and type I receptors ALK1, ALK2, ALK3 and ALK6. The activated type I receptors start intracellular signal transduction by phosphorylating specific R-Smads. In contrast, the activation of ALK1, ALK2, ALK3 and ALK6 results in the phosphorylation of Smad1, Smad5 and Smad8, whilst Smad2 and 3 are phosphorylated by ALK4, ALK5 and ALK7 respectively. This results in the formation of heteromeric compounds that accrue within the nucleus, thereby controlling specific expression of genes by binding to DNA together with other DNA binding transcription factors (Adapted from Goumans *et al*, 2009).

1.7.4 Functions of transforming growth factor-beta

The functions of TGF-β proteins are widespread. These include regulating the production, differentiation, migration and existence of various cell types; tissue remodelling and reproductive function (Ghosh *et al.*, 2005, Ten Dijke and Arthur, 2007, Sporn and Roberts, 1992, Jones *et al.*, 2006, Bertolino *et al.*, 2005, Hu *et al.*, 1998). Additionally, they allow for the production of extracellular matrix and the development of cellular cycles, apoptosis, cellular differentiation and migration, and their ability to implement homeostatic effects in response to cellular or tissue injury and repair mechanisms.

Thus, inadequate expression or signalling of TGF-β results in a myriad of pathologies including arthritis, cancers, atherosclerosis, fibrotic diseases of the liver, lung and kidneys, human hereditary telangiectasia, autoimmune disorders, and vascular anomalies (Nakagawa *et al.*, 2004). TGF-β stimulates pro- and anti-angiogenic factors via Smad3 and 2 pathways respectively. It has also been implicated in pre-eclampsia development by stimulating complex intracellular signalling pathways via the receptor-activated Smads2 and 3 and inhibitory Smads6 and 7.

1.7.4.1 Role of Transforming Growth Factor βeta in normal placental development

Various ligands of the TGF-β family are expressed by the placental villi, especially the syncytio- and the cytotrophoblast cells (Jones *et al.*, 2006). This expression may modify the regulation of hormonal production of estrogen, progesterone and placental lactogen (Jones *et al.*, 2006). Furthermore, the maternal decidua expresses activin A and macrophage inhibitory cytokine, supporting the stromal cell differentiation into decidualised cells (**Figure 1.22**).

Hence these growth factors in concert with TGF-β_2, regulate the invasion of extravillous cytotrophoblast, whilst TGF-β_3, produced by the differentiating cytotrophoblasts, prevents this invasion. Moreover, decidual TGF-β may act on the uterine natural killer cells thereby decreasing their cytotoxicity and generating uterine-specific phenotypes (Jones *et al.*, 2006). The tissue macrophages on the other hand, generate various cytokines, including TGF-β and activins, within the implantation site and in the non-pregnant endometrial layer that tightly regulate matrix metalloproteinase expression and activity.

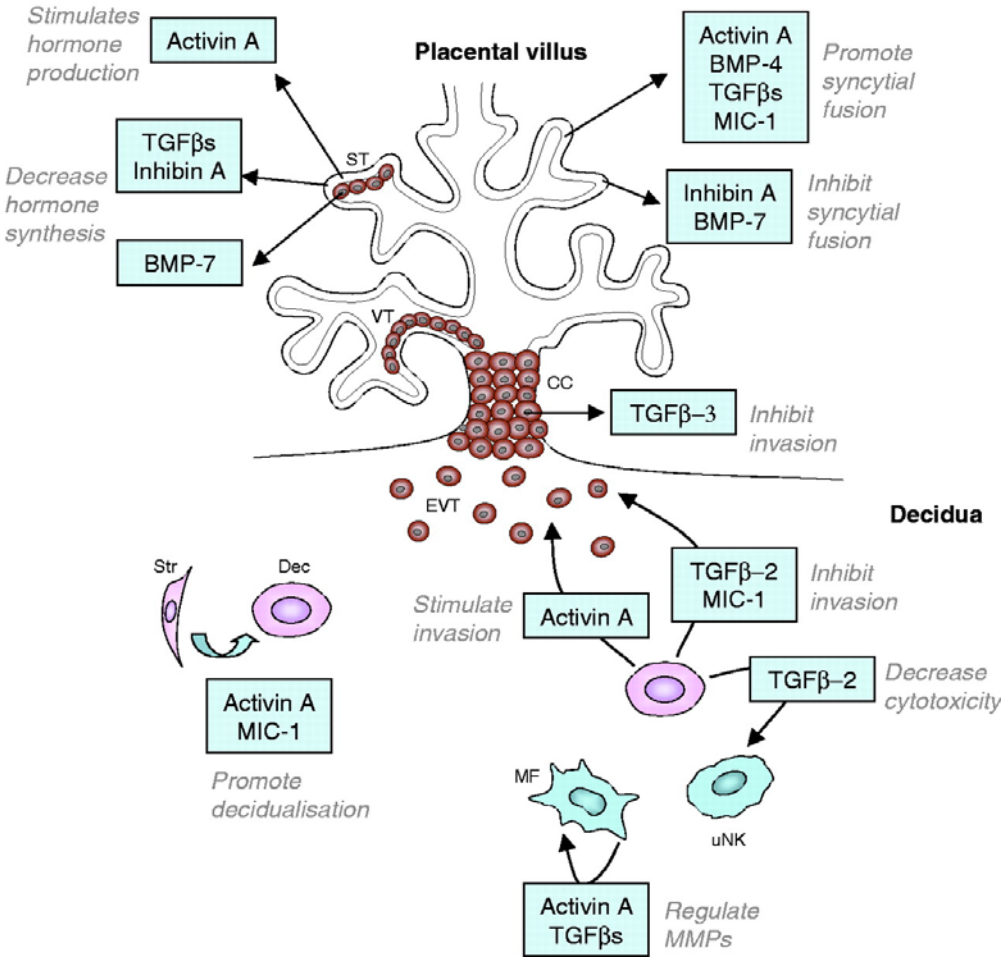


Figure 1.22: Role of TGF-β at the maternal–fetal interface

The syncytiotrophoblast (ST) and inner villous cytotrophoblast cells (VT) expresses many TGF-β ligands which may alter syncytial fusion or the production of hormones such as estrogen, progesterone or placental lactogen. Growth factors such as Activin A and macrophage inhibitory cytokine (MIC-1), expressed by the maternal decidua, support stromal cells (Str) differentiation into decidualised cells (Dec). Activin A + MIC-1 + TGF-β_2, support the decidual regulation of the invasive extravillous cytotrophoblast (EVT). TGF-β_3 produced by the differentiating cytotrophoblast cells located within the cell column (CC) prevents this invasion. This decidual TGF-β is likely to act on the uterine natural killer cells (uNK) to reduce their cytotoxicity thereby generating the uterine-specific phenotype. During implantation the tissue macrophages (MF) produces both TGF-β and activins that operate opposite to each to support the expression and function of matrix metalloproteinase (Adapted from Jones *et al*., 2006).

1.8 Inhibitors of placental angiogenesis

Endothelial function is dependent on the effects of pro-angiogenic factors, suggestive of their central role in the development of the placental vasculature, in addition to their regulation of trophoblast differentiation and development. Angiogenic proteins are abundantly produced by the placenta, and their subsequent release into the maternal circulation could impact vascular adaptation to pregnancy (Cross, 2006).

Recent detection of placental anti-angiogenic proteins sFlt-1 and soluble endoglin (sEng), endogenous inhibitors of VEGF and TGF-β, has been implicated in pre-eclampsia development (Levine *et al.*, 2006, Powe *et al.*, 2011). It is thus plausible that the endothelial dysfunction that characterises pre-eclampsia may be due to the placental discharge of these anti-angiogenic factors into maternal circulation. Hence, a correlation between sFlt-1 and sEng with maternal endothelial dysfunction and disrupted nitric oxide formation is postulated (Levine *et al.*, 2006, Powe *et al.*, 2011). Other placental derived anti-angiogenic proteins such as endostatin, angiostatin, prolactin and thrombospondin-1 may also be implicated in placentation, but their role remains uncertain (Bdolah *et al.*, 2004, Klagsbrun and Moses, 1999).

1.9 Pre-eclampsia

1.9.1 Clinical characteristics

Pre-eclampsia is a human, pregnancy-specific, multisystem disease of unknown origin (Noris *et al.*, 2005, Hubel, 1999, Rajakumar *et al.*, 2012, Agarwal and Karumanchi, 2011). It is defined by clinical diagnostic findings as a new onset of hypertension (systolic blood pressure ≥140mmHg or diastolic blood pressure ≥90mmHg) and the presence of proteinuria (≥300mg of

protein every 24hrs) and/or edema, presenting late after the 20th week of gestation. The endothelium may thus be regarded as the target of this maternal disorder as a result of the hypertension and proteinuria (Powe *et al.*, 2011). Additionally, this resulting hypertension is categorised by peripheral vasoconstriction and reduced arterial compliance. Pre-eclampsia is thus characterised by an irregular vascular response to placentation which is linked with enhanced systemic vascular resistance, platelet aggregation, activation of the coagulation system and endothelial cell dysfunction (Agarwal and Karumanchi, 2011, Vitoratos *et al.*, 2012).

1.9.2 Risk factors of pre-eclampsia

Pre-eclampsia occurs more frequently in conditions where there is greater demand for placental oxygen, such as multiple and molar pregnancies, or those with reduced oxygen transfer due to pre-existing vascular alterations (Maynard *et al.*, 2005). This therefore defines its greater prevalence in primigravidae, whose uterine vasculature is less developed than those of multiparous or women with microvascular alterations secondary to chronic hypertension.

The risk factors are diverse and exclusive to pre-eclampsia. The factors that enhance the risk of developing pre-eclampsia include primipaternity (Skjaerven *et al.*, 2002), obesity and insulin resistance (Walker, 2000), pre-gestational diabetes, maternal infection, pre-existing thrombophilia (Kupferminc *et al.*, 1999), maternal susceptibility genes, family history of pre-eclampsia and placental hydropic degeneration (Noris *et al.*, 2005, Sibai *et al.*, 2005).

A methodical study conducted by Duckitt and Harrington, (2005) showed that in pregnancies following donor insemination; oocyte and embryo donation as well as the protective effect of partner change in case of previous pre-eclamptic pregnancy increases the risk of pre-eclampsia development. In addition, maternal age (≥40 years), pre-eclampsia in previous pregnancy, chronic hypertension or renal failure, rheumatic disease, presence of antiphoshoplipid antibodies and maternal and low birth weight amplified the possibility of the advancement of pre-eclampsia (Duckitt and Harrington, 2005). In addition, women with a previous history of pre-eclampsia are at a greater risk of recurrence. Furthermore, multiple gestations also incur greater risk due to increased placental mass. However, the exact role that genetic factors play in the development of pre-eclampsia still remains to be elucidated. Unexpectedly, cigarette smoking during pregnancy has a protective effect against pre-eclampsia (Conde-Agudelo *et al.*, 1999, Ahmed, 2011).

1.9.3 Human Immunodeficiency Virus and pre-eclampsia

Earlier studies report that triple antiretroviral therapy (ART) reconstitutes the immune system in HIV infected women, thereby placing them at a greater risk of developing pre-eclampsia, whilst those with compromised immunity and are untreated have a lower rate (Wimalasundera *et al.*, 2002). However, the increased incidence of pre-eclampsia development in treated HIV individuals is dependent on the magnitude of immune reconstitution (ie the incidence is greater amongst those that are on triple ART than mono-or dual ART. Similarly, recent longitudinal studies revealed that HIV infected women on HAART prior to pregnancy was linked with a greater risk for the development of pre-eclampsia and subsequent fetal demise (Suy *et al.*, 2006). In contrast, the risk of pre-eclampsia development is not lowered in untreated HIV

infected women (Frank *et al.*, 2004), whilst others have shown that HIV infection may prevent the development of pre-eclampsia, even whilst on treatment (Mattar *et al.*, 2004). Thus it is likely that HIV infection possibly 'protects' against eclampsia and to a lesser degree, pre-eclampsia

1.9.4 Pathogenesis of pre-eclampsia

The cause of pre-eclampsia is historically referred to as the "*disease of theories*" and is currently a major area of research (Karumanchi and Lindheimer, 2008, Young *et al.*, 2010). Many factors are responsible for the maladaptation and dysfunctional invasion of uteroplacental arteries typical of pre-eclampsia (Irminger-Finger *et al.*, 2008). The pathogenesis of this maternal disorder thus encompasses various molecular mechanisms, including systemic inflammation, angiogenic imbalance, abnormal renin-angiotensin system functioning, and placental hypoxia and ischaemia (Vitoratos *et al.*, 2012). However, the manner in which these mechanisms operate (ie. independently or synergistically) still remains unclear.

Earlier studies demonstrated a sequential interaction between intrinsic factors such as the unusual biology of extravillous trophoblasts with extrinsic maternal uterine factors may play a role in pre-eclampsia (Moffett-King, 2002). The maternal uterine factors include poor decidual remodelling, impaired role of uterine natural killer (uNK) cells, and the ineffective maternal endothelial expression of adhesion molecules. The molecular mechanism that ensures trophoblastic differentiation requires the conversion of an ectoderm to a vascular mesoderm (Mohaupt, 2007). This contact area between migrating trophoblast cells with the adjacent

extracellular matrix is central for the trophoblastic invasion and the eventual transformation of maternal arterioles (Mohaupt, 2007). In addition, surface molecules such as integrins and epithelial (E)-cadherin, play a significant role in this matrix-cell interaction and the eventual cell migration during angiogenesis (Irminger-Finger *et al.*, 2008, Redman and Sargent, 2009).

In normal pregnancies, studies by Zhou *et al* (1997) indicate a down-regulation of epithelial-like receptors (VE-cadherin and α6β4 integrins) by the invasive cytotrophoblasts. These epithelial-like receptors are replaced by endothelial adhesion molecules such as platelet–endothelial cell adhesion molecule (PECAM), vascular endothelial (VE)-cadherin, vascular cell-adhesion molecule 1 (VCAM1), and α4 and αVβ3 integrins (Zhou *et al.*, 1997). However, in pre-eclampsia, these cells have reduced levels of VE-cadherin, VCAM1 and αVβ3 integrin, indicating their failure to acquire an endothelial adhesion phenotype (Zhou *et al.*, 1997).

Hence, this multi-factorial disease occurs only in the presence of a placenta and is attributed to insufficient invasion by trophoblast cells and maternal endothelial dysfunction (Young *et al.*, 2010, Hertig and Liere, 2010). Studies have implicated increased levels of anti-angiogenic placental derived sFlt-1 and sEng in maternal blood as a cause of endothelial dysfunction and the consequential signs and symptoms of pre-eclampsia (Levine *et al.*, 2006, Maynard *et al.*, 2003). However, the exact role these anti-angiogenic factors play *in vivo* during early placental development remains unsolved (Huppertz, 2008). Whilst the placenta may be the major disease contributor, it is obvious that both the maternal and fetal mechanisms should be taken into consideration when assessing the ratio of danger.

1.9.4.1 Role of the placenta

Whilst the cause of pre-eclampsia remains inexplicable, speculations on its origins grow. The placenta is identified as the source of the disease (Maynard *et al*., 2008a, Redman and Sargent, 2003, Myatt and Webster, 2009). The disease resolves rapidly postpartum as effective treatment requires the delivery of both fetus and placenta (Ahmed, 2011). This rapid disease resolution is evident in hydatiform moles where fetal presence is unnecessary for the development of pre-eclampsia (Chun *et al.*, 1964, Koga *et al.*, 2009).

Molar pregnancies are thus risk factors for the development of early-onset pre-eclampsia which may result from the overproduction of the anti-angiogenic proteins secreted by trophoblast cells (Kanter *et al.*, 2010, Koga *et al.*, 2009). Notable, however, is the fact that premature delivery (<32 weeks gestation) negatively affects the health of the baby, whilst delaying delivery to enable *in utero* maturation of fetal lung increases maternal risk.

Several factors such as immunologic, proangiogenic, anti-angiogenic, genetic and environmental factors (**Fig. 1.23**), have been implicated in the pathogenesis of pre-eclampsia (Maynard and Karumanchi, 2011, Wang *et al.*, 2009). Furthermore, most of these factors may influence the expression of both sFlt-1 or sEng expression, by exerting modulatory effects of their eventual circulating levels.

The culmination of this disease is described in a two stage disease process (**Fig. 1.23**) that is summarised below (Wang *et al.*, 2009, Young *et al.*, 2010):

- Stage 1 (1^{st} and 2^{nd} trimester): Feto-placental or asymptomatic stage, which is characterised by the failure of cytotrophoblasts to adopt the invasive endothelial phenotype resulting in abnormal placentation. This subsequently leads to incomplete or shallow invasion of the spiral arteries that remain as small calibre, high resistance vessels leading to a reduced placental perfusion, and the release of placental soluble factors that ultimately enter the maternal circulation (**Fig. 1.23**)

- Stage 2 (3^{rd} trimester): Maternal stage which manifests by the occurrence of increased blood pressure and proteinuria (**Fig. 1.23**).

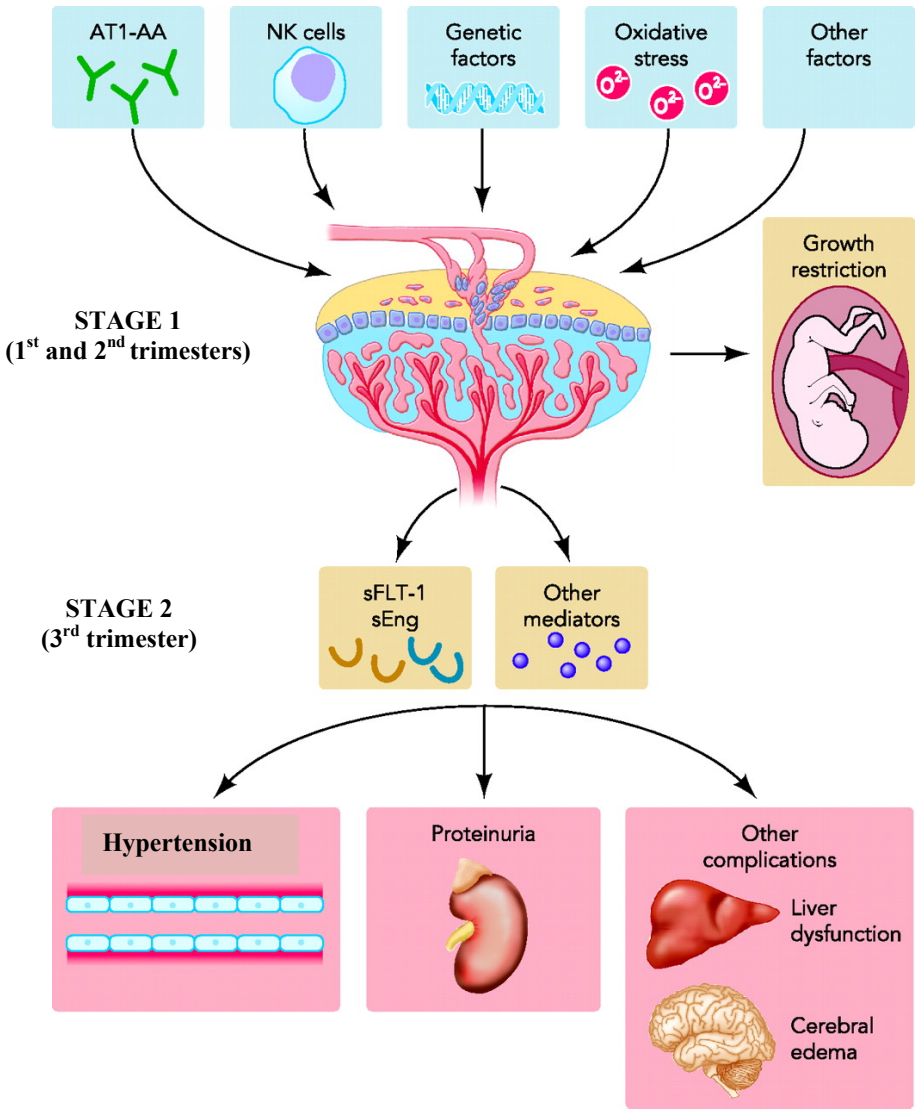


Figure 1.23 Pathogenesis outline of pre-eclampsia

Immune factors [such as Angiotensin I autoantibodies (AT1-AA)], oxidative stress, natural killer (NK) cell abnormalities, and other factors may result in placental dysfunction, resulting in the placental release of sFlt-1 and sEng (with permission from Wang *et al.*, 2009).

1.9.4.1.1 *The feto-placental stage*

Placental derangement in hypertensive pregnancies begins with invasion of the vasculature by trophoblast cells that does not proceed beyond the decidual portion of the spiral arteries (Pijnenborg *et al.*, 1991). Furthermore, the second wave of endovascular trophoblast invasion into the myometrial segments of spiral arteries, at 15th gestational week is severely reduced (Pijnenborg *et al.*, 1991). As a result, the spiral arteries of the myometrium remain anatomically intact and do not dilate, in other words they do not undergo the "physiological change" observed in normal pregnancies (**Fig. 1.24**). The arteries remain responsive to circulating vasopressors and dilate to only 40% of the diameter to that which occurs in normal pregnancy. Consequently, there is reduced flow through the arteries with resultant underperfusion of the developing placenta, placing the growing fetus at risk of oxygen and nutrient deprivation (Pijnenborg *et al.*, 2006, Naicker *et al.*, 2003).

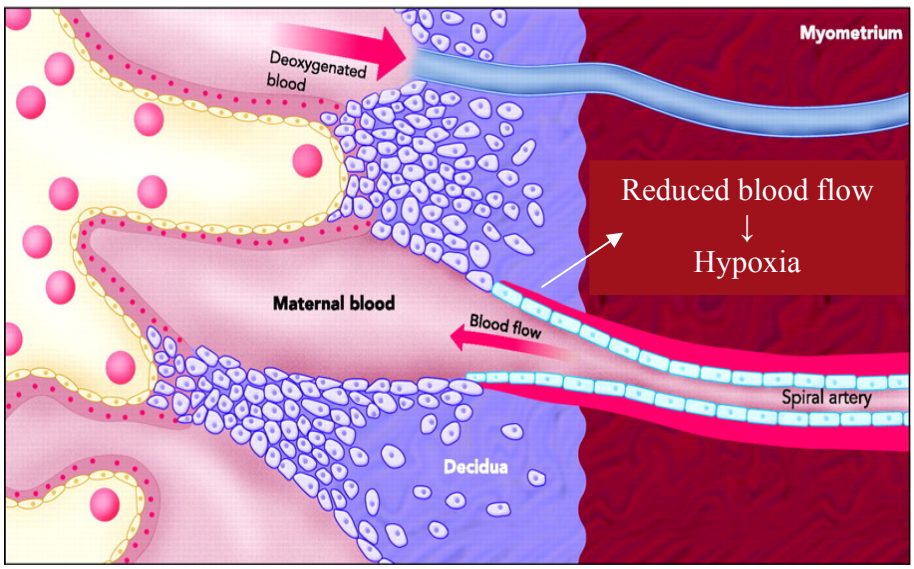


Figure 1.24: Abnormal placental development in pre-eclampsia (with permission from Wang *et al.*, 2009)

1.9.4.1.2 *The maternal stage*

During pre-eclampsia, the inadequate physiological conversion of spiral arteries decreases placental perfusion. This reduced placental perfusion interacts with genetic, behavioral, and environmental maternal factors, resulting in increased inflammatory response (Zhou *et al.*, 1997, Zhou *et al.*, 2002, Young *et al.*, 2010). Moreover, conditions such as diabetes or obesity, (**Fig. 1.25**), increase the risk of pre-eclampsia development and the possibility of later cardiovascular disease (Roberts and Gammill, 2005).

In addition, pre-eclampsia can rapidly develop into a clinical setting of convulsions (eclampsia), continuous headaches, visual alterations, epigastric pains, weight gain, oedema, acute renal failure, cerebral oedema & haemorrhages, pulmonary edema, thrombocytopaenia, haemolytic anaemia, coagulopathy, and the syndrome of haemolysis, elevated liver enzymes and low platelet count (HELLP) (Young *et al.*, 2010). Thus, following a suspected diagnosis of pre-eclampsia, analyses of maternal blood is done to monitor thrombocytopaenia, HELLP or renal impairment, whilst fetal management occurs via ultrasound surveillance and fetal heart rate testing.

Mild pre-eclampsia, however, can be controlled until 37 weeks of gestation (Young *et al.*, 2010). Additionally, those requiring treatment are managed with magnesium for approximately 24 hours to reduce or prevent the possibility of eclampsia. Whilst the acute effects of pre-eclampsia resolves postpartum, this disorder may be a risk factor for future cardiovascular disease in the mother long after her reproductive years are completed (Ray *et al.*, 2005).

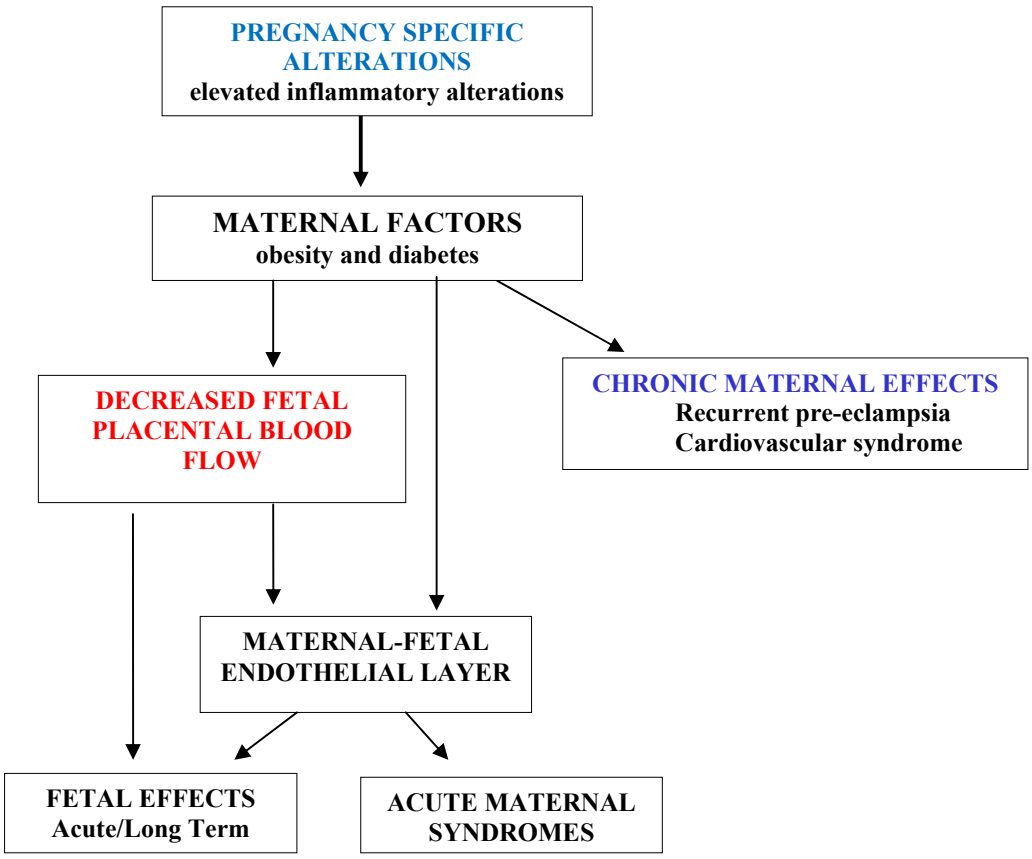


Figure 1.25: Outline of the maternal fetal /placental interactions in pre-eclampsia (Adapted from Roberts and Gammill, 2005)

1.9.4.2 Target organ effects of pre-eclampsia

Glomerular endotheliosis, enhanced vascular permeability inflammatory reactions and the consequent end-organ deterioration, are features of pre-eclampsia (**Table 1.5**) (Roberts and Lain, 2002). Decreased perfusion is probably secondary to the enhanced sensitivity of the blood vessels to any pressor agent, and leads to the establishment of the coagulation cascade with formation of microthrombi, and fluid loss from the intravascular compartment (Rodrigo *et al.*, 2005, Lain and Roberts, 2002, Roberts and Lain, 2002).

However, the concept of endothelial dysfunction and placental hypoxia as mediators of inducing the expression and release of pro- and ant-angiogenic factors in pre-eclampsia remains challenging. This may induce and accelerate angiotensin II mediated hypertension, proteinuria and other systemic manifestations. Ischeamic lesions in the placenta may occur as a result of this accelerated process (Rodrigo *et al.*, 2005, Lain and Roberts, 2002, Roberts and Lain, 2002).

Recent morphological and morphometric studies have suggested that placental transport and haemodynamics may be influenced by a reduced villous carrying capacity (Ducray *et al.*, 2011). This, in turn, exacerbates the ischeamia that arises from the decreased maternal flow. The materno-fetal exchange is consequently intensified due to the subsequent fetal maladaptation (Ducray *et al.*, 2011).

Table 1.5: End-organ involvement (Adapted from Roberts and Lain, 2002)

End-organ Involvement	Description
Hematologic	Platelets <100 x 10^3/μL
	Microangiopathic hemolysis (increased lactate dehydrogenase or bilirubin)
Hepatic	Epigastric or right upper quadrant pain
	Elevated liver enzymes (aspartate aminotransferase ≥2 x normal)
Neurologic	Cerebral disturbances
	Visual disturbances & Persistent headaches
Placental	Intrauterine growth retardation and
	Oligohydramnios
	Abnormal Doppler studies of umbilical artery
Pulmonary	Pulmonary oedema
Renal	Oliguria (≤500 mL/24 h)
	Creatinine level (>1.2 mg/dL [106.1 μmol/L])

1.9.4.2.1 Endothelial dysfunction

The vascular endothelium, an extremely selective permeable layer, is a key component of the intima, exerting major circulatory effects via its ability to release various vasoactive molecules (Contreras *et al.*, 2003). It mediates the ability of the vasculature to modify their architecture in response to heamodynamic alterations. Within a functional endothelium, the endothelial cells mediate immune and inflammatory responses, maintain the integrity of the vascular compartment, prevent intravascular coagulation, and modify the contractile function of underlying smooth muscle cells (Roberts and Redman, 1993, Hayman *et al.*, 1999, Contreras *et al.*, 2003). However, activated or injured endothelial cells lose these functional capacities and release pro-coagulants, vasoconstrictors and mitogens that promote platelet aggregation and the coagulation cascade (Hayman *et al.*, 1999, Roberts and Redman, 1993, Redman and Sargent, 2003, Irminger-Finger *et al.*, 2008).

Endothelial mediators include nitric oxide, endothelin & matrix metalloproteinases, relaxin, arachidonic acid, placental ischeamia, lipoprotein-induced toxicity, renin-angiotensin system, increased reactive oxygen species (ROS), anti-angiogenic effects, immune maladaptation and the eventual production and release of proinflammatory cytokines (La Marca *et al.*, 2008, Rodrigo *et al.*, 2005). Endothelial nitric oxide is demonstrated as the largest contributor to the physiological remodelling in the vasculature, by its negative exertion over the proliferation of vascular smooth muscle cells in response to pressor stimuli (Contreras *et al.*, 1999).

This highly selective and permeable layer is also important during normal pregnancy. Features such as decreased vascular reactivity in the presence of vasoconstrictors, decreased peripheral

vascular resistance and elevations in the production of nitric oxide, characterise the development of normal pregnancy. In contrast, widespread endothelial dysfunction due to placental and renal origin is speculated to lead to the clinical manifestations of pre-eclampsia (Todros *et al.*, 2007, Alladin and Harrison, 2012). Abnormal placentation evident in pre-eclampsia affects the flow of gas and nutrient exchange at the feto-maternal interface (Redman and Sargent, 2005). This results in a relatively hypoxic environment which contribute to the increased discharge of anti-angiogenic factors such as sFlt-1 and sEng into the maternal circulation (Redman and Sargent, 2005). The resultant angiogenic imbalance becomes evident prior to the clinical signs of this maternal disorder (Redman and Sargent, 2005, Karumanchi *et al.*, 2005). Vascular growth is subsequently is obstructed with a consequential reduction in endothelial receptor binding and the discharge of procoagulant and vasoconstricting substances (Karumanchi *et al.*, 2005).

Furthermore, the hypertension that characterises pre-eclampsia is accompanied by an irregular regulation of vascular tone; retention of fluid and the elevated permeability of endothelial cells (Alladin and Harrison, 2012, Todros *et al.*, 2007). Additionally, the clotting mechanism is affected with altered production of coagulation factors. Thus, it is possible that the endothelial dysfunction combined with the activation of the coagulation cascade during pre-eclampsia contributes to the development of future cardiovascular disease in the mother.

1.9.4.2.2 Renal pathology

Glomerular endotheliosis is the pre-eminent morphological anomaly in pre-eclamptic patients whereby endothelial cells enlarge and obstruct the capillary lumen (Wang *et al.*, 2009, Khedun *et al.*, 2000). The resultant loss of endothelial integrity, with a subsequent enhanced vascular permeability, is characteristic of pre-eclampsia. Moreover, the ensuing podocyturia has been suggested as a possible risk indicator for the early detection of pre-eclampsia development (Garovic *et al.*, 2007, Ramsuran *et al.*, 2012) however, whether this is the source or result of proteinuria remains unclear .

1.9.4.2.3 Cerebral edema

One of the most fatal consequences of pre-eclampsia is eclampsia which is characterised by the occurrence of seizures (Sibai, 2005, Sibai *et al.*, 2005). Treatment of eclamptic patients include magnesium sulphate prophylaxis (ACOG, 2002). Autopsy reports from eclamptic women often include cerebral oedema and intracerebral parenchymal haemorrhage as brain related injuries (Wang *et al.*, 2009). Hence, cerebrovascular injuries such as cerebral hemorrhage and stroke are linked to most of the eclampsia-associated deaths (Sibai, 2005). There is, however, a lack of correlation of these pathologies with the severity of hypertension, indicative that the oedema may emanate from endothelial dysfunction rather than a direct result of blood pressure elevation.

1.9.4.3 Mechanisms of pre-eclampsia

1.9.4.3.1 Altered angiogenic balance

The recent implication of circulating anti-angiogenic factors (sFlt-1 and sEng) in the pathogenesis of pre-eclampsia has gained much interest (Maynard *et al*., 2003, Levine *et al*., 2006, Karumanchi and Epstein, 2007, Maynard *et al*., 2008a, Wang *et al*., 2009, Powe *et al*., 2011). Subsequent endothelial dysfunction inclusive of the reduced production of prostacyclin, nitric oxide and procoagulant proteins occur due to the antagonistic effects on the signalling pathways of VEGF, PlGF, and TGF-β (**Figs. 1.26-1.27**).

The vascular endothelium during normal pregnancy has receptor sites for both VEGF and PlGF which mediates pro-angiogenic effects essential for the maintenance of vascular homeostasis (Karumanchi and Epstein, 2007). However, during pre-eclampsia, the signalling pathways are greatly impaired since sFlt-1 binds to several VEGF and PlGF proteins with sEng altering the signaling pathways of TGF-β_1 (**Figs. 1.26-1.27**).

Several studies have explored the antagonistic effect of sFlt-1 (Maynard and Karumanchi, 2011, Young *et al.*, 2010). The breakthrough research conducted by Maynard and colleagues launched the anti-angiogenic pre-eclamptic theory due to their demonstration of increased levels of sFlt-1, both systemically and from the pre-eclamptic placenta (Maynard *et al.*, 2003). These investigators further documented reduced circulating levels of free VEGF and PlGF, resulting in endothelial dysfunction *in vitro* that could be rescued by exogenous VEGF and PlGF. These reports subsequently steered the proposal that sFlt-1 probably contributes to the aetiology of pre-eclampsia (**Figs. 1.26-1.27**) (Karumanchi *et al.*, 2005). This group later

established the anti-angiogenic effects of sEng in pre-eclampsia (Venkatesha *et al.*, 2006, Levine *et al.*, 2006, Karumanchi and Epstein, 2007).

The molecular basis for placental dysfunction of these anti-angiogenic factors in early gestation is still a dynamic area of investigation. Thus the evidence that links the angiogenic theory to pre-eclampsia has stimulated intense clinical discussion and is likely to renovate the potential detection and management of pre-eclampsia.

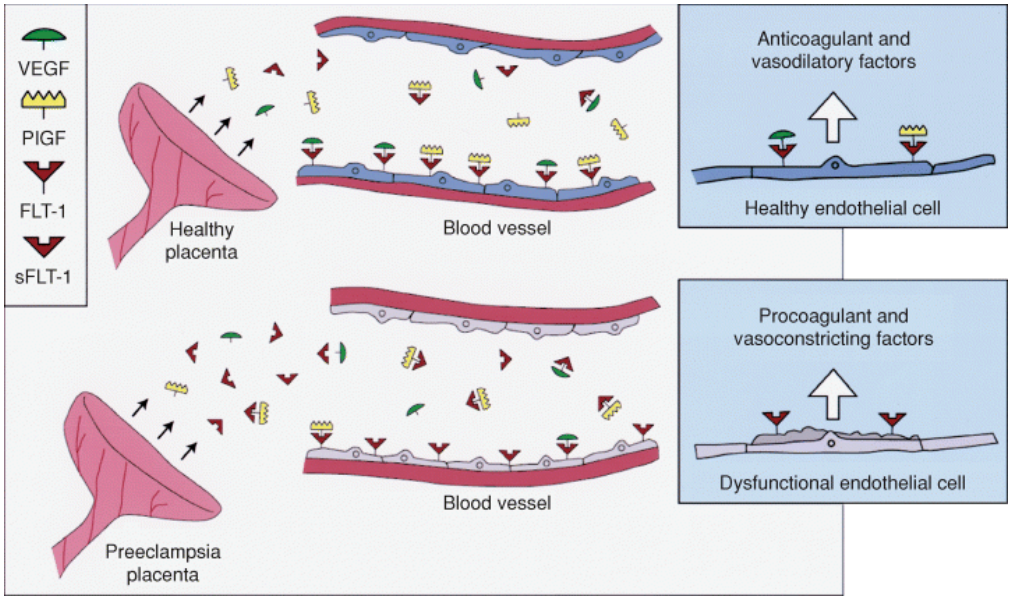


Figure 1.26: Role of sFlt-1 in endothelial dysfunction (with permission from Karumanchi *et al*., (2005)

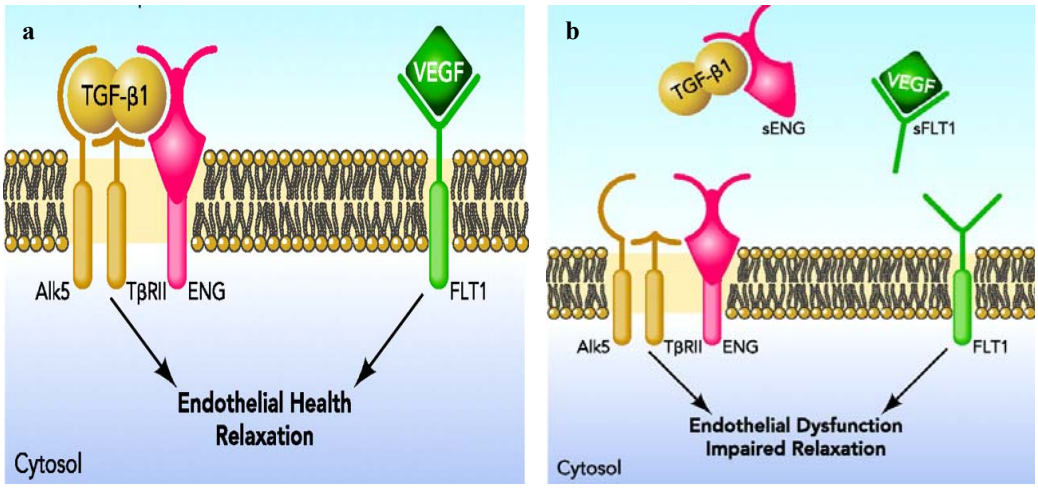


Figure 1.27: Role of sFlt-1 and sEng in (a) normotensive pregnancy and (b) pre-eclampsia.

(with permission from Karumanchi & Epstein, 2007).

1.9.4.3.2 Hemodynamic changes/oxidative stress and nitric oxide

Normal pregnancy is characterised by reduced peripheral vascular resistance and arterial blood pressure, whilst in pre-eclampsia these effects are deficient or reversed (Powe *et al.*, 2011, Maynard and Karumanchi, 2011). Hence, this lack of normal physiological function subsequently leads to an increased systemic vascular resistance and reduced cardiac output plus an inflated sensitivity to vasopressors, for example angiotensin II and norepinephrine (Roberts *et al.*, 1989). Moreover, females that are predisposed to developing pre-eclampsia may reflect an endothelium-dependent vasorelaxation and slight elevations in blood and pulse pressure prior to the development of this disease, indicative that alterations within the endothelium are present early in its progression (Khan *et al.*, 2005).

Reactive oxygen species (ROS) such as superoxide anion is produced by hypoxia, which serves as a powerful stimulator (Rodrigo *et al.*, 2005). In addition, superoxide anions are produced in cells by enzymes such as NADPH oxidase and xanthine oxidase. Xanthine oxidase may play a significant role in placental injury as well as an enhanced ability to produce ROS. Thus if ROS production in the placenta is greater than the ability of the antioxidant resistance, then this oxidative stress leads to injury to the placenta with the subsequent syncytial necrosis and infarction (Rodrigo *et al.*, 2005). The macrophage enriched placenta contains Hofbauer cells and is a major source of nitric oxide (NO), thereby contributing to added supply of free radicals and the subsequent endothelial dysfunction (Rodrigo *et al.*, 2005).

Nitric oxide is formed through a reaction catalysed by endothelial NO synthase (eNOS). It plays a major role in maintaining vascular homeostasis, and functions as a powerful

physiological vasodilator during normal pregnancy (Rodrigo *et al.*, 2005, Mohaupt, 2007). However, pre-eclamptic pregnancies are characterised by a deficiency of NO, due to its rapid degradation into peroxy nitrite ($ONOO^-$), a powerful vasoconstrictor and oxidant (Rodrigo *et al.*, 2005, Mohaupt, 2007).

A review analyses conducted by Noris *et al*., (2005) indicates that the release of NO mediates angiopoietin-induced development and movement of extravillous trophoblast cell *in vitro*. Additionally, NO mediates VEGF-induced up-regulation of VE-cadherin in endovascular trophoblasts. Thus, NO is significant in promoting cytotrophoblast endovascular invasion during normal placentation, due to its high calibre vasodilatory and angiogenic/ vasculogenic properties. It is likely that the placental modifications that occur in pre-eclampsia reflect a reduced ability of cytotrophoblast cells to form NO.

1.9.4.3.3 Renin-angiotensin-aldosterone system (RAAS)

The RAAS is a significant regulator of blood pressure during normal physiological functions, whilst its role in the aetiology of hypertension including pre-eclampsia is still under investigation (Nejatizadeh *et al.*, 2008). It is postulated that pre-eclampsia may possibly be due to gene mutations of the RAAS (**Fig. 1.28**). Accordingly, the bradykinin released during normotensive pregnancy induces the generation of NO, prostacyclin, and ROS, which results in the inactivation of angiotensin 1 (AT1)-receptor followed by a decreased angiotensin II response, and thus homeostasis (Nejatizadeh *et al.*, 2008).

The activation of the AT1-B2 receptor formation is present during pre-eclampsia, resulting in an increased angiotensin II response (**Fig. 1.28**). This thereby stimulates vascular NADPH oxidase, which produces ROS that joins with NO to form peroxynitrite ($ONOO^-$), a powerful vasoconstrictor and oxidant. Pre-eclampsia thus arises due to the absence of endothelial-dependent vasodilatory and inflammatory effects (**Fig. 1.28**) as a result of endothelial and smooth muscle cellular use of nitric oxide (Nejatizadeh *et al.*, 2008).

In addition, blood flow regulation is dependent on the source of renin, with tissue-based RAAS being identified as the key regulation factor (Nejatizadeh *et al.*, 2008). Previous studies have revealed that pre-eclamptic placentae have higher levels of both active renin and renin mRNA than placentae from normal pregnancies (Sowers *et al.*, 1993), whilst differential expression assays reported elevated decidual expression of AT1 receptors concurrent with the production of angiotensin II and AT1 autoantibodies (Herse *et al.*, 2007).

Similarly, AT1 receptor autoantibodies isolated from the maternal circulation of pre-eclamptic women are capable of mimicking characteristics of pre-eclampsia, thereby elevating the levels of both sFlt-1 and sEng in pregnant mice (Zhou *et al.*, 2008). Hence it is possible that AT1-AA is possibly one of the many factors that contribute to the placental dysfunction associated with the production of anti-angiogenic factors.

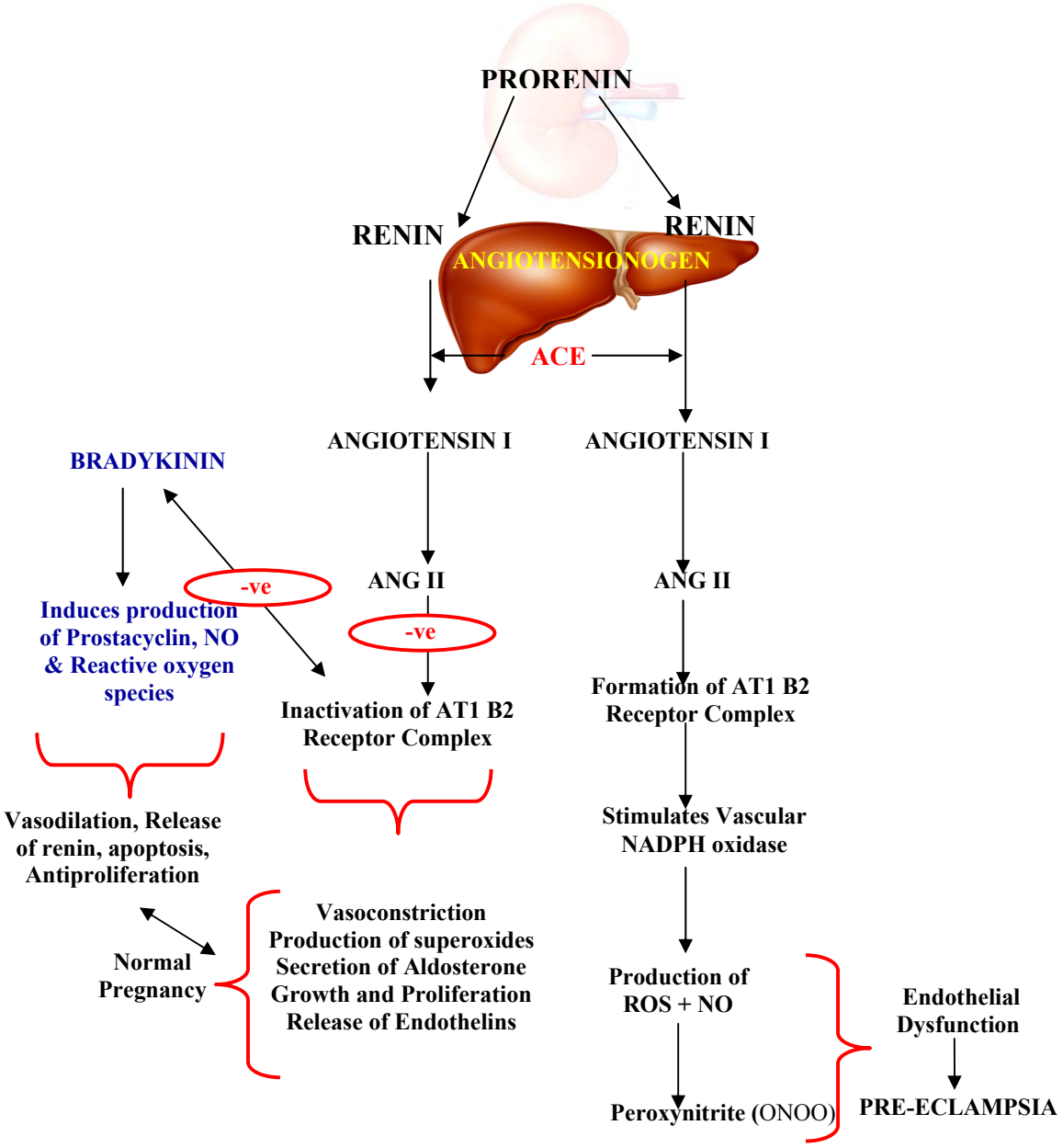


Figure 1.28: Postulated mechanism of RAAS in pre-eclampsia (Adapted from Nejatizadeh *et al.*, 2008)

1.9.4.3.4 Genetic effects

Genome wide studies, whole genome exon capture and sequencing are currently underway to identify genetic changes in pre-eclampsia (Agarwal and Karumanchi, 2011). Several polymorphisms in sFlt-1 and VEGF is linked with severity of pre-eclampsia (Ranheim *et al.*, 2001). Furthermore, VEGF 936C/T genotype has been shown to lower VEGF plasma levels compared to women carrying the VEGF936C/C genotype (Banyasz *et al.*, 2006). The G-allele of the 405G/C compared to the A allele of the VEGF 2578C/A polymorphism have a lower risk for developing severe pre-eclampsia (Widmer *et al.*, 2007). Paternal imprinting at the STOX1 gene locus is implicated in pre-eclampsia development (van Dijk *et al.*, 2005).

1.9.4.3.5 Immune maladaptation

The inadequate placentation that characterises pre-eclampsia when combined with an elevated inflammatory response affects the endothelium and leads to both endoplasmic reticulum and oxidative distress (Redman and Sargent, 2009). This leads to the increased placental discharge of sFlt-1, which binds and reduces the availability of VEGF in response to widespread systemic inflammatory responses into the maternal circulation (Maynard and Karumanchi, 2011, Silasi *et al.*, 2010). As a consequence, the VEGF deprived endothelium becomes dysfunctional and leads to the classical pre-eclamptic symptoms (Redman and Sargent, 2003, Irminger-Finger *et al.*, 2008, Saito *et al.*, 2007).

Uterine natural killer (uNK) cells are the main population of decidual lymphocytes and form part of the innate immune system (Kopcow and Karumanchi, 2007). The interaction of killer

cell immunoglobulin-like receptors (KIRs) on the maternal uNK cells with trophoblast indicators co-ordinates the invasive nature of cytotrophoblastic cells (Kopcow and Karumanchi, 2007, Irminger-Finger *et al.*, 2008). Subsequently, the invading cytotrophoblast expresses atypical arrangements of major histocompatibility complexes such as human leukocyte antigens HLA-C, HLA-E and HLA-G.

Human leukocyte antigen C (HLA-C), a class I molecule, interacts with KIRs and CD 94 receptors found on the surface of decidual NK cells thereby regulating the interaction of extravillous trophoblast cells and the NK cells (Irminger-Finger *et al.*, 2008, Kopcow and Karumanchi, 2007). Placentation during normal pregnancy has to be balanced in terms of inhibition and activation of uNK cells, however, in pre-eclampsia this contact between HLA-C molecules and KIRs may result in decreased cytotrophoblastic invasion.

1.9.5 Clinical implications and biomarkers of pre-eclampsia

To date, early delivery of fetus and placenta is the only cure for pre-eclampsia. Current diagnostic tools include the measurement of blood pressure and sampling of urine protein, which are known to have little sensitivity and specificity about the disease progression or maternal and perinatal effects (Verlohren *et al.*, 2012). Thus an appropriate diagnosis will assist in reducing both maternal and fetal morbidity and mortality.

Identification of a biomarker for the early detection of pre-eclampsia will add to the perceptive of this disease. This will aid clinical management by identifying women at high risk who may need careful follow-up and delivery at an early stage in pregnancy, or reduce the symptoms so that the pregnancy continues thereby improving neonatal morbidity and mortality. Additionally, it will contribute to an improved perspective of the association between this maternal disorder and the increased risk for cardiovascular disease in later life. On the other hand, unnecessary interventions may also be avoided in women at low risk of pre-eclampsia development.

Four major biomarkers enable early detection of pre-eclampsia (**Fig. 1.29**) (Carty *et al.*, 2008). Whilst the current major prevention strategy is a low dose of aspirin as a form of prophylactic therapy, its effect can only be determined if it is administered during early gestation (Bates *et al.*, 2004). To date, angiogenic imbalance in maternal circulation is greatly correlated with the aetiology of this disorder and the diagnostic utility of sFlt-1 and sEng have been widely highlighted (Verlohren *et al.*, 2012, Hagmann *et al.*, 2012, Lapaire *et al.*, 2010). Thus it would seem that combined with use of low dose aspirin therapy, the evaluation of the sFlt1/PlGF ratio

as a predictable anti-angiogenic indicator may provide added clinical data on the development of this disorder (De Vivo *et al.*, 2008, Verlohren *et al.*, 2010).

Several studies have explored the use of pro-angiogenic factors in the prediction of pre-eclampsia (Verlohren *et al.*, 2010, De Vivo *et al.*, 2008). Inconsistencies prevail, however, on the utility of these factors during the first trimester of pregnancy. Whilst elevated sFlt-1 concentrations have been shown to be present during the first trimester (Baumann *et al.*, 2008), other studies have negated this elevation (Thandani *et al*., 2004b, Rana *et al*., 2007). Reduced concentrations of maternal blood levels of PlGF, together with uterine arterial Doppler measurements, have been explored as a useful candidate for the detection of early onset pre-eclampsia (Thandani *et al*., 2004b). The use of maternal blood levels of sFlt-1, PlGF and sEng may serve as useful diagnostic tools for detection during early stages of the second trimester (Levine *et al.*, 2006, Venkatesha *et al.*, 2006).

Additionally, the use of automated prototype assays from Beckman Coulter, Inc. to R&D systems microplate assays was investigated for the quantification of pro-angiogenic factor analytes in the detection of pre-eclampsia (Wothe *et al.*, 2011). Thus, the use of angiogenic markers as a clinical tool is likely to influence the decision-making of the medical staff, improve the quality of maternal and fetal life, and/or decrease healthcare expenditure (Verlohren *et al.*, 2012, Hagmann *et al.*, 2012).

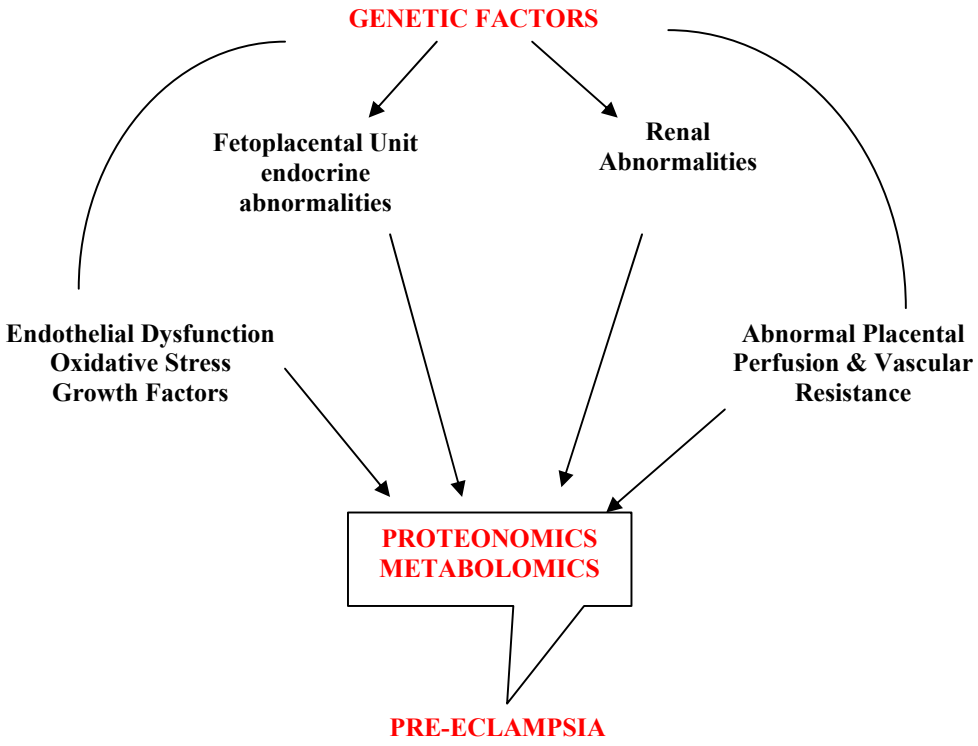


Figure 1.29: Biomarkers of pre-eclampsia (Adapted from Carty *et al*., 2008)

1.10 Null Hypothesis

Variations in gene expression, immunoexpression, regulation and the subsequent role of sFlt-1 and sEng and their antagonists VEGF and PlGF does not occur in pre-eclampsia. HIV status does not affect the expression of sFlt-1 and sEng and their antagonists VEGF and PlGF in normotensive and pre-eclamptic pregnancies.

1.11 Aims and objectives of this study

The aim of this study is to determine the effect of HIV status on pro-angiogenic vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), and anti-angiogenic soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng) growth factors in pre-eclampsia and normotensive pregnancies.

The objectives of the study were to evaluate the:

- immunolocalisation of VEGF, PlGF, sFlt-1 and sEng in HIV negative normotensive and pre-eclamptic versus HIV positive normotensive and pre-eclamptic placentae using immunohistochemistry interfaced with morphometrical image analyses;
- ultrastructural immunolocalisation of VEGF, PlGF, sFlt-1 and sEng in HIV negative normotensive and pre-eclamptic versus HIV positive normotensive and pre-eclamptic placentae using immunoelectron microscopy;
- placental expression of VEGF, PlGF, sFlt-1, and sEng in HIV negative normotensive and preeclamptic versus HIV positive normotensive and preeclamptic placentae using real time polymerase chain reactions (RT-PCR)
- circulating concentrations of serum VEGF, PlGF,sFlt-1 and sEng in HIV negative normotensive and pre-eclamptic versus HIV positive normotensive and pre-eclamptic pregnancies using enzyme linked immunosorbant assays (ELISA) techniques.

2.1 Ethical approval

This prospective study was conducted in the Optics and Imaging Centre, Doris Duke Medical Institute, College of Health Sciences, University of KwaZulu-Natal (PG009/07; Addendum I). Institutional ethical clearance (BF 037/07; Addendum II) was obtained from the Biomedical Research Ethics Committee, University of KwaZulu-Natal. Permission to conduct research at the Obstetric Unit, King Edward VIII Hospital (KEH) was granted by the Hospital Manager (Addendum III).

The study population consisted of patients from the Obstetric Unit at KEH, recruited by a professional research nurse between September 2008 and June 2010. Patients recruited were informed by the research nurse of the procedures and their role in the study. Informed consent was obtained from all patients participating in the study (example of consent document; Addendum IV). Thereafter, a structured data sheet was completed by the research nurse to collate relevant maternal, demographic, clinical and fetal information (Addendum V). Maternal and infant records were also reviewed for data regarding labour, delivery and the clinical condition of the newborn.

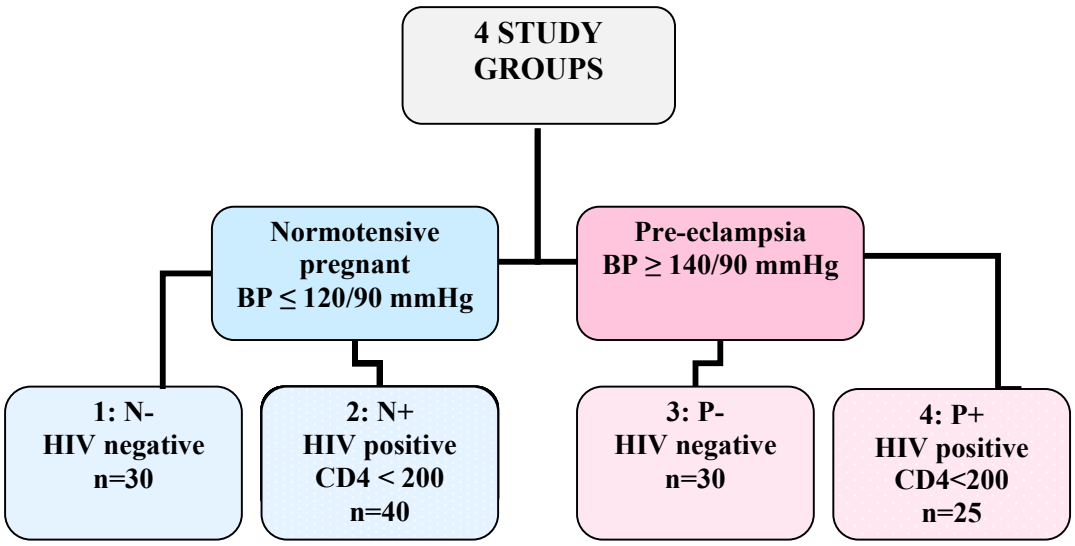
2.2 Patient recruitment and consent

2.2.1 Patient selection and inclusion criteria

Pre-eclampsia was defined by

- persistent systolic blood pressure greater than 140 mmHg and diastolic blood pressure greater than 90mmHg taken at least 6hrs apart, after 20 weeks' gestation in a previously normotensive patient, and
- new onset proteinuria defined as urine protein concentration of ≥ 30 mg/dl or 1+ on a urine dipstick in at least 2 random specimens collected at least 4hrs apart.

Patients recruited were divided into **four study groups** based on the following inclusion criteria:



Normotensive groups were matched for maternal and gestational age with the pre-eclamptic groups ie. group 1 (control) vs group 3; group 2 (control) vs group 4. For HIV, group 1 (control) vs group 2; group 3 (control) vs group 4.

2.2.2 Exclusion criteria

Women with the following disorder were excluded from the study: chorio-amnionitis, chronic hypertension, eclampsia, abruptio placentae, intra-uterine death, chronic diabetes mellitus, gestational diabetes, chronic renal disease, connective tissue disease, treatment with aspirin, warfarin, non-sterooidal anti-inflammatory drugs, lipid lowering or anti-hypertensive disease, systemic lupus erythematous, sickle cell disease and anti-phospholipid antibody syndrome; thyroid disease, cardiac disease and active asthma requiring medication during pregnancy and pre-existing seizure disorders.

2.3 Sample collection

Placental tissue was obtained post-delivery from women undergoing normal vaginal or caesarean section modes of delivery. Macroscopic normal areas of placental tissue were obtained, with care taken to avoid areas showing evidence of necrosis and infarctions. This tissue was divided into 4 pieces for histology, immunohistochemistry, imunoelectron microscopy as well as for genetic analyses.

Maternal blood samples were collected antenatally in commercially available plain gel tubes. Serum was obtained by centrifugation of blood samples at 3500rpm for 10min at 4°C. Serum was then carefully aliquotted into labelled cryotubes and stored at -70°C until further investigation.

2.4 Histological evaluation of the placenta

2.4.1 Hematoxylin and Eosin (H&E)

2.4.1.1 Sample preparation

Full thickness of placental tissue from the central region of the placenta was dissected and immersed in 10% buffered formaldehyde (Polychem, KZN, SA). Fixed tissue was subsequently dehydrated, infiltrated with paraffin wax and embedded in cassettes using an automated tissue processor and tissue embedding station, respectively (Leica ASP 200S tissue processor; Leica EG 1160 embedding station; Germany). This automated protocol is outlined in **Table 2.1**.

2.4.1.2 Microtomy

Tissue sections (3µm thickness) were cut using low profile sterile disposable blades (Leica 819; Germany) on a rotary microtome (Leica Jung RM 2035; Germany). They were floated in a water bath (Leica HI210; Germany) and collected onto poly-l-lysine coated glass slides (Thermo Scientific, Germany) and stained with Mayer's haematoxylin and eosin for evaluation of placental morphology (**Table 2.2**). Additionally, corresponding sections were collected for immunohistochemistry. All sections were baked overnight at 60°C before dewaxing in xylene and rehydrated according to the schedule outlined in **Table 2.3**.

2.4.1.3 Image archival

Histopathological assessment of H & E stained sections across all groups were qualitatively evaluated and archived on a Zeiss Axioscope A1 (Carl Zeiss, Germany).

2.5 Immunolocalisation of pro- and anti-angiogenic factors in placental tissue

2.5.1 Specificity of antibodies used

The primary and secondary antibodies utilised for the immunohistochemical evaluation of the pro-angiogenic and anti-angiogenic factors within the placentae were obtained from R&D Systems, Minneapolis, USA.

2.5.1.1 Primary antibodies

i. Mouse monoclonal anti-human PlGF antibody (MAB264, R&D Systems, USA)

PlGF is a member of the VEGF family of growth factors which binds with great affinity to VEGFR-1. It detects rhPlGF and shows no cross reactivity with rhVEGF, rhPDGF-AA; rhPDGF-AB or rhPDGF-BB. This commercially produced antibody was produced from a hybridoma that was extracted from a mouse immunised with a lysate that expressed recombinant human PlGF. This fraction of the IgG of the tissue culture supernatant was purified by protein G affinity chromatography (Huse *et al.*, 2002).

ii. Mouse monoclonal anti-human VEGF antibody (MAB 293, R&D Systems, USA)

This commercially produced antibody has a capacity to recognise $rhVEGF_{165}$ and neutralize its biological activity. It was produced from a hybridoma that was produced from the fusion of a mouse myeloma with B cells obtained from a mouse immunised with purified, insect cell line Sf 21-derived, recombinant human $VEGF_{165}$ amino acid isoforms ($rhVEGF_{165}$). This fraction of the IgG of the tissue culture supernatant was purified by protein G affinity chromatography (Huse *et al.*, 2002).

iii. Goat anti-human VEGFR-1 antibody (AF321, R&D Systems, USA)

This commercially available VEGFR-1 antibody was produced in goats immunised with purified, insect cell line Sf 21-derived, recombinant human VEGFR-1/Fc chimera extracellular domain (rhVEGFR-1/Fc chimera). Its specific IgG was purified by human VEGFR-1 affinity chromatography. It has the capacity to identify human VEGFR-1 in immunohistochemistry and prevent the binding of rhPlGF to immobilised rhVEGFR-1/Fc.

iv. Goat anti-human Endoglin antibody (AF1097, R&D Systems, USA)

This commercially produced antibody was produced in goats that were immunised with purified, recombinant human endoglin (rhEndoglin) extracellular domain. Its specific IgG was purified by human Endoglin affinity chromatography (Huse *et al.*, 2002).

2.5.1.2 Biotinylated secondary antibody (anti-rabbit, anti-mouse and anti-goat immunoglobulins (K0679, Dako Glostrup, Denmark)

Biotinylated anti-rabbit, anti-mouse and anti-goat immunoglobulins were diluted in phosphate buffered saline (PBS) which contained stabilising proteins and 0.015mol/L sodium azide (preservative).

2.5.2 Antigen retrieval and blocking

Antigen retrieval for all antibodies were performed in a commercial antigen retrieval solution viz., Target Retrieval Solution (S169984, DakoCytomation, Glostrup, Denmark) for 11min at medium high power, in a conventional microwave oven with a power consumption of 1200W (LG MS2524W). Endogenous peroxidase blocking involved incubation in 3% H_2O_2 for 5min

and additional protein blocking was carried out with 10% bovine serum albumin (A4503, Sigma, USA) solution for 30min.

2.5.3 Immunohistochemical staining procedure for VEGF, PlGF, sVEGFR-1/sFlt-1 and sEng

Post-blocking, sections were washed in PBS: 0.1% Tween 20 followed by a single PBS wash on a flatbed rocking station. Thereafter, slides were incubated overnight in the following primary antibodies at 4°C:

- 100mg/ml mouse anti-human PlGF (1:10 dilution) antibody (R&D Systems, MAB264),
- 50mg/ml mouse anti-human VEGF (1:20 dilution) antibody (R&D Systems, MAB 293),
- 5mg/ml goat anti-human VEGFR-1 (1:200 dilution) antibody (R&D Systems, AF321), and
- 5mg/ml goat anti-human Eng (1:200 dilution) antibody (R&D Systems, AF1097).

Subsequently, sections were washed thoroughly in PBS: 0.1% Tween 20 and incubated with biotinylated anti-rabbit, anti-mouse and anti-goat secondary antibodies to amplify the signal from the primary antibody (K0679, LSAB+System-HRP, Dako Glostrup, Denmark), for 30min at room temperature. Following PBS washes, sections were incubated for 30min at room temperature with LSAB+Streptavidin (K0679, LSAB+System-HRP, Dako Glostrup, Denmark).

The reaction was visually detected within 10min with diamino-benzidine (DAB) as the chromogen. Nuclei were counterstained with Mayer's haematoxylin (5min). Sections were subsequently rehydrated and mounted in dibutylphthalate xylene (DPX).

Negative controls were performed by replacing the primary antibody with non-immune sera of the same IgG isotype as the primary antibody. Buffer controls were performed by replacement of the primary antibody with PBS. The placental tissue was used as a positive control due to its ease of availability and its elevated content of both the pro-angiogenic and anti-angiogenic factors (Nevo *et al.*, 2006). This protocol is outlined in **Fig. 2.1**, **Table 2.1** and **2.2** and **2.3**.

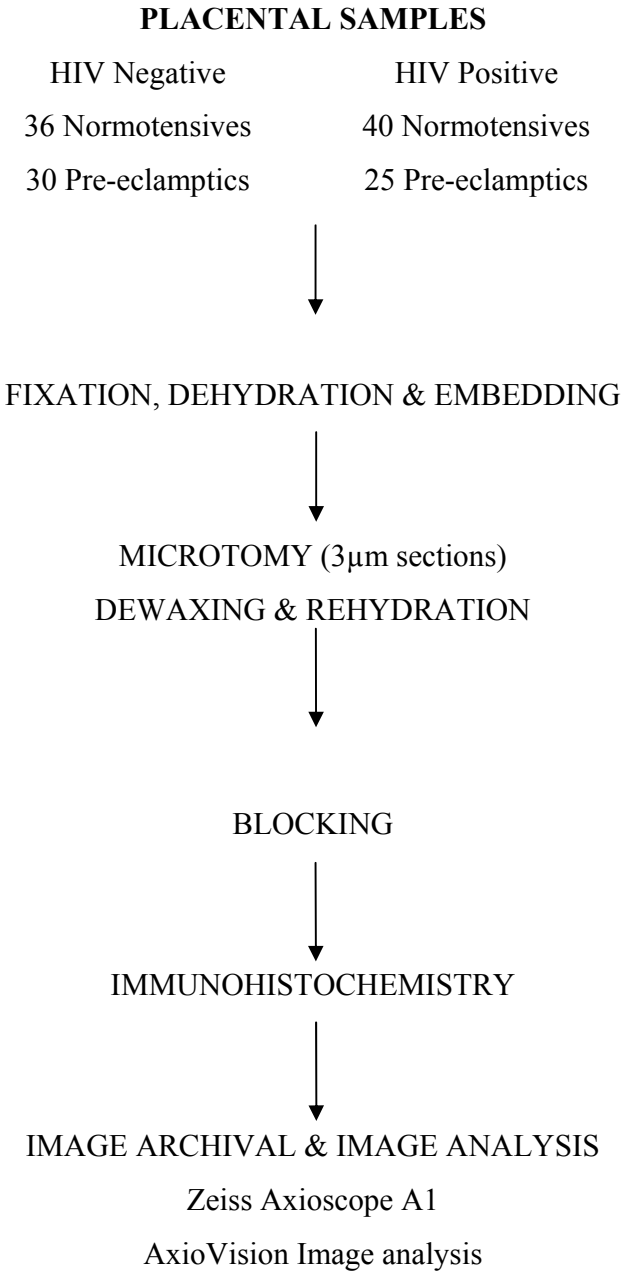


Figure 2.1: Summary of immuno-histochemical staining procedure

Table 2.1: Procedure for processing of placental tissue for light microscopy

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

Table 2.2: Procedure for Mayers Hematoxylin & Eosin Staining

STEP	TREATMENT	SOLUTION	TIME
1	Dewaxing	Xylene	5min X2
2	Rehydration	100% Ethanol 90% Ethanol 70% Ethanol H_20	1min X2 1min 1min 1min
3	Nuclei Stain	Mayer's Haematoxylin Blue: Rinse under running tap H_20	5min 5min
4	Cytoplasmic Stain	Immerse in 0.5% alcoholic eosin (BDH Lab supplies)	2min
5	Dehydration	95% ethanol 100% ethanol Xylene	30sec 1min 1min
6	Mounting	Dibutylphthalate xylene (DPX)	

Table 2.3: Procedure for immuno-histochemical localization of pro- and anti-angiogenic factors

NO	STEP	CHEMICAL SOLUTION	TIME
		Slides were baked overnight at 60°C	
1	Dewaxing	Deparaffinize in Xylene	5min X2
2	Rehydration	100% Ethanol 95% Ethanol 70% Ethanol Picric acid d H_20 PBS On Rocker	5min X2 5min 5min 30min 5min 5min
3	Antigen Retrieval Pretreatment	Target retrieval solution (Citrate buffer, pH 6) • Microwave at 90°C for 5min • Top up & boil for a further 5min • Cool sections to 65°C (10-20min) • Wash buffer (PBS-Tween 20) • PBS	 10min X2 5min X3 5min
		Hydrophobic Barrier: Circle specimen with a pap pen	
4	Endogenous peroxidise	3% H_20_2	5min
5	Protein blocking	Wash Buffer (TBS-Tween 20) 10% serum albumin solution	30min
6	Primary antibody/ 2% BSA	• sFlt1 1:200 sEng 1:200 • PlGF 1:10 VEGF 1:20	overnight 4°C
7	Biotinylated Link	3 YELLOW drops	30min RT
8	Streptavidin Peroxidase	3 RED drops of Streptavidin -HRP Substrate-Chromogen solution	30min RT 10min RT
9	Nuclei stain	• Mayer's haematoxylin • "Blued" in running tap water	5min 5min
10	Dehydration	• 70% alcohol • 80% alcohol • 95% alcohol • 100% alcohol • Xylene	5min 5min 5min X2 5min X2
11	Mountant	Mount using DPX permanent mounting medium	

* All washes were performed in 3X PBS-Tween 20 wash followed by a single PBS wash on a flatbed rocking station.

* Excess buffer was tapped off & wiped around specimen between steps 1-11.

* Volume of reagents utilised during incubation = 120µl.

2.5.4 Morphometric image analysis of antibody expression

Image analysis is a method of obtaining quantitative data from images. The immunoexpression of the various antibodies were evaluated with the Zeiss Axioscope A1, using AxioVision Image analysis software package version 4.8.3 in combination with the auto-measurement module (Carl Zeiss, Germany).

Five areas of the placental villi per specimen (exchange villi) and placental stem villi per specimen (conducting villi) were randomly selected and archived at an initial magnification of X20 and X10, respectively. The size of the frame was defined as the scaled area of the measurement frame and expressed as μm². For the exchange villi, the entire frame area was selected. This measurement corresponded to a mean area frame of 312483.68μm². However, the frame area for stem villi varied due to single object analyses. Regions of interest (ROI) (with/without immunoreactivity) for both exchange and stem villi were automatically segmented and expressed as a field area percentage (%/μm^2) (**Figure 2.2**). Intensity of immunoreactivity was expressed on a grey scale with a range from 0-255.

Thus, image recordings provided data for the following defined parameters, outlined below:

- Area frame (μm^2): Area of the scaled measurement frame.
- Field area percent (%): Percentage area of all regions in measurement frames.
- Densitometric mean: Mean grey value of all regions; unit: grey scale.
- Densitometric minimum: Minimum grey value in all regions; unit: grey scale.
- Densitometric maximum: Maximum grey value in all regions; unit: grey scale.

Specific predefined measurement parameters were utilised to create a database for data analyses. An excel database using the predefined parameters was then created for both the exchange and stem villi immunostained with sVEGFR-1/sFlt-1, Eng, PlGF and VEGF antibodies across all study groups, respectively.

2.5.5 Statistical analysis

Two-way analyses of variance (ANOVA) tests were used to measure the effects of HIV on pregnancy (normotensive and pre-eclamptic) simultaneously, thereby assessing whether there was an interaction between our parameters. The two-way ANOVA test generated three p-values, one for each parameter independently, and one measuring the interaction between the two parameters.

Hence, the two-way ANOVA was done to examine the effect of sFlt-1, sEng, PlGF and VEGF on HIV-associated pregnancy. All data were assessed for their distribution by the Shapiro-Wilk test. Additionally, the homogeneity of variance between groups was assessed by the Levene's test for equality of error variances. Post-hoc subgroup analyses were done for multiple comparisons. The Mann-Whitney test was also used to compare the means of CD4 counts between groups. Descriptive statistics were utilised and outcome variables are presented as median (inter-quartile range). A probability level of $p<0.05$ was considered statistically significant. All statistical analyses were conducted using GraphPad Prism® version 5.01 and IBM SPSS Statistics version 20.

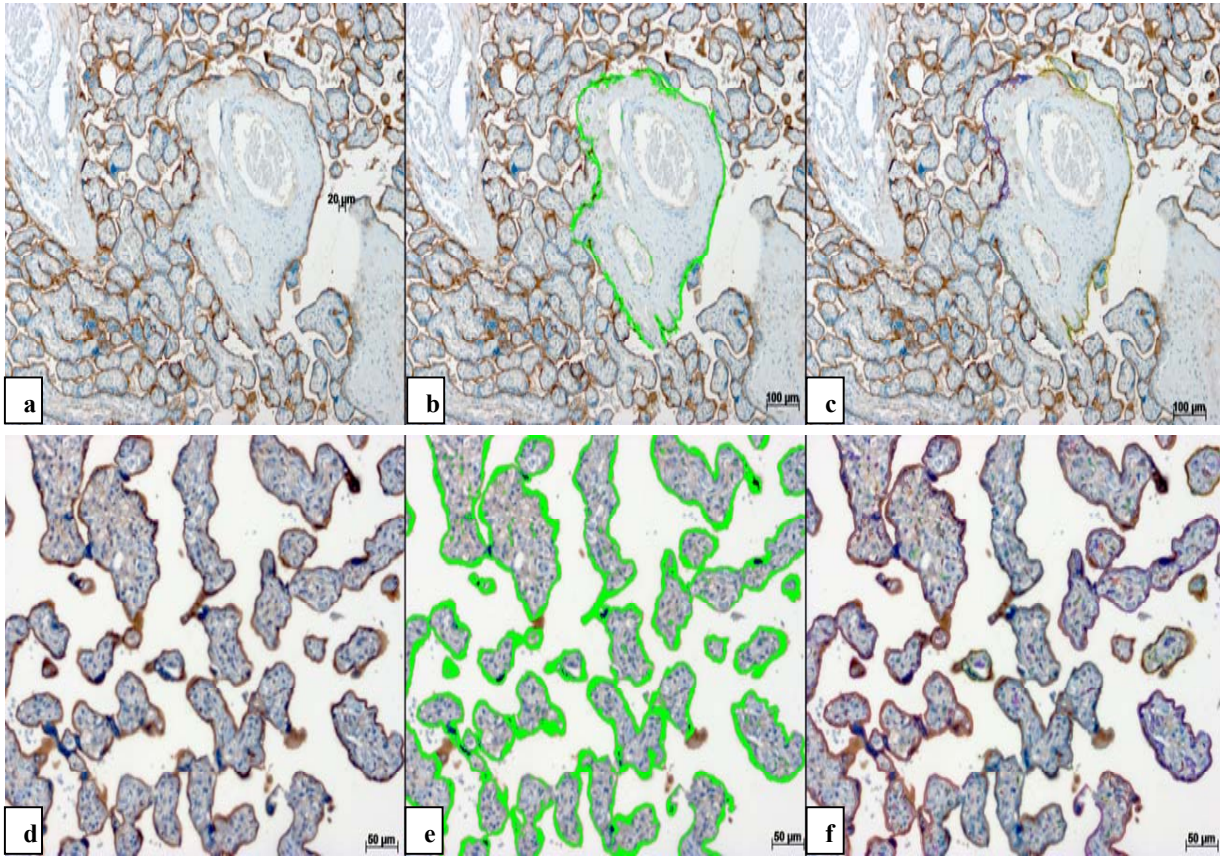


Figure 2.2: Sequential steps in image analyses of conducting (a-c) and exchange (d-f) villi. Figures (a and d) represent initial images, (b and e) show areas of segmentation whilst (c and f) represent mean densitometric values in different colours.

2.6 Immunoelectron microscopy (IEM)

2.6.1 Specimen processing

Post-delivery, a small piece of placental tissue was immediately immersed into 0.1% glutaraldehyde/4% paraformaldehyde fixative in 0.2M phosphate buffer overnight at 4°C (pH 7.2). Specimens were then minced into 1mm^3 cubes and re-immersed into fresh fixative. Thereafter the tissue was washed in 0.2M phosphate buffer (pH 7.2; 4°C). The tissue was subsequently dehydrated through an ascending series of ethanol, via propylene oxide as the intermediate solvent and finally infiltrated with a low viscosity Spurr's epoxy (Sigma, USA) resin (Spurr, 1969) prior to embedding in capsules (size 00, BEEM, USA) and polymerised for 48hrs at 60°C (**Table 2.4**).

2.6.2 Ultramicrotomy

Semi-thin placental sections (1µm) were cut with glass knives using an ultramicrotome (Reichert S/Ultracut R, Leica, Germany). Sections were collected onto glass slides, heat-fixed, stained with 1% toluidine blue and examined with a Leica microscope (Leica DM500, Lecia, Germany). Fields of interest containing placental villi were selected, identified on the resin block face, and then trimmed to produce a trapezoidal shaped face. Ultrathin pale-gold sections (50-60nm) were cut and collected onto uncoated 200 mesh nickel grids (Sigma, UK) prior to immunoelectron labelling.

2.6.3 Specificity of antibodies used

The following primary and secondary antibodies were utilised for the immunohistochemical evaluation of required pro- and anti-angiogenic factors within placental tissue samples:

2.6.3.1 Primary antibodies (refer to 2.3.2.5)

i. Mouse monoclonal anti-human PlGF antibody (MAB264, R&D Systems, USA,

ii. mouse monoclonal anti-human VEGF antibody (MAB293, R&D Systems, USA,

iii. goat antihuman VEGFR-1 antibody (AF321, R&D Systems, USA),

iv. goat anti-human Endoglin antibody (AF1097, R&D Systems, USA).

2.6.3.2 Secondary antibodies

i. Goat anti-mouse IgG conjugated with 10nm colloidal gold particles (G7652, Sigma, United Kingdom)

This anti-mouse IgG molecule was produced in goat using an immunogen mouse IgG that was purified from normal mouse serum. It was isolated by immunospecific methods of purification to remove all goat serum proteins, inclusive of immunoglobulins that do not bind specifically to mouse IgG. The affinity isolated antibody was conjugated to 10nm gold particles and the excess antibody was removed. The identity of this antibody was established by the Dot Blot Assay prior to conjugation (Brada and Roth, 1984).

ii. Rabbit anti-goat conjugated with 10nm colloidal gold particles (G5402, Sigma, United Kingdom)

This anti-goat IgG molecule was produced in the rabbit using a goat IgG that was purified from normal goat serum as the immunogen. It was isolated by immunospecific methods of purification to remove all rabbit serum proteins, inclusive of immunoglobulins that do not bind specifically to goat IgG. The affinity isolated antibody was conjugated to 10nm gold particles and the excess antibody was removed. The identity of this antibody was established by the Dot Blot Assay prior to conjugation (Brada and Roth, 1984).

2.6.4 Immunohistochemical staining procedure

Ultrathin sections were etched in 5% H_20_2 for 5min and incubated in PBS containing 10% bovine serum albumin (A4503, Sigma, USA) to block non-specific protein binding sites (**Table 2.5**). Following washing, 100mg/ml of PlGF (1:10 dilution) antibody (mouse monoclonal anti-human PlGF, MAB264, R&D Systems, USA), 50mg/ml of VEGF (1:20 dilution) antibody (mouse monoclonal anti-human VEGF, MAB 293, R&D Systems, USA), 33.3mg/ml of VEGFR-1 (1:30 dilution) antibody (goat anti-human E, AF321, R&D Systems, USA) and 6.7mg/ml of Endoglin (1:150 dilution) antibody (goat anti-human Endoglin, AF1097, R&D Systems, USA), respectively, were incubated with the sections overnight at 4°C.

Sections were then incubated for 2hrs in goat anti-mouse IgG and rabbit anti-goat IgG conjugated with 10nm colloidal gold particles (1:15 dilution), respectively (Sigma, USA) at room temperature for the detection of the primary antibody binding sites. Sections were subsequently washed in PBS and post fixed in 2% glutaraldehyde. All sections were contrast

enhanced with an ethanolic saturated solution of uranyl acetate and Reynold's lead citrate (Reynolds, 1963) and viewed on the Jeol 1011 transmission electron microscope (Jeol 1011, Japan).

NB: The primary and secondary antibody solutions were diluted with PBS containing 1% and 0.1% bovine serum albumin, respectively (A4503, Sigma, USA). Negative controls received the same treatment, except for replacement of the primary antibody with PBS.

Table 2.4: Processing schedule for immunoelectron electron microscopy

STEP	TREATMENT	SOLUTION	TEMPERATURE	TIME
1	Fixation	0.1 % Glutaraldehyde/4% Paraformaldehyde (pH 7.2)	4°C	Overnight
2	Wash	Phosphate buffer (pH 7.2) Phosphate buffer (pH 7.2)	4°C 4°C	10min X2
3	Dehydration	Dehydration: 70% Ethanol 90% Ethanol 100% Ethanol	RT RT RT	30min 30min 15min X4
4	Intermediate solvent	100% ethanol: propylene oxide	RT	30min
5	Infiltration	50:50 Propylene oxide: Resin Spurr Resin	RT 60°C	30min 60min X2
13	Polymerisation	Spurr Resin	60°C	48hrs

Table 2.5: Procedure for immunoelectron staining

STEP	PROCEDURE	TIME
*Etching	5% H_20_2	5min RT
Washing	dH_20 (10ml)	RT
Blocking	Phosphate buffered saline pH 7.2	30min RT
*Primary antibody	Primary antibody in 1% BSA Phosphate buffered saline • sFlt-1: 1/30 dilution sEng: 1/150 dilution • PlGF: 1/10 dilution VEGF: 1/20 dilution	Overnight 4°C
Washing	10ml Phosphate buffered saline, pH 7.2 per grid	5min RT
Blocking	Phosphate buffer 0.1% BSA/PBS pH 7.2 5ml/grid	5min RT
Secondary antibody	• Secondary antibody (conjugated to 10 nm gold particle) 0.1% BSA in Phosphate buffered saline (1:15 dilution)	2hrs RT
Washing	• Phosphate buffer 0.1% BSA 5ml per grid (w/v), pH 7.2 • Phosphate buffer 5ml per grid, pH 7.2 • dH_20 (5ml per grid)	RT
Post fix	2% glutaraldehyde PBS	5min RT
Washing	dH_20 (5 ml per grid)	RT
*Staining	Saturated uranyl acetate dH_20 (10ml per grid)	2min RT
	Reynold's lead citrate dH_20 (10ml per grid)	2min RT

* Volume of reagents utilised during incubation = 40µl

2.7 Genetic analysis of pro- and anti-angiogenic factors

2.7.1 Placental preparation

Post-delivery, central and peripheral regions of placental tissue (1cm) were dissected and immersed into cryotubes containing RNA*later* stabilising solution (Ambion, Austin, USA) and allowed to penetrate into tissue overnight at 4°C and then stored at -70°C until RNA extraction.

2.7.2 RNA Extraction

Equimolar quantities of central and peripheral regions of placental tissue equivalent to 50mg was homogenised using a Tissue Tearor (Biospec Products Inc., USA) in 1ml of Trizol® for approximately 60sec. Homogenised samples were incubated for 10min at room temperature to allow for complete dissociation of the nucleoprotein complexes. Chloroform (0.2ml) was then added per 1ml of Trizol reagent.

Sample tubes were shaken vigorously using a vortex, and further incubated for 10min at room temperature. Incubated samples were then centrifuged at 10 000rpm for 15min at 4°C. Following centrifugation, the mixture separated into 3 phases viz., a lower red phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. The aqueous phase, which was exclusively RNA, contained approximately 60% of the volume of the Trizol reagent used for homogenisation.

The aqueous phase was transferred to a freshly labelled tube and RNA was precipitated by mixing with 0.5ml isopropyl alcohol per 1ml Trizol reagent. The samples were then gently mixed and incubated for 10min at room temperature. Thereafter, the samples were centrifuged

at 10 000rpm for 15min at 4°C. Following centrifugation, the RNA precipitate was visible as a gel-like pellet on the side or bottom of the eppendorf tube. The supernatant was then carefully decanted and the RNA pellet was washed with 1ml of 70% RNase free ethanol. Samples were mixed using a vortex and further centrifuged at 10000rpm for 15min at 4°C. The supernatant was carefully decanted and the RNA pellet was air-dried for 5-10min and then re-dissolved in 30µl diethyl pyrocarbonate (DEPC) treated water.

2.7.3 Spectrophotometric analysis

The RNA (1µl) was thereafter diluted in 1ml of DEPC treated water. The optical density (OD) was determined at 260nm and 280nm using the Beckman Coulter UV Visible Spectrophotometer (DU 800, Beckman Coulter, USA). The OD values were used to determine the concentration and purity of RNA at a A260/A280 ratio ≥ 1.6.The principle that 1 OD at 260nm = 40µg/ml RNA was applied across all samples. The purified RNA product (1µg) was then run on a 1.5% agarose gel to determine the quality of the product.

2.7.4 DNA removal

All RNA samples were treated with a DNA Removal kit (DNase; AM 1096: Applied Biosystem, Foster City, USA) to remove DNA contamination. The final reaction volume for DNase treatment was 50µl. The DNase treatment reagents (5µl of 10X buffer and 2µl of rDNaseI) were added to 20µg/µl RNA and brought to reaction volume with DEPC or nuclease free water (**Table 2.6**). Treated samples were then incubated at 37°C for 20-30min. Vortexed DNase inactivation reagent (5µl) was then added into each sample tube and incubated at room temperature for 2min. Samples were then centrifuged at 10 000G for 1.5min and the

supernatant was carefully transferred into a new RNase free tube for reverse transcription. The RNA was then stored at -70°C until use for reverse transcription.

2.7.5 Reverse transcription

Single stranded DNA was synthesised from DNA free RNA using the high-capacity cDNA Reverse Transcription Kit with RNase Inhibitor (4374966, Applied Biosystem, Foster City, USA), with a final reaction volume of 20µl. The kit included reagents that when mixed, produced a 2X Reverse Transcription (RT) master mix. The 2X RT master mix was prepared on ice according to the manufacturer's instructions (**Table 2.7**). The cDNA RT reactions were prepared by adding 10µl of 2X RT mastermix into individual reaction tubes

The RNA sample (10µl) was then added into each reaction tube and pipetted up and down two times to ensure adequate mixing of reagents. The tubes were then briefly centrifuged to spin down the contents and eliminate any air bubbles. The tubes were then loaded onto the thermal cycler programme using the optimised thermal cycling conditions (**Table 2.8**), according to the manufacturer's instructions. The reaction volume on the thermal cycler was set to 20µl and the reverse transcription reaction was run. The quality of cDNA was evaluated by running the product on a 2% agarose gel. The cDNA was then stored at -20°C until use for real-time PCR.

Table 2.6: Procedure for DNA removal

DNase TREATMENT REAGENTS	QUANTITY (µl)
10X Buffer ($1/10^{th}$ of reaction volume)	5
rDNaseI	2

Table 2.7: Procedure for preparation of RT mastermix

COMPONENT	VOLUME (µl) PER REACTION KIT
10 X RT Buffer	2.0
25 X dNTP Mix (100mM)	0.8
10 X RT Random Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease-free H_2O	3.2

Table 2.8: Procedure for thermal cycling conditions

	STEP 1	STEP 2	STEP 3	STEP 4
Temperature (°C)	25	37	85	4
Time (min)	10	120 (2x 60 min)	5	∞

2.7.6 Real Time PCR

The TaqMan® Fast Universal PCR Master Mix (2X), No AmpErase® UNG (PN 4352042, Applied Biosystems, Foster City, USA) was used to run TaqMan® gene expression assays on the Applied Biosystems 7500 Fast Real-Time PCR System for 40min. The plate document was prepared and set-up using the 7500 Software version 2.0.1 ABI SDS program. The real-time PCR reaction mixture for the Applied Biosystems 7500 Fast Real-Time PCR System was aligned for 20µl. The thermal cycling conditions for the real time PCR reaction was set at the following temperatures and time periods viz., 95°C: 0:20min; 60°C: 0:30min and 95°C: 0:03min.

2.7.6.1 Genes utilised

Taqman PCR inventoried gene expression assays (Applied Biosystems, Foster City, USA) were used for this study. The following genes were used:

- Flt-1 and sFlt-1 assay id Hs01052962_g1;
- sEng assay id: Hs00164438_m1;
- VEGF assay id: Hs00173626_m1;
- PlGF assay id: Hs01119262_m1; and
- 18S ribosomal RNA (Applied Biosystem, Foster City, USA).

The reaction plates also included 18S rRNA primers and probes. The relative expression level of the gene of interest was calculated as a ratio with respect to the 18S rRNA to standardise for any quantity of input cDNA.

The PCR reaction mix (**Table 2.9**) component for each target gene was prepared in RNase free thin walled frosted lid PCR tubes (AM12225, Applied Biosystems, Foster City, USA) according to the manufacturer's instructions. This was followed by transferring 16µl of the PCR reaction mix into a sterile microamp optical 96 well 0.1ml fast reaction plate (4346906, Applied Biosystems, Foster City, USA). Single stranded cDNA samples (100ng) were diluted (1:50) and 4µl were then pipetted onto reaction plates.

Table 2.9: Procedure for reparation of PCR reaction mix

RTPCR REACTION MIX COMPONENT	**VOLUME PER 20µl REACTION**
20X Taqman Gene Expression assay	1
2X Taqman Fast Universal PCR Master Mix	10
RNase free H_20	5
Total volume (µl)	16

The PCR plates were then sealed with microamp optical adhesive films and briefly centrifuged at 10 000rpm for 2min at 4°C. Sealed plates were then loaded on the ABI 7500 Fast Real-Time PCR System. Once the reaction cycle was completed, the data from gene expression was viewed on the amplification plots, baseline and threshold values were set and analysed. The PCR reaction plates were then removed from the machine and the PCR product specificity was evaluated by 1.5% agarose gel using 5µl from each reaction. The relative concentration of each gene for each sample was calculated as the gene concentration/the housekeeping gene (18sRNA)

2.7.7 Statistical analysis

A two-way ANOVA was done to examine the effect of HIV status on the expression of sFlt-1, sEng, PlGF and VEGF on HIV-associated pregnancy. All data were assessed for their distribution by the Shapiro-Wilk test. The homogeneity of variance between groups was assessed by the Levene's test for equality of error variances. The Bonferroni post-hoc test was done for multiple comparisons. Descriptive statistics were utilised and outcome variables are presented as median (inter-quartile range) for non parametric data. A probability level of $p<0.05$ was considered statistically significant. All statistical analyses were conducted using GraphPad Prism® version 5.01 and IBM SPSS Statistics version 20.

2.8 Quantisation of serum pro- and anti-angiogenic factors using enzyme-linked immunosorbent assay (ELISA) techniques

2.8.1 Collection of serum

Maternal blood samples were collected antenatally in plain gel tubes and centrifuged at 3500rpm for 10min at 4°C. The serum was then carefully aliquotted into labelled cryotubes and stored at -70°C until further investigation.

2.8.2 The principle of enzyme-linked immunosorbent assay (ELISA) techniques

The quantikine immunoassay kit (R&D Systems, Minneapolis, USA) was employed to estimate the levels of all cytokines under study. This kit utilised the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for sVEGFR-1/sFlt-1, and polyclonal antibodies specific for sEng, TGF-β_1, VEGF and PlGF was pre-coated onto microplates. Standards and samples are pipetted into the wells and any analyte present was bound by the immobilised antibody.

Following washing of unbound substances, an enzyme-linked polyclonal antibody specific for the growth factors under study was added to the wells. This was followed by subsequent washes to eliminate any unbound antibody-enzyme reagents. Substrate solution was then added to the wells and a colour developed in proportion to the amount of analyte bound in the initial step. The colour development was stopped and the intensity of the colour was measured using an ELISA reader. The microplate was read at the appropriate wavelength (450nm) with a reference filter of 650nm within 30min.

2.8.3 Procedure for the estimation of VEGF, PlGF, TGF-β_1, sVEGFR-1 and sEng

The immunoassays for VEGF, PlGF, TGF-β_1, sVEGFR-1/sFlt-1, and sEng were performed in triplicate with the use of commercial kits as previously described (Maynard *et al*., 2003) (R&D Systems, Minneapolis, USA). ELISA for human VEGF and PlGF assays detect free, but not bound, forms of the growth factors. The lowest standard concentrations of the VEGF and PlGF kits were 31.2pg/ml and 15.6pg/ml respectively. Results of the assays are given in pg/ml for serum sFlt-1, PlGF, VEGF and TGF-β_1 and in ng/ml for sEng.

Serum was collected for protein appraisal for the following antibodies conjugated to horseradish peroxidise:

- Human VEGF polyclonal antibody (DVE00, R&D Systems, Minneapolis, USA);
- Human PlGF polyclonal antibody (DPG00, R&D Systems, Minneapolis, USA;
- Human TGF-β_1 polyclonal antibody (DB100B, R&D Systems, Minneapolis, USA);
- Human endoglin mouse monoclonal antibody (DNDG00, R&D Systems, Minneapolis, USA);
- Human sVEGF R-1/Flt-1 polyclonal antibody (DVR100B, R&D Systems, Minneapolis, USA).

Estimation of all serum samples for VEGF, PlGF, TGF-β1, sVEGFR-1/sFlt-1, and sEng were diluted in their respective calibrator diluents, viz., VEGF (1:2), PlGF (1:2), TGF-β_1 (1:40), sVEGFR-1/sFlt-1 (1:10) and sEng (1:5). Centrifugal filter units (Amicon Ultra-0.5, Millipore, Ireland) were only utilised for concentration, ultra-purification and recovery of serum VEGF levels.

All serum samples were incubated in a 96-well plate pre-coated with a capture antibody directed against VEGF, PlGF, TGF-β_1, sVEGFR-1/sFlt-1 and sEng for 2hrs at room temperature (**Fig. 2.3**). The wells were then washed three times in wash buffer (0.05%Tween 20/PBS) and incubated with the respective secondary antibodies against VEGF, PlGF, TGF-β_1, sVEGFR-1 and sEng conjugated to horseradish peroxidase for 2 hrs at room temperature. The plates were then washed three times and a substrate solution containing H_2O_2 and tetramethylbenzidine (50µl) was then added. Optical density was determined within 30min at 450nm and a reference filter at 595nm using the BioRad microplate reader (Model 3550, BioRad, UK). The protein levels were calculated using a standard curve derived from known concentrations of the respective recombinant proteins.

2.8.4 Analyses

The amount of substrate hydrolysed was determined colorimetrically by measuring the absorbance at 450nm. The mean of triplicate determinations was calculated and a standard curve was plotted. By interpolation of the sample values, the concentrations of the extracted samples of all cytokines under study were determined from the reference curve.

2.8.5 Statistical Analysis

A two-way ANOVA was done to examine the effect of HIV status on the expression of maternal circulating levels of sFlt-1, sEng, PlGF, VEGF and TGF-$\beta_1$in HIV-associated pregnancy. All data were assessed for their distribution by the Shapiro-Wilk test. Additionally, the homogeneity of variance between groups was assessed by the Levene's test for equality of error variances. Bonferroni post-hoc subgroup analyses were done for multiple

comparisons. Descriptive statistics were utilised and outcome variables are presented as means and SD for parametric data or median (inter-quartile range) for non parametric data. A probability level of $p<0.05$ was considered statistically significant. All statistical analyses were conducted using GraphPad Prism® version 5.01 and IBM SPSS Statistics version 20.

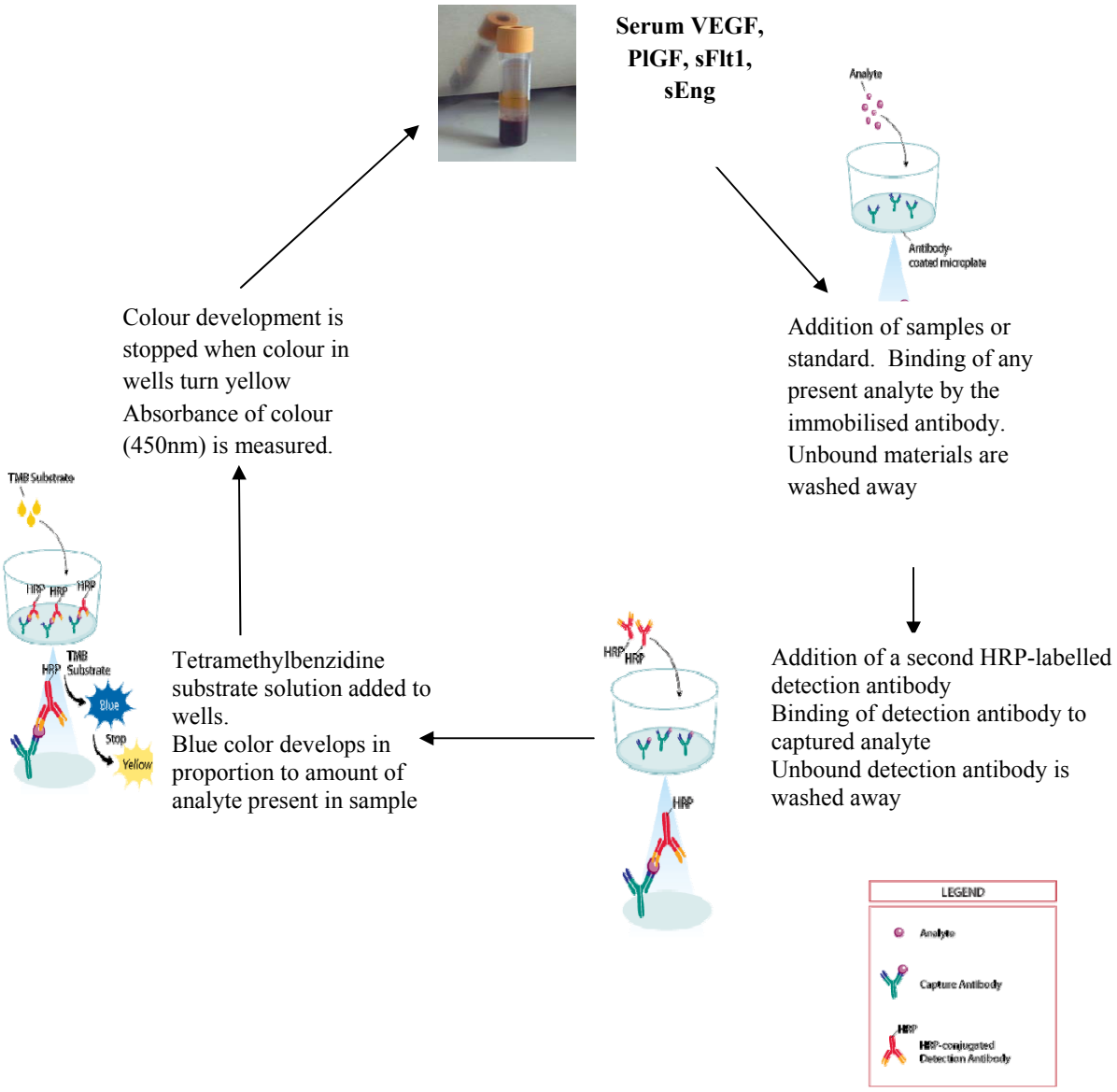


Figure 2.3: Schematic representation of ELISA technique

(R & D Systems, Inc, 2012, www.rndsystems.com).

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CHAPTER 3

Results

3.1 Clinical characteristics

The maternal demographics of the study population are shown in **Table 3.1**. Maternal age ranged between 24-32 years whilst gestational age ranged between 36-40 weeks (**Table 3.1**). A significant effect of type of pregnancy (normotensive and pre-eclamptic) on maternal (p=0.008) and gestational (p=0.000) age across all 4 groups was noted (p<0.05; **Table 3.1**). Bonferroni post hoc analyses of the effect of type of pregnancy (normotensive and pre-eclamptics) on gestational age showed a significant effect between N+ (39±1.45) vs P+ (37±2.56, p=0.000). Preterm delivery (<37 weeks gestation) occurred in both the P+ and P- groups. However, there was no significant effect of HIV status on type of pregnancy for maternal age [F(1.123)=0.127, p=0.722] or gestational age [F(1.123)=2.48, p=0.117].

In addition, a significant effect of type of pregnancy (normotensive and pre-eclamptic) on both systolic (p=0.000) and diastolic (p=0.000) blood pressures was shown across all study groups (**Table 3.1**). Bonferroni post hoc analyses revealed a significant effect of type of pregnancy (normotensive and pre-eclamptics) on systolic pressure between the N- (114.84 ± 10.66 mmHg) *vs* P- (156.63 ± 13.91 mmHg) and N+ (113.45 ± 10.55 mmHg) *vs* P+ (152.6 ± 9.03 mmHg). A similar pattern was observed for diastolic blood pressure (**Table 3.1**). Irrespective of HIV status, blood pressure was higher in the pre-eclamptic compared to the normotensive pregnancies [N- vs P- (p< 0.0001) and the N+ vs P+ (p< 0.0001)]. There was however no significant effect of HIV status on type of pregnancy for systolic [F(1.123)=0.428, p=0.514] or diastolic [F(1.123)=0.175, p=0.676] blood pressure.

Similarly, there was a significant effect of type of pregnancy (normotensive and pre-eclamptic) on placental weight (p=0.001) and on parity (p=0.003), across all 4 groups (p<0.05; **Table 3.1**). Bonferroni post hoc tests of the effect pregnancy (normotensive and pre-eclamptic) on placental weight revealed a significant difference between N- vs P- (p=0.000) and N+ vs P+ (p=0.000). However, there was a significant effect of HIV status on type of pregnancy for placental weight [F(1.118)=10.32, p=0.002]. The pregnancies complicated by pre-eclampsia have larger placentas compared to normotensive pregnancies (p=0.001).

The HIV positive pre-eclamptic cohort showed an elevation of CD4 counts compared to the HIV positive normotensives (Mann-Whitney test, p=0.035; **Table 3.1**).

Table 3.1: Clinical profile of patients

	Normotensive Pregnant Women		**Pre-eclamptic Women**		
	HIV Negative (N-)	**HIV Positive (N+)**	**HIV Negative(P-)**	**HIV Positive(P+)**	**P value**
n	36	40	30	24	
Systolic BP (mmHg)	114.84±10.66	113.45±10.55	156.63±13.91	152.6±9.03	*p*=0.000
Diastolic BP (mmHg)	70.59±6.85	70±6.78	94.37±12.75	92±9.67	*p*=0.000*
Maternal age (years)	24±5.2	27±4.7	26±6.9	30±6	*p*=0.008
Gestation(wks)	39±1.04	39±1.4	38±2.95	37±2.5	*p*=0.000
***Parity**	0(0-1)	1(1-2)	1(0-1)	1(1-3)	*p*=0.003
Placental weight (g)	359±64	455±98	461±80.5	459±85	*p*=0.000
#CD 4 counts	-	274(160-331)	-	350(250-449)	p=0.035*

significance p<0.05; n=130; Summary statistics shown as mean ± SD,

* median + inter-quartile range; #Mann-Whitney U test was used for statistical analyses

3.2 Histological assessment of placental villi

Qualitative histological analyses of H&E stained sections of placental tissue across all study groups confirmed the presence of four different types of villi viz., stem, intermediate (immature and mature) and terminal villi. Additionally, amniotic epithelia (**Fig. 3.1a**), chorion (**Fig. 3.1b**), basal plate with extravillous trophoblasts cells (**Fig. 3.1c-d**), anchoring villi (**Fig. 3.1c**) and septa were observed. Also, deposition of Langhans and Rohr fibrinoids at the chorionic and basal plate was observed, respectively (**Fig. 3.1a-d**).

Structurally, the villi lying within the intervillous space were covered by a multi-nucleated syncytiotrophoblast epithelial layer (**Fig. 3.2a-d**). This syncytiotrophoblast layer was interrupted by syncytial bridges, knots and fibrin deposition (**Fig. 3.2e**). Beneath this surface layer, intermittent cytotrophoblast cells were present (**Fig. 3.2f**). The syncytiotrophoblastic and cytotrophoblastic layer was separated from the stromal region by a basement membrane. The stromal core comprised fetal blood vessels, connective tissue cells, fibres and ground substance.

Stem villi were identified by arteries and veins lying within a dense connective tissue stroma (**Fig. 3.2a**). The arteries displayed typical tunica adventitia, media and endothelial cells lining the lumen.

Immature intermediate villi were identified by a reticular-like stroma, with occasional cytotrophoblasts below the syncytiotrophoblast region (**Fig. 3.2b**). Mature intermediate villi were identified according to their characteristic dilated blood vessels (sinusoids and capillaries) (**Fig. 3.2c**). The stromal core consisted of loose collagen fibers and connective tissue cells.

Terminal villi were identified by their large dilated sinusoids and fetal capillaries (**Fig. 3.2d**) within a stromal core containing various connective tissue cells.

Qualitative histopathological anomalies observed across all study groups is summarised below:

- Syncytial knots were observed by the focal accretion of nuclei from the syncytium into the intervillous space (**Fig. 3.3a-d**). The frequency of this knotting was more pronounced in the pre-eclamptic compared to the normotensive groups.

- Perivillous fibrinoid deposition was observed (**Figs. 3.3b and 3.4a**) occurring more frequently in the pre-eclamptic compared to the normotensive groups.

- Intravillous fibrinoid was noted. Total occlusion of the intravillous mesenchyme by fibrinoid deposition forming avascular villi was observed across all groups (**Figs. 3.3b, c and 3.4b**).

- The occurrence of cytotrophoblasts was prominent in the pre-eclamptic placenta (**Fig. 3.4c**).

- Large immature intermediate villi were prominent in pre-eclampsia (**Fig. 3.4d**). In addition, a thin vasculosyncytial membrane devoid of nuclei was noted due to bulging of fetal blood vessels against the syncytiotrophoblast surface.

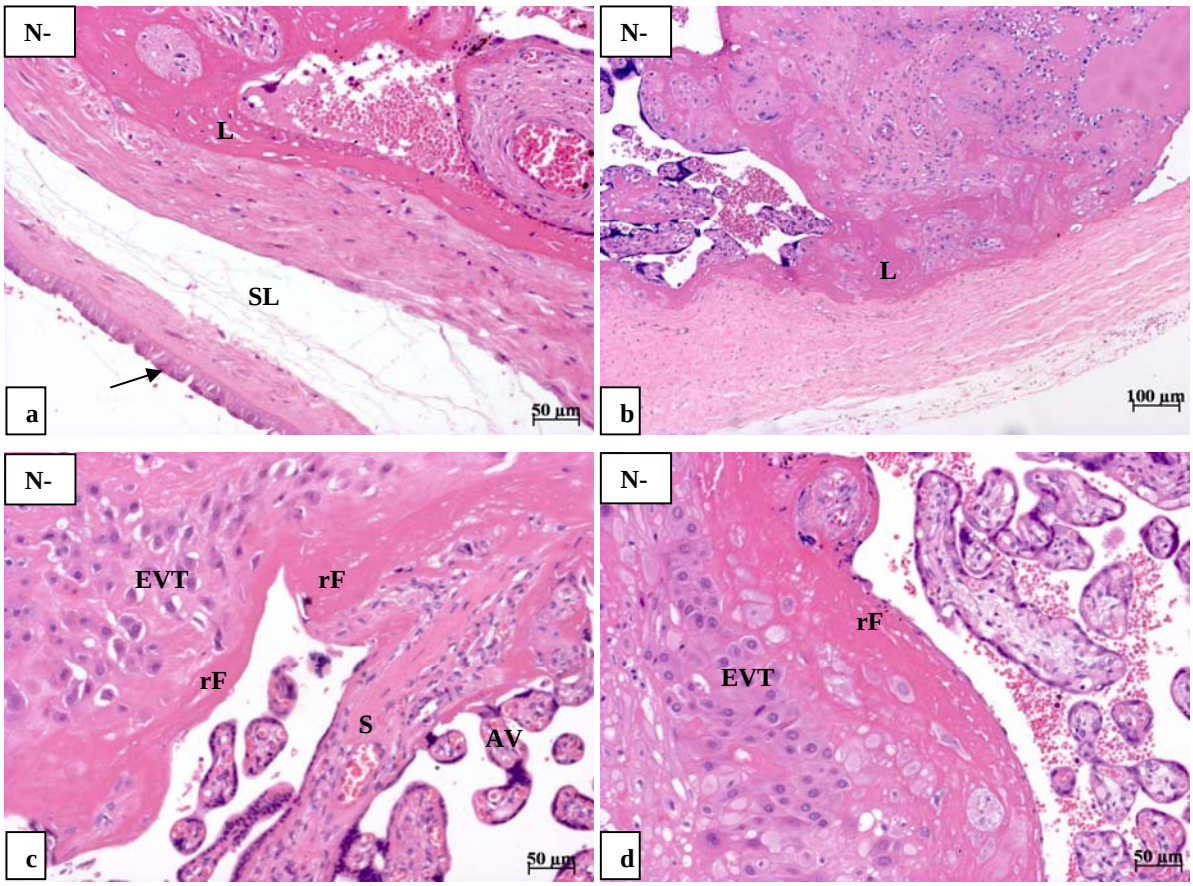

Figure 3.1: Light micrographs depicting (a) amnion (arrow); (b) chorion and (c-d) extravillous trophoblasts (EVT) within fibrinoid of basal plate within N-groups. Note spongy layer (SL), septum (S) and anchoring villi (AV). Note Langhans (L) and Rohr fibrinoid (rF).

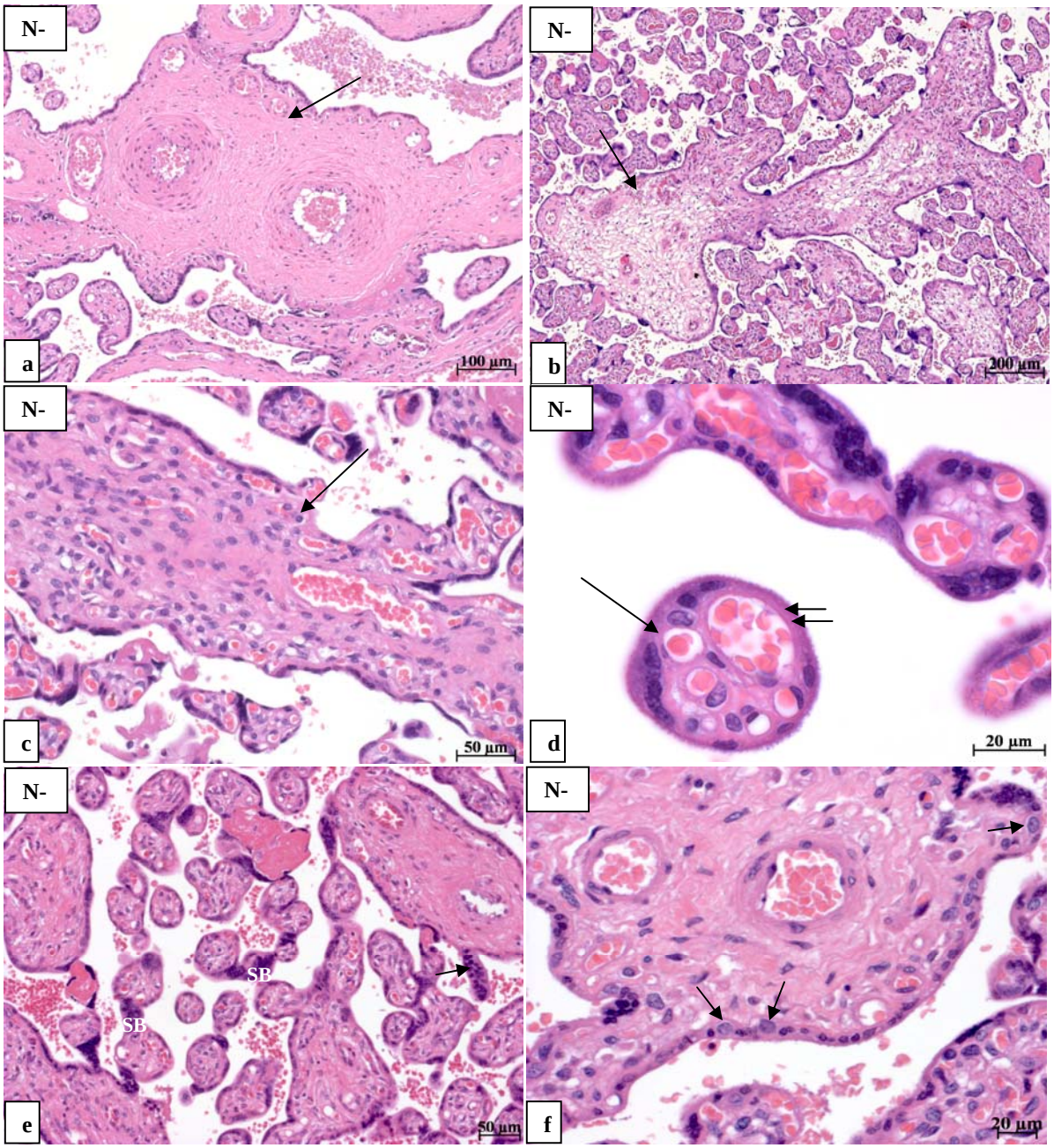

Figure 3.2: Light micrographs depicting (a) stem villus (arrow); (b) immature intermediate villus (arrow); (c) mature intermediate villus (arrow); (d) terminal villus (arrow); (e) syncytial knots (arrow) and syncytial bridges (SB) and (f) cytotrophoblasts (arrow) within the N- groups. Note vasculosyncytial membrane (double arrow).

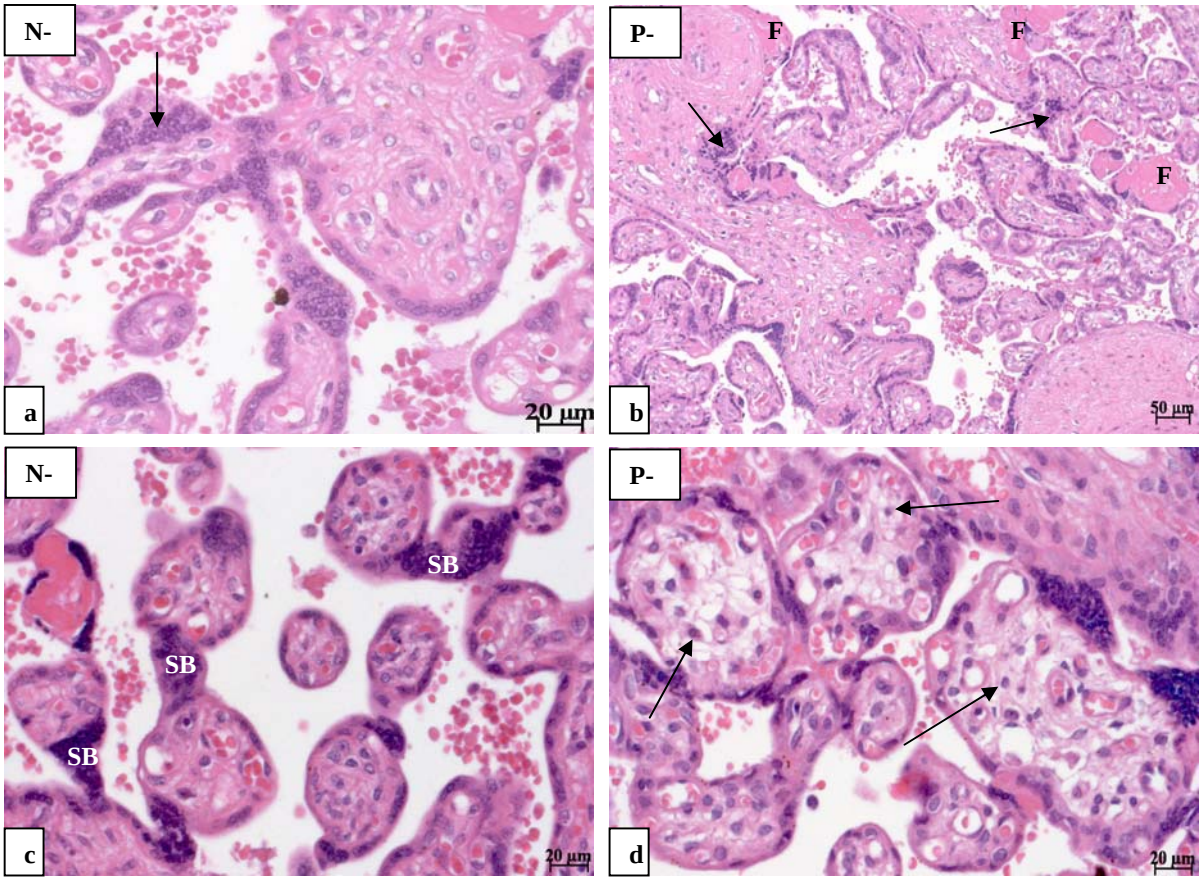

Figure 3.3: Light micrographs depicting (a-d) syncytial knotting (arrows) and syncytial bridges (SB) within (a,c) N- and (b,d) P- groups. Note perivillous and intravillous fibrin (F) and immature intermediate villi (arrow).

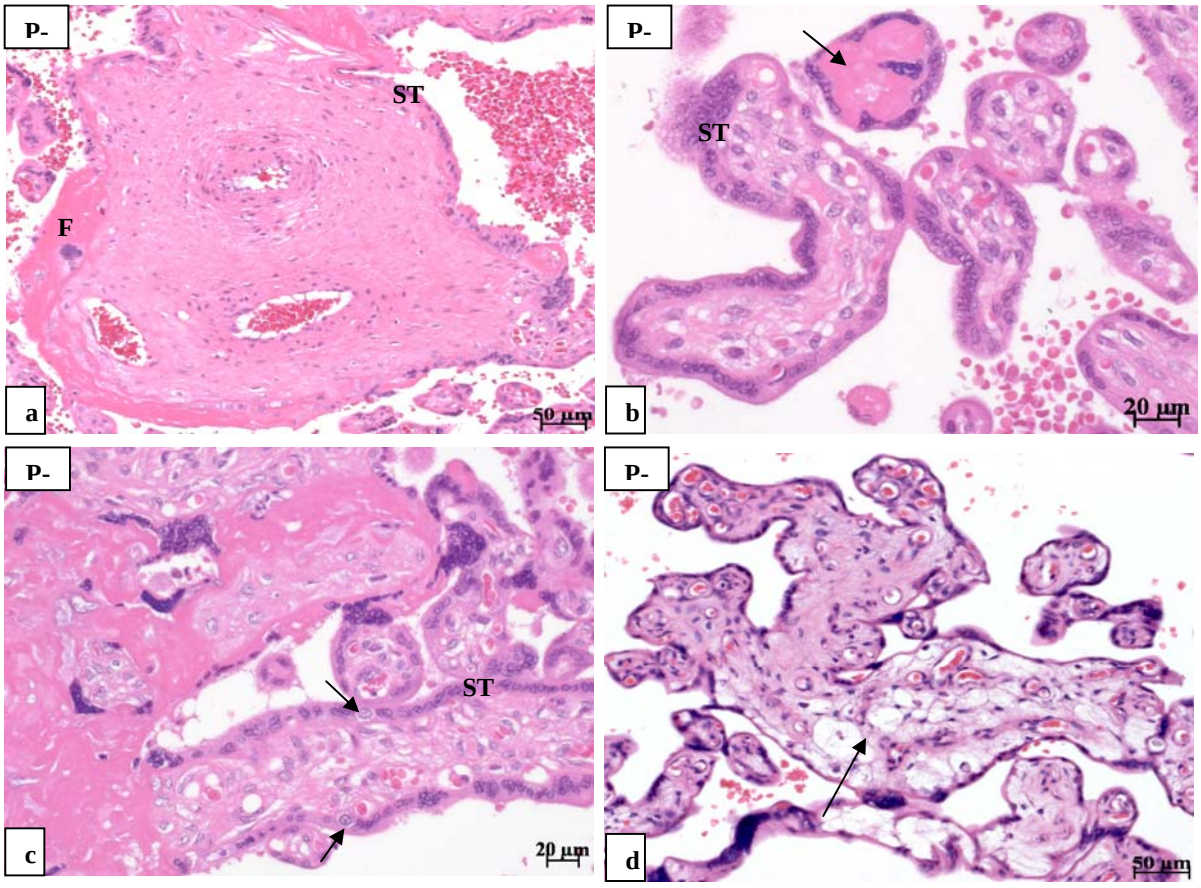

Figure 3.4: Light micrographs depicting (a) perivillous fibrin (F) around stem villi; (b) total obliteration of terminal villi and stroma with intravillous fibrin (arrow); (c) cytotrophoblast proliferation (arrows) and (d) immature intermediate villi within the P- groups. Note syncytiotrophoblast layer (ST).

3.3 Immunohistochemical localisation of antibody expression

3.3.1 sVEGF-R1/sFlt-1

3.3.1.1 Immunolocalisation

The distribution of sFlt-1 immunoreactivity within conducting and exchange villi across all study groups (N-, N+, P- and P+) was noted. The syncytiotrophoblast layer expressed variable immunoreactivity for sFlt-1 across all groups (**Figs. 3.5-3.10**). In addition, strong immunoreactivity was located within the syncytial knots (**Figs. 3.5c, 3.6c, 3.7-9**). The mesenchymal tissue of stromal core displayed weak-absent immunoprecipitation. Additionally, the endothelial cells lining the vessels in the stem villi were immuno-positive across all study groups (**Figs. 3.5a-d; 3.6a-d**).

Assessment of sFlt-1 within syncytiotrophoblast of exchange villi showed mild to strong immunoreactivity across all groups (**Fig. 3.7-3.9**). However, the immunoreactivity of endothelial cells lining the dilated fetal blood vessels within exchange villi displayed variable immunostaining across the N- and P- (**Fig. 3.8**) and N+ and P+ (**Fig. 3.9**). Strong sFlt-1 immunoexpression of endothelial cells (**Figs. 3.8a,c; 3.9a,d**) and weak-absent immunoexpression (**Figs. 3.8b,d; 3.9b,e**) were noted. The syncytial brush border and perivillous fibrinoid was also immunopositive for sFlt-1. Stromal core displayed weak-absent immunoreactive labelling. Variable immunostaining of cytotrophoblasts was observed (**Fig. 3.9e-f**). The extravillous trophoblasts cells were positive for sFlt-1 (**Fig. 3.10a-d**).

The negative control in which the primary antibody was replaced with PBS was non-reactive (**Fig. 3.10e**). Replacement of the primary antibody with non-immune sera of the same IgG class as the primary produced no immunoreactivity. The positive control (placenta) was immunopositive (**Fig. 3.10f**).

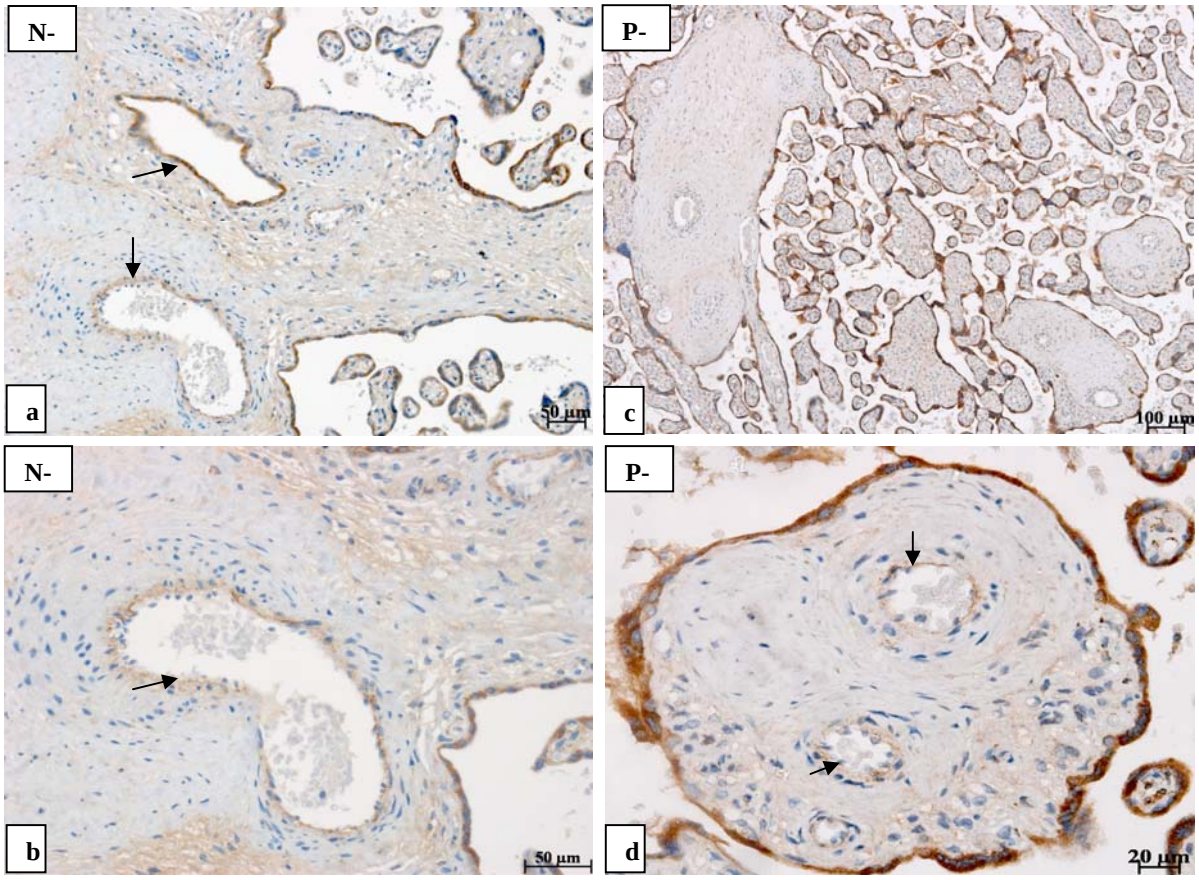

Figure 3.5: Light micrographs depicting immunoreactivity of sFlt-1 within (a-d) stem villi within N- (a,b) and P- (c,d) groups. Note endothelial cells (arrows).

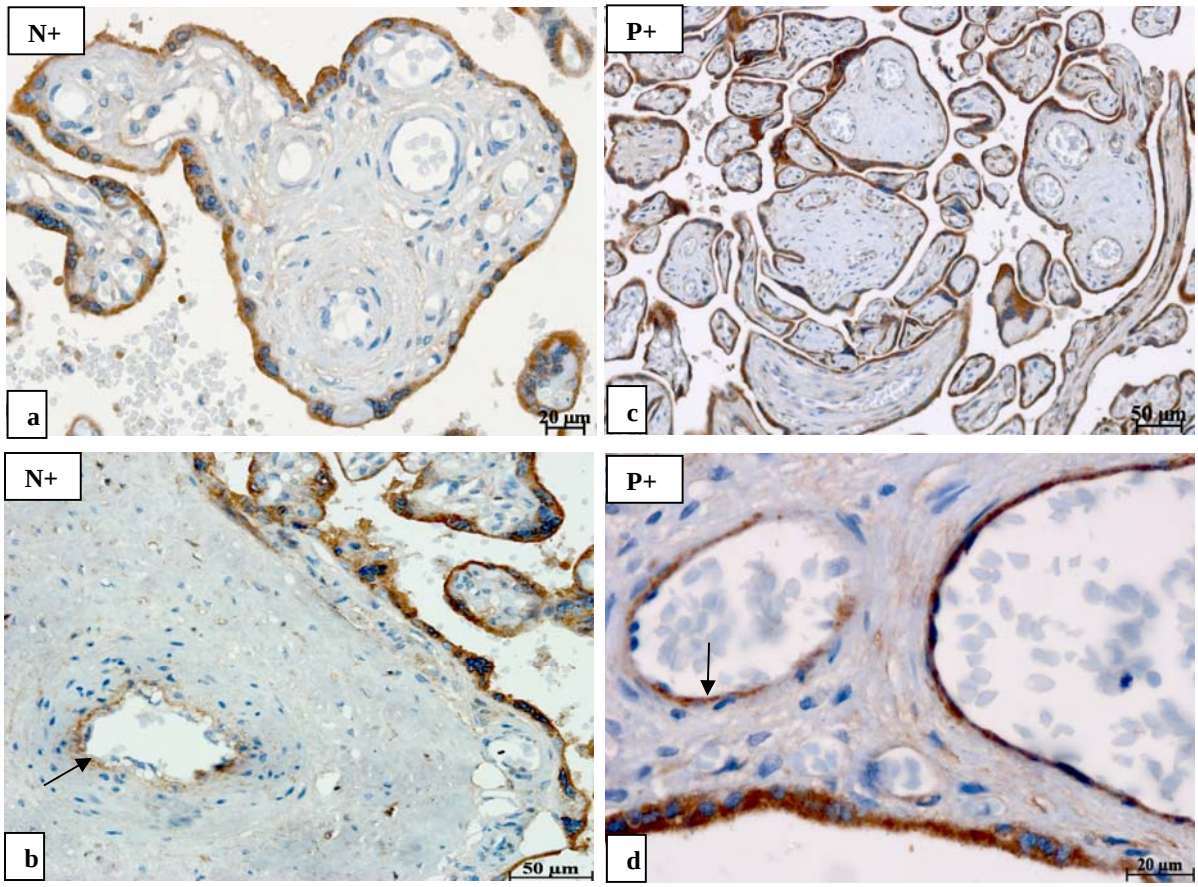

Figure 3.6: Light micrographs depicting immunoreactivity of sFlt-1 within (a-d) stem villi within N+ (a,b) and P+ (c,d) groups. Note endothelial cells (arrows).

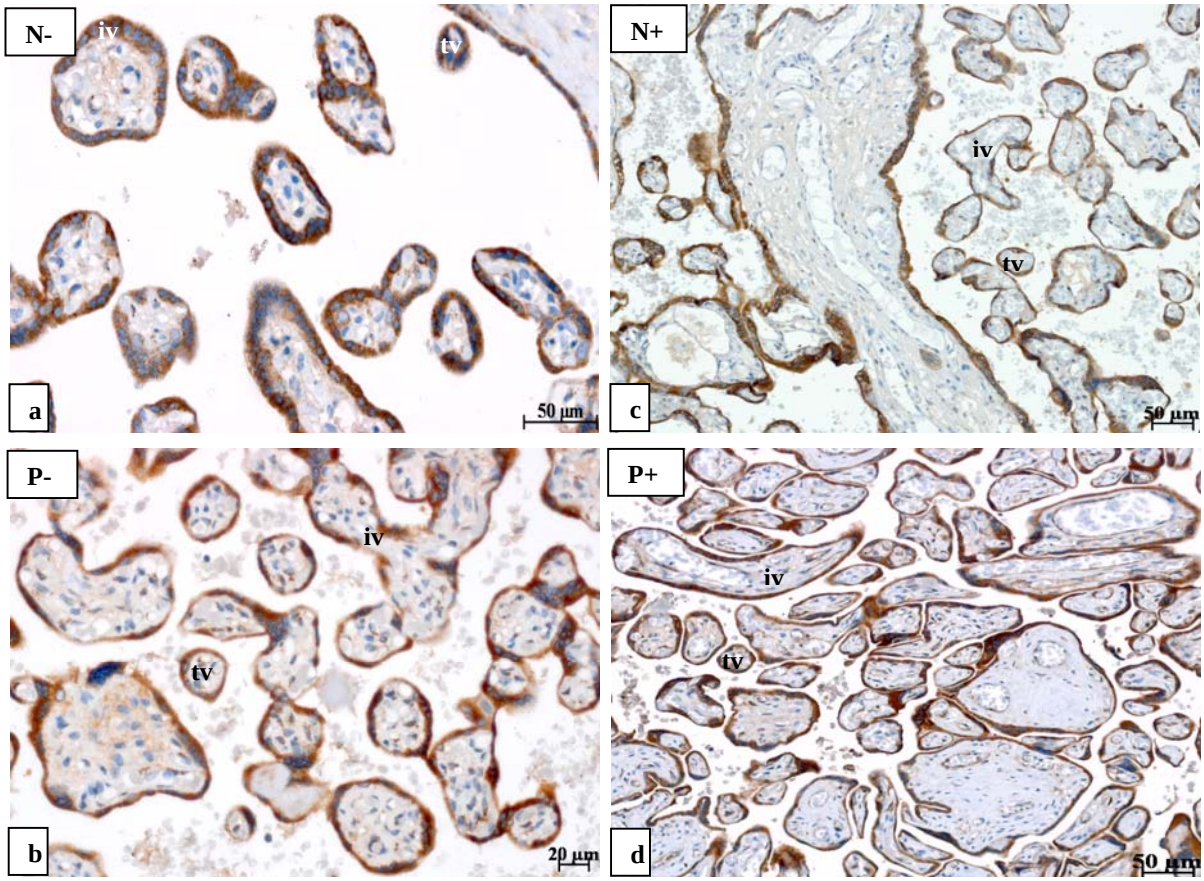

Figure 3.7: Light micrographs depicting immunoreactivity of sFlt-1 within (a-d) exchange vessels within N- (a), P- (b), N+ (c) and P+ (d) groups. Note intermediate (iv) and terminal villi (tv).

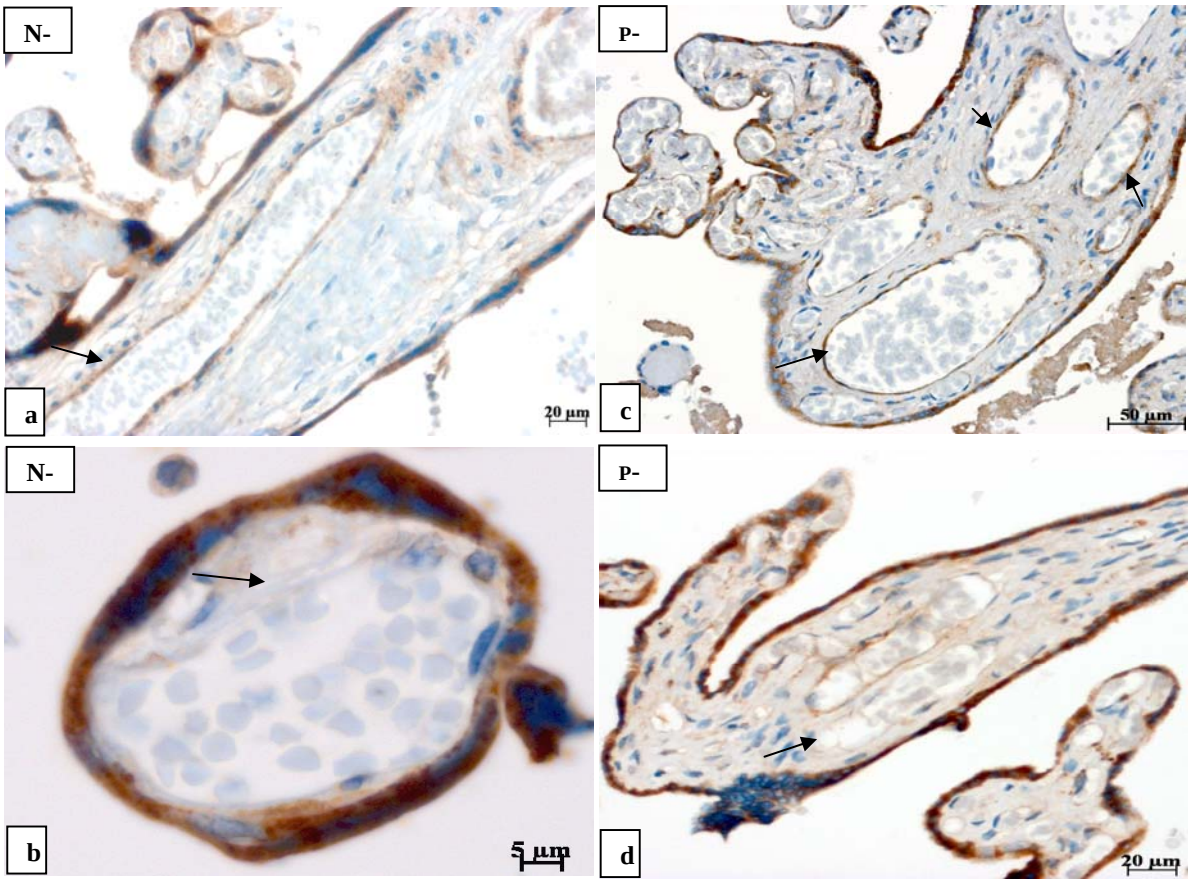

Figure 3.8: Light micrographs depicting (a, c) positive and (b, d) negative immunoreactivity of sFlt-1 within fetal endothelial cells in N- (a,b) and P- (c,d) groups.

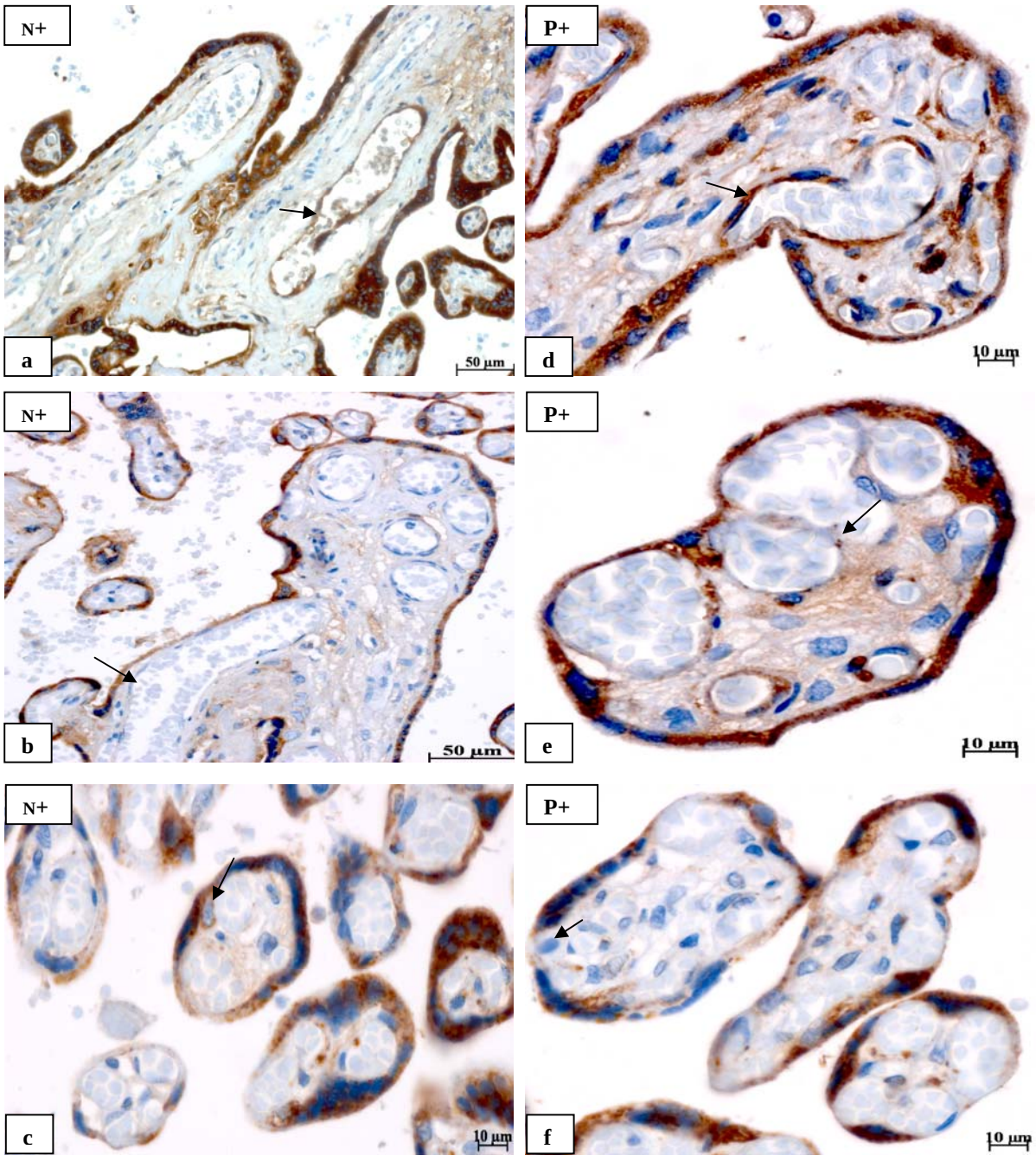

Figure 3.9: Light micrographs depicting (a, c, d) positive and (b, e, f) negative immunoreactivity of sFlt-1 within fetal vessels and cytotrophoblasts respectively within N+ (a-c) and P+ (d-f) groups.

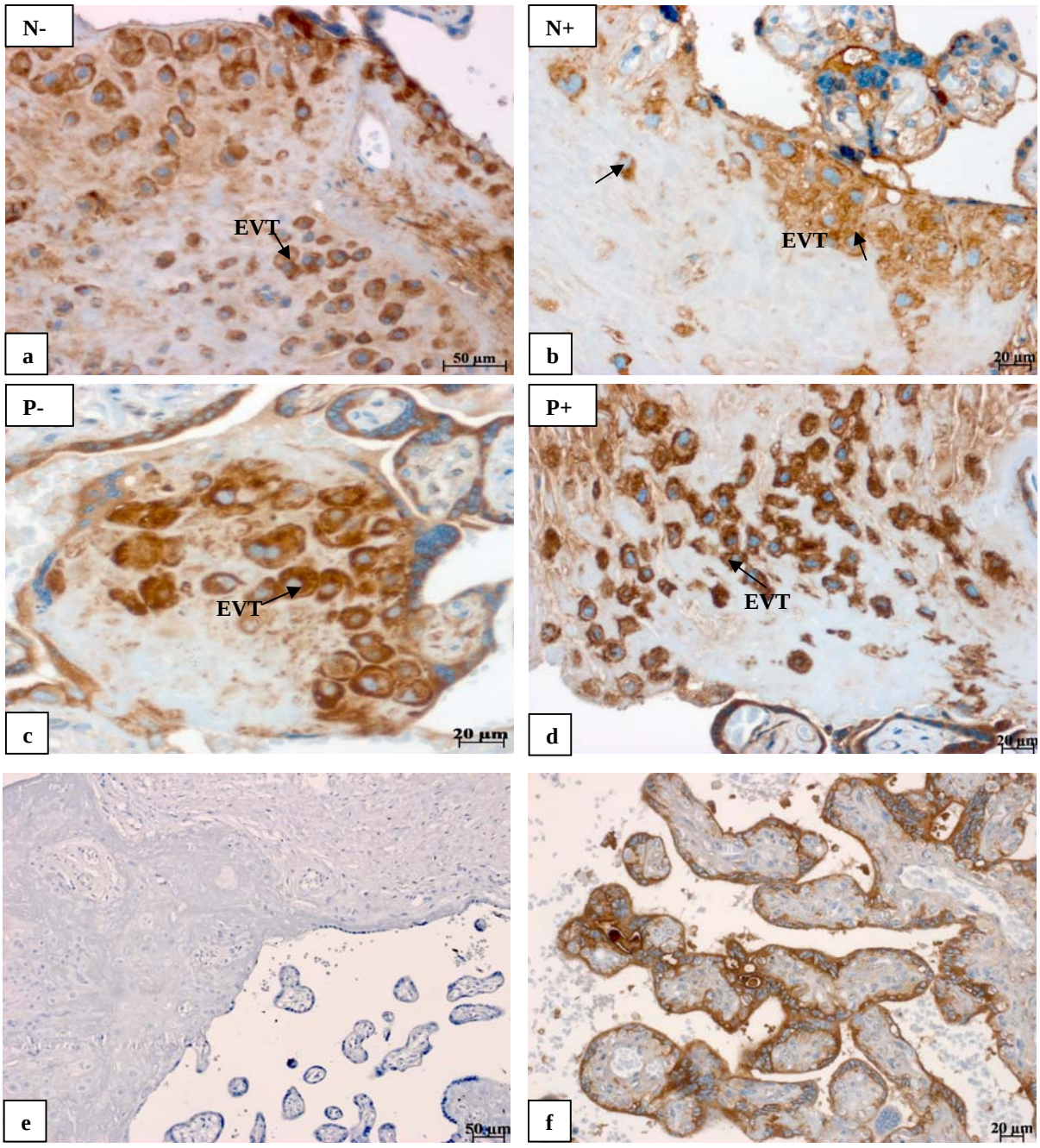

Figure 3.10: Light micrographs depicting immunoreactivity of sFlt-1 within (a-d) extravillous trophoblast cells (arrows EVT) within N-(a), P- (b), N+ (c) and P+ (d) groups; (e) negative control and (f) positive control.

3.3.1.2 Morphometric image analysis of sFlt-1 within placental tissue

3.3.1.2.1 Two-way Analyses of Variance

i. Exchange villi (intermediate and terminal villi)

Quantitative image analysis of sFlt-1 immunoreactivity based on the intensity within the intermediate and terminal villi populations across all groups are shown (**Table 3.2; Fig. 3.11).** The mean area percentage occupied by exchange villi within the N-, N+, P- and P+ groups was 49.57±7.01%; 42.08±9.3%; 46.92±10.22% and 45.98±9.35% respectively. Consequently, the mean percentage area of the intervillous space within the N-, N+, P- and P+ groups were 50%; 58%; 53% and 55% respectively.

There was no significant effect of HIV status on the immunoexpression (field area percent of the chromogen) of sFlt-1 within exchange villi [$F(1,123)=0.39$, $p=0.53$] across all groups. However, a significant effect of type of pregnancy (normotensive and pre-eclamptic) on sFlt-1 immunoexpression within exchange villi [$F(1,123)=9.199$, $p=0.003$] was noted, indicating .that pre-eclamptic have higher sFlt-1 immunoreactivity (mean=15.949, 95% CI: 14.830-17.069,) compared to the normotensive pregnant (mean=13.691, 95% CI: 12.733-14.649, $p=0.003$) pregnancies. Bonferroni post hoc analyses revealed a significant effect of sFlt-1 immunoexpression within exchange villi between N- *vs* P- ($p=0.003$) groups (**Table 3.2, Fig. 3.11**).

The mean intensity of immunoreactivity (chromogen) within the N-, N+, P- and P+ groups expressed as a densitometric grey value was 13(10-16); 14(11-17); 16(14-18) and 16(13-17) respectively, with a range varying between 12 (8-13) to 88 (77-99).

Finally, there was no significant effect between HIV status and type of pregnancy (normotensive and pre-eclamptic) on sFlt-1 immunoexpression within exchange villi [$F(1,123)=2.76$, $p=0.09$].

ii. Conducting villi (stem villi)

Quantitative image analyses of sFlt-1immunoreactivity within the stem villi across all groups are shown (**Table 3.3 and Fig. 3.12).** There was no significant effect of HIV status on the immunoexpression of sFlt-1 within stem villi [$F(1,123)=3.866$, $p=0.052$] across all groups. Likewise, no significant effect of type of pregnancy (normotensive and pre-eclamptic) on sFlt-1 immunoexpression was noted within stem villi [$F(1,123)=0.02$, $p=0.89$].

Despite the lack of significance, the mean intensity of sFlt-1 immunoreactivity across the N-, N+, P- and P+ groups expressed as a densitometric grey value was 14.2 (12-18.6); 13.9 (11.5-19.3); 16.2 (13.8-19.9) and 13.9 (10.5-16) respectively.

Finally, no interaction was noted between HIV and type of pregnancy on sFlt-1 immunoexpression within stem villi [$F(1,123)=2.24$, $p=0.121$].

3.3.1.2.2 Correlation of sFlt-1 immunoexpression between exchange and stem villi

A Pearson correlation coefficient was computed to assess the relationship between exchange and stem villi immunoexpression of sFlt-1 across all study groups. There was no correlation between exchange and stem villi immunoexpression of sFlt-1 ($r=0.052$, $p=0.563$).

Table 3.2: Morphometric image analysis of sFlt-1 immunoexpression in exchange villi

	Normotensive Pregnant Women		Pre-eclamptic Women		
	HIV Negative (N-)	**HIV Positive (N+)**	**HIV Negative (P-)**	**HIV Positive (P+)**	**P value**
n	36	40	30	24	
Area Frame µm^2	312843.68	312843.68	312843.68	312843.68	
Field Area of villi %	49.57±7.01	42.08±9.3	46.92±10.22	45.98±9.35	*p*=0.005
Immunoreactivity %	12.84±4.5	14.54(±4.72)	16.34(±3.32)	14.94(±4.2)	*p*=0.003
Intensity of immunoreactivity (median and interquartile range)					
Mean	56(46-64)	36(31-42)	39(29-45)	37(28-44)	
Min	24(15-30)	12(10-17)	12(8-13)	12(8-16)	
Max	88(77-99)	64(55-77)	60(54-73)	58(50-65)	

Significance:*p<0.05; n=130; parametric-data (mean ± SD)
Densitometry (grayscale 0-255)

Table 3.3: Morphometric image analysis of sFlt-1 immunoexpression in stem villi

	Normotensive Pregnant Women		Pre-eclamptic Women		
	HIV Negative (N-)	**HIV Positive (N+)**	**HIV Negative (P-)**	**HIV Positive (P+)**	**P value**
n	36	40	30	24	
Immunoreactivity %	15.78±4.58	15.29±5.41	17.01±5.26	13.95±3.66	*p*=0.89
Intensity of Immunoreactivity (median and interquartile range)					
Mean	91(66-104)	52(44-56)	52(43-61)	49(40-60)	
Min	27(22-33)	18(15-21)	17(11-21)	18(11-29)	
Max	121(98-136)	75(68-80)	76(63-81)	71(65-78)	

Significance:*p<0.05; n=130; parametric-data (mean ± SD) Densitometry (grayscale 0-255)

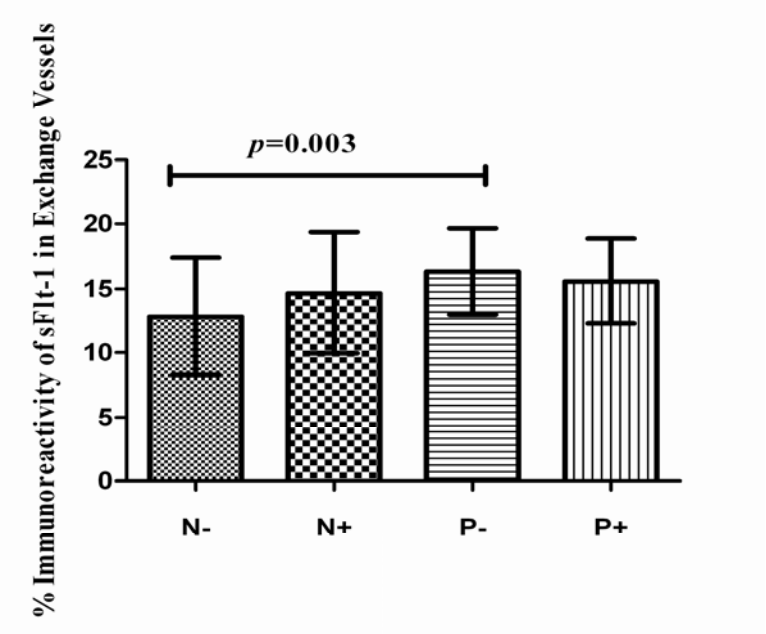

Figure 3.11: Percentage immunoreactivity of sFlt-1 in exchange villi (mean ± SD) in HIV positive pre-eclamptic (P+); HIV negative pre-eclamptic (P-); HIV negative normotensive pregnant (N-) and HIV positive pregnant women (N+).

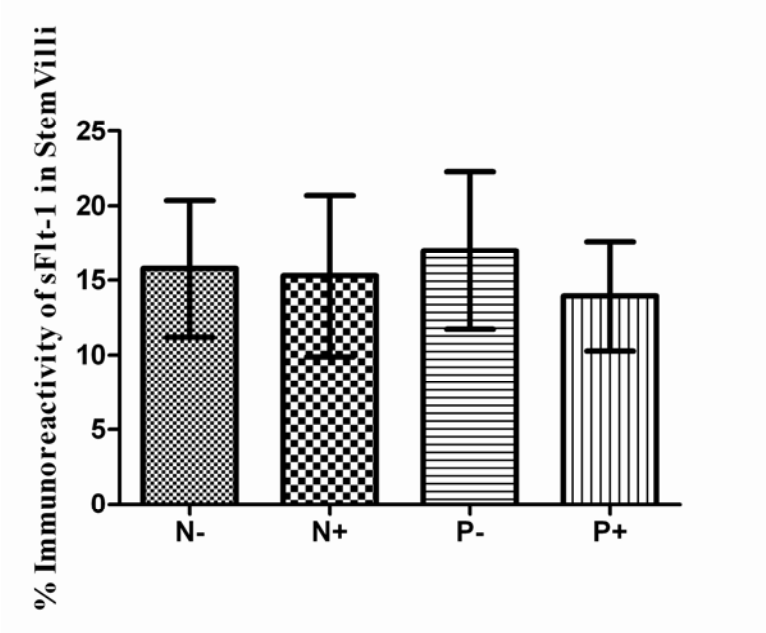

Figure 3.12: Percentage immunoreactivity of sFlt-1 in stem villi (mean± SD) within HIV positive pre-eclamptic (P+); HIV negative pre-eclamptic (P-); HIV negative normotensive pregnant (N-) and HIV positive pregnant women (N+).

3.3.2 sEndoglin (sEng)

3.3.2.1 Immunolocalisation

The immunolocalisation of sEng within conducting and exchange villi across all study groups (N-, N+, P- and P+) was variable. The syncytiotrophoblast layer showed mild-strong immunoreactivity (**Figs. 3.13-3.17)**. Its brushborder displayed pronounced immunoreactivity (**Fig. 3.13e-f**). In addition, strong immunoreactivity was located within the syncytial knots (**Figs. 3.13c, 3.15-16**). The stromal core displayed weak-absent immunoprecipitation. Additionally, the endothelial cells lining both the fetal veins and arteries of stem villi were immunopositive across all study groups (**Figs. 3.13a-d; 3.14a-d**).

Evaluation of sEng within the syncytiotrophoblast layer of exchange villi showed mild to strong immunoreactivity across the N- and P- (**Fig. 3.15a-f**) and the N+ and P+ groups (**Fig. 3.16a-d**). sEng was immunopositive within endothelial cells lining dilated fetal blood vessels of exchange villi across all groups (**Figs. 3.15-3.16**). Additionally, mild-strong sEng immunoreactivity of the syncytial brush border (**Fig. 3.14e-f**) was observed. The stromal core displayed weak-absent sEng immunoreactivity. All extravillous trophoblast cells were immunopositive for sEng (**Fig. 3.17a-d**).

The negative control in which the primary antibody was replaced with PBS was non-reactive (**Fig. 3.17e**). Replacement of the primary antibody with non-immune sera of the same IgG class of the primary produced no immunoreactivity. The positive control (placenta) was immunopositive (**Fig. 3.17f**).

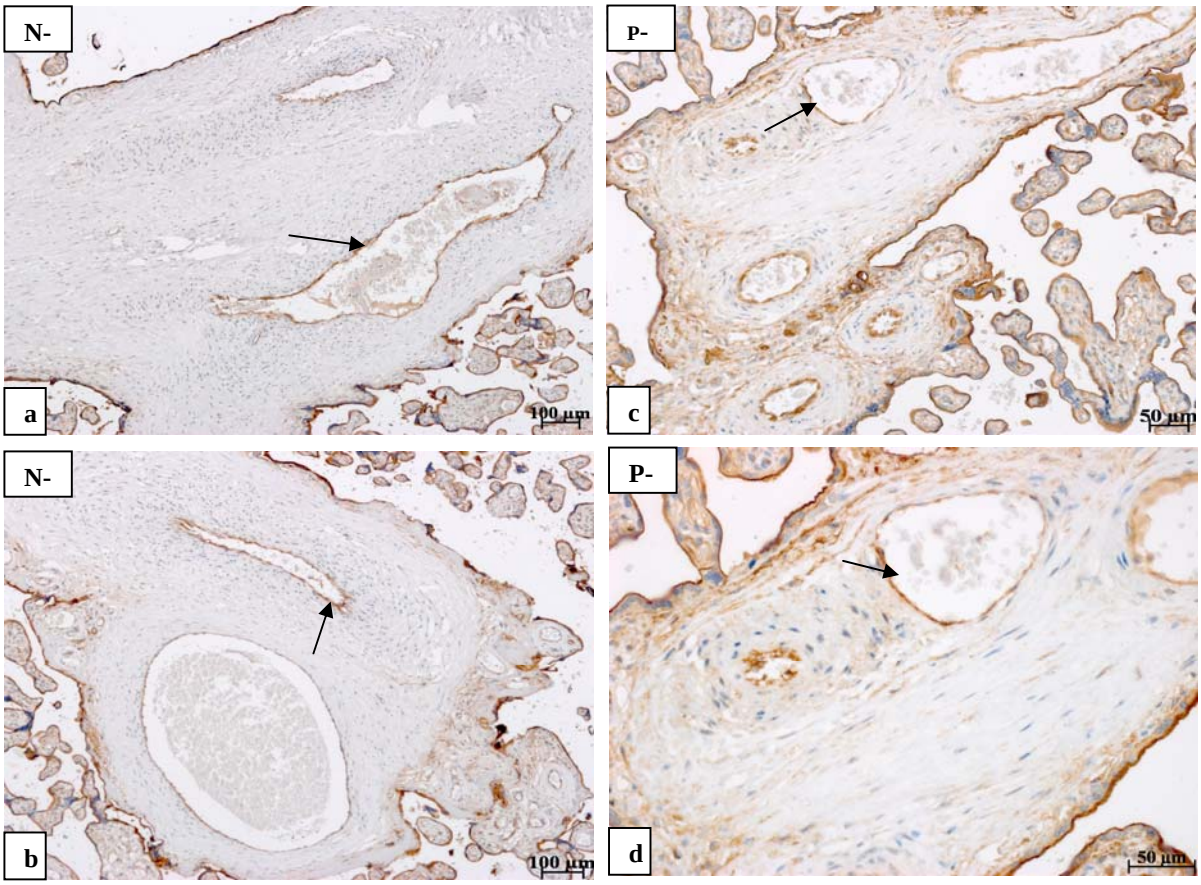

Figure 3.13: Light micrographs depicting immunoreactivity of sEng within (a-d) stem villi within N-(a,b) and P- (c,d) groups. Note endothelial cells (arrows).

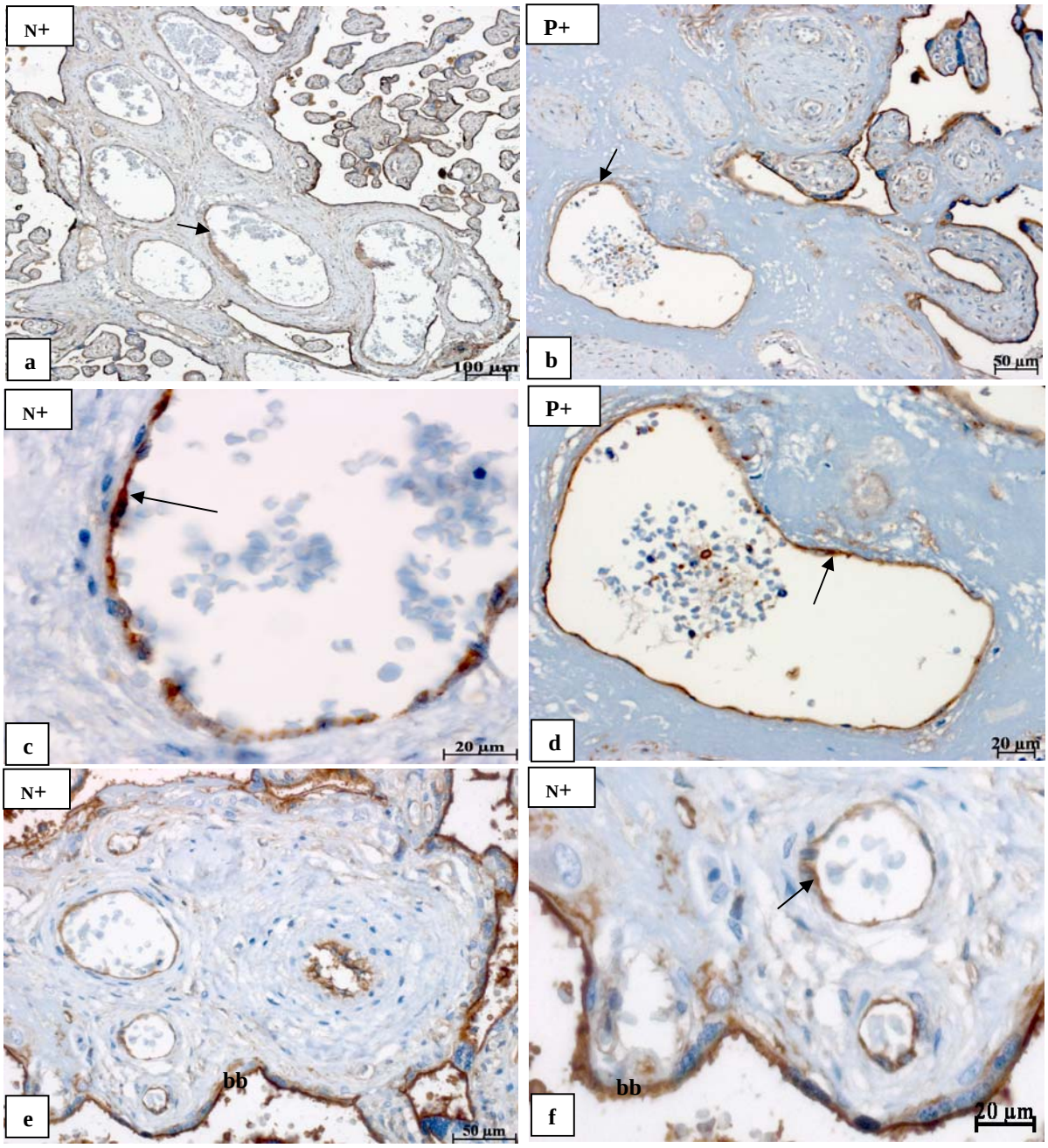

Figure 3.14: Light micrographs depicting immunoreactivity of sEng within (a-d) stem villi and (e-f) syncytial brushborder (BB) within N+(a,c,e) and P+ (b,d,f) groups. Note endothelial cells (arrows).

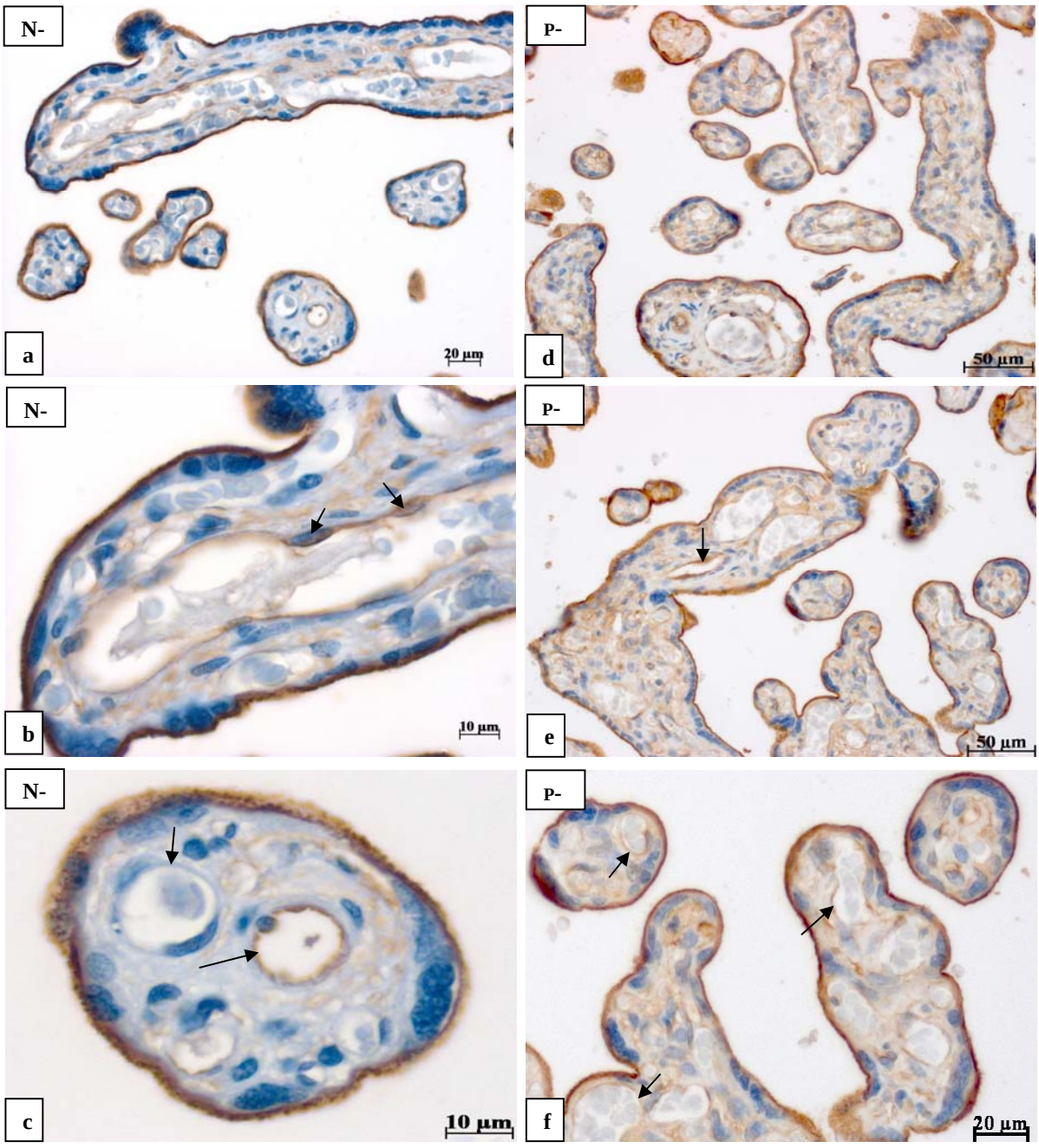

Figure 3.15: Light micrographs depicting immunoreactivity of sEng within (a-f) fetal vessels within N-(a-c) and P- (d-f) groups. Note endothelial cells (arrows).

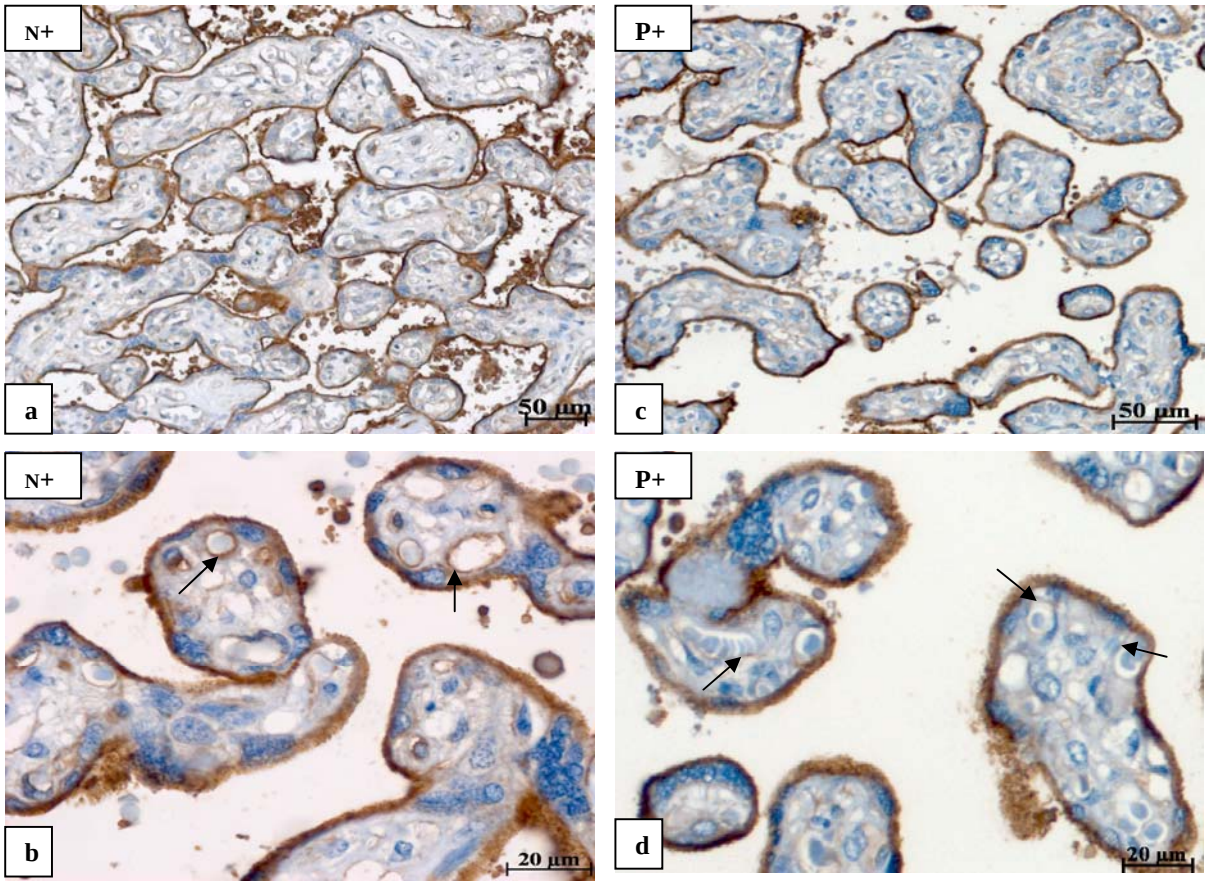

Figure 3.16: Light micrographs depicting immunoreactivity of sEng within (a-d) fetal vessels within N+(a,b) and P+ (c,d) groups. Note endothelial cells (arrows).

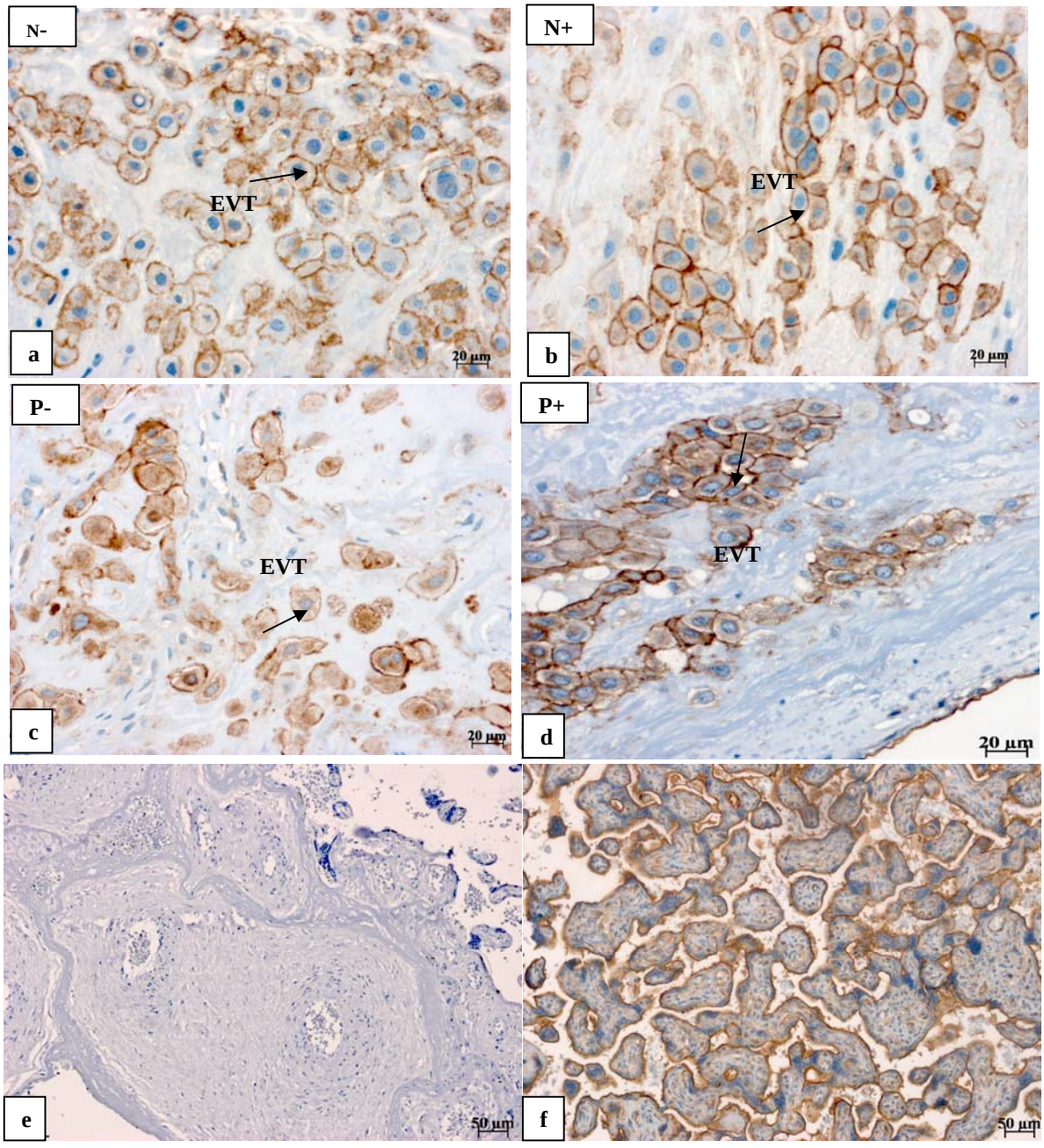

Figure 3.17: Light micrographs depicting immunoreactivity of sEng within (a-d) extravillous trophoblasts cells (EVT) within N-(a), P- (c), N+ (b) and P+ (d) groups; (e) negative control and (f) positive control.

3.3.2.2 Morphometric image analysis of sEng within placental tissue

3.3.2.2.1 Two-way Analyses of Variance

i. Exchange villi (intermediate and terminal villi)

Quantitative image analyses of sEng immunoreactivity based on the intensity within the exchange villi across all groups are shown (**Table 3.4; Fig. 3.18)**. The mean area percentage occupied by villi among the N-, N+, P- and P+ groups was 37.49±6.23%; 30.28±10.66%; 34±7.47% and 38.2±10.9% respectively. Hence, the mean percentage area of the intervillous space was 62%; 71%; 67% and 64 among the N-, N+, P- and P+ groups.

There was no significant effect of HIV status on sEng immunoexpression (field area percent of the chromogen) within exchange villi [$F(1,123)=2.964$, $p=0.088$] across all groups. However, a significant effect of type of pregnancy (normotensive and pre-eclamptic) on sEng immunoexpression in exchange villi [$F(1,123)=5.545$, $p=0.020$] was noted. This showed higher sEng expression in the pre-eclamptic (mean=14.037, 95% CI: 13.249-14.825) compared to the normotensive (mean=12.803, 95% CI: 12.129-13.477) groups. The bonferroni post hoc test showed a significant difference between the N- *vs* P- ($p=0.04$) and P- *vs* N+ ($p=0.013$) groups (**Table 3.4; Fig. 3.18)**

The mean intensity of immunoreactivity (chromogen) for sEng expressed as a densitometric grey value within the N-, N+, P- and P+ groups was 12.5 (10.9-14.5); 12.5 (11.4-14.2); 14.6 (13-16.2) and 12.7 (10.9-14.6) respectively, with a range of 10 (8-12) to 76 (68-89).

Finally, no interaction was noted between HIV status and type of pregnancy on sEng immunoexpression within exchange villi [$F(1,123)=1.61$, $p=0.207$].

ii. Conducting villi (stem villi)

Quantitative image analyses of sEng immunoreactivity within stem villi across all groups are shown (**Table 3.5; Fig. 3.19)**. A significant effect between HIV status and sEng immunoexpression in stem villi was demonstrated between HIV positive and HIV negative groups [$F(1,123)=11.978$, $p=0.001$, mean=13.674, 95%CI: 12.441-14.908 *vs* mean=16.655, 95% CI: 15.478-17.832].

In addition, a significant effect of type of pregnancy (normotensive and pre-eclamptic) on sEng immunolocalisation within stem villi [$F(1,123)=13.161$, $p<0.001$] was noted, indicating higher sEng immunoreactivity in the pre-eclamptic (mean=16.7272, 95% CI: 15.431-18.022) compared to normotensive (mean=13.602, 95% CI: 12.494-14.711) groups. The bonferroni post hoc test showed a significant difference between the N- *vs* N+ ($p=0.005$), P- *vs* N+ ($p=0.000$) and N+ *vs* P+ (p=0.01) groups (**Table 3.5; Fig. 3.19)**.

Mean intensity of sEng immunoreactivity among N-, N+, P- and P+ groups expressed as a densitometric grey value was 14.5(11.1-18.3); 11.4(8.7-13.8); 17.5(13.4-22.3) and 14.9(12.1-19.4) respectively, with a range of 13 (12-15) to 140 (115-161).

Finally, there was no significant effect between HIV status and type of pregnancy (normotensive and pre-eclamptics) on sEng immunoexpression within stem villi [$F(1,123)=0.65$, $p=0.421$].

3.3.2.2.2 Correlation of sEng immunoexpression between exchange and stem villi

A Pearson correlation coefficient was computed to assess the relationship between exchange and stem villi immunoexpression of sEng across all study groups. There was no correlation between the exchange and stem villi immunoexpression of sEng ($r=0.157$, $p=0.07$)

Table 3.4: Morphometric image analysis of sEndoglin immunoexpression in exchange villi

	Normotensive Pregnant Women		Pre-eclamptic Women		
	HIV Negative (N-)	**HIV Positive (N+)**	**HIV Negative (P-)**	**HIV Positive (P+)**	**P value**
n	36	40	30	24	
Area Frame µm^2	312843.68	312843.68	312843.68	312843.68	
Field Area of villi %	37.49±6.23	30.28±10.66	34±7.47	38.2±10.9	p=0.001
Immunoreactivity %	12.92(±2.64)	12.68(±3.2)	14.82(±2.92)	13.25(±2.75)	p=0.020
Intensity of immunoreactivity (median + interquartile range)					
Mean	36(27-43)	37(33-44)	44(37-53)	29(26-36)	
Min	13(8-16)	13(9-15)	15(13-18)	10(8-12)	
Max	60(43-73)	67(55-78)	76(68-89)	56(43-71)	

Significance: $^*p<0.05$; n=130; parametric-data (mean ± SD)
Densitometry (grayscale 0-255)

Table 3.5: Morphometric image analysis of sEndoglin immunoexpression in stem villi

	Normotensive Pregnant Women		Pre-eclamptic Women		
	HIV Negative (N-)	**HIV Positive (N+)**	**HIV Negative (P-)**	**HIV Positive (P+)**	**P value**
n	36	40	30	24	
Immunoreactivity %	15.46(±5.02)	11.76(3.45)±	17.86(5.76)±	15.58(±4.86)	p<0.001
Intensity of immunoreactivity (median + interquartile range)					
Mean	51(42-74)	63(51-71)	87(69-107)	49(42-56)	
Min	19(13-22)	24(18-32)	25(21-34)	13(12-15)	
Max	74(68-121)	100(84-113)	140(115-161)	83(75-84)	

Significance: $^*p<0.05$; n=130; parametric-data (mean ± SD)
Densitometry (grayscale 0-255)

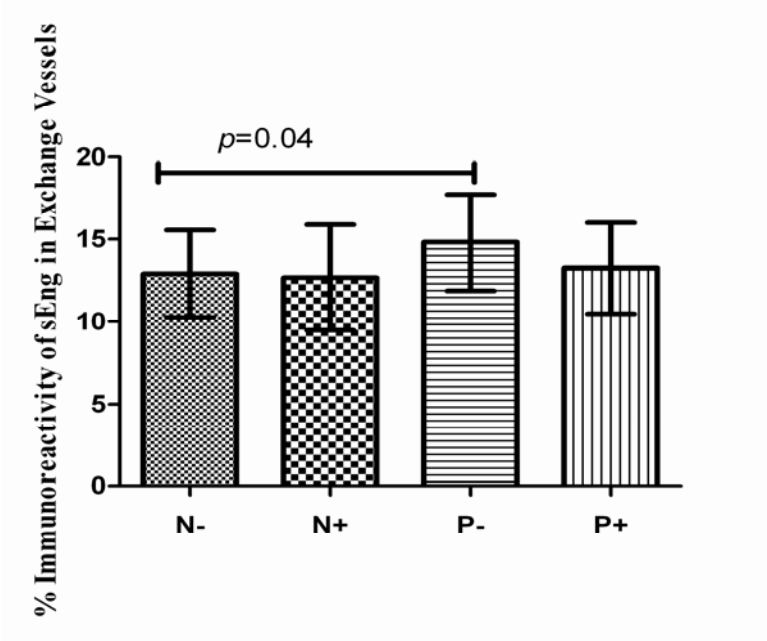

Figure 3.18: Percentage sEng immunoreactivity within exchange villi (mean ±SD) across HIV positive pre-eclamptic (P+), HIV negative pre-eclamptic (P-), HIV negative normotensive pregnant (N-) and HIV positive pregnant women (N+).

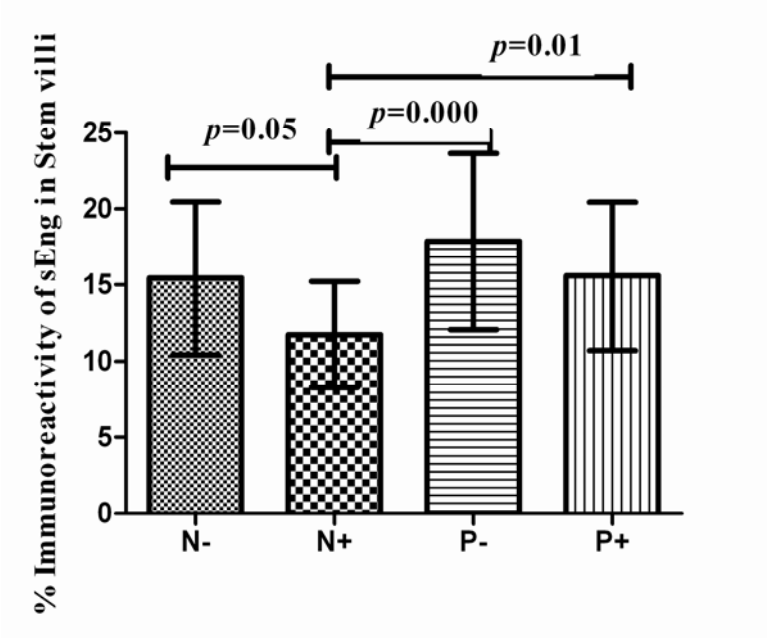

Figure 3.19: Percentage sEng immunoreactivity within stem villi (mean ± SD) across HIV positive pre-eclamptic (P+), HIV negative pre-eclamptic (P-) HIV negative normotensive pregnant (N-) and HIV positive pregnant women (N+).

3.3.3 Placental growth factor (PlGF)

3.3.3.1 Immunolocalisation

The immunoreactive distribution of PlGF within conducting and exchange villi across all study groups (N-, N+, P- and P+) are shown (**Figs. 3.20-3.24**). The syncytiotrophoblast layer displayed weak immunoreactivity across all groups, whilst, PlGF immunoreactivity of endothelial cells lining blood vessels of stem villi varied (**Figs. 3.20a-d; 3.21a-d**).

Assessment of syncytiotrophoblasts within exchange villi showed mild to weak immunoreactivity across all groups (**Figs. 3.22-3.23**). Evaluation of endothelial cells lining the dilated blood vessels within intermediate and terminal villi across all groups was immunopositive for PlGF (**Figs. 3.22-3.23**). All extravillous trophoblast cells were immunopositive for PlGF (**Fig. 3.24a-d**).

The negative control in which the primary antibody was replaced with PBS was non-reactive (**Fig. 3.24e**). Replacement of the primary antibody with non immune sera of the same IgG class of the primary produced no immunoreactivity. The positive control (breast carcinoma) was immunopositive for PlGF (**Fig. 3.24f**).

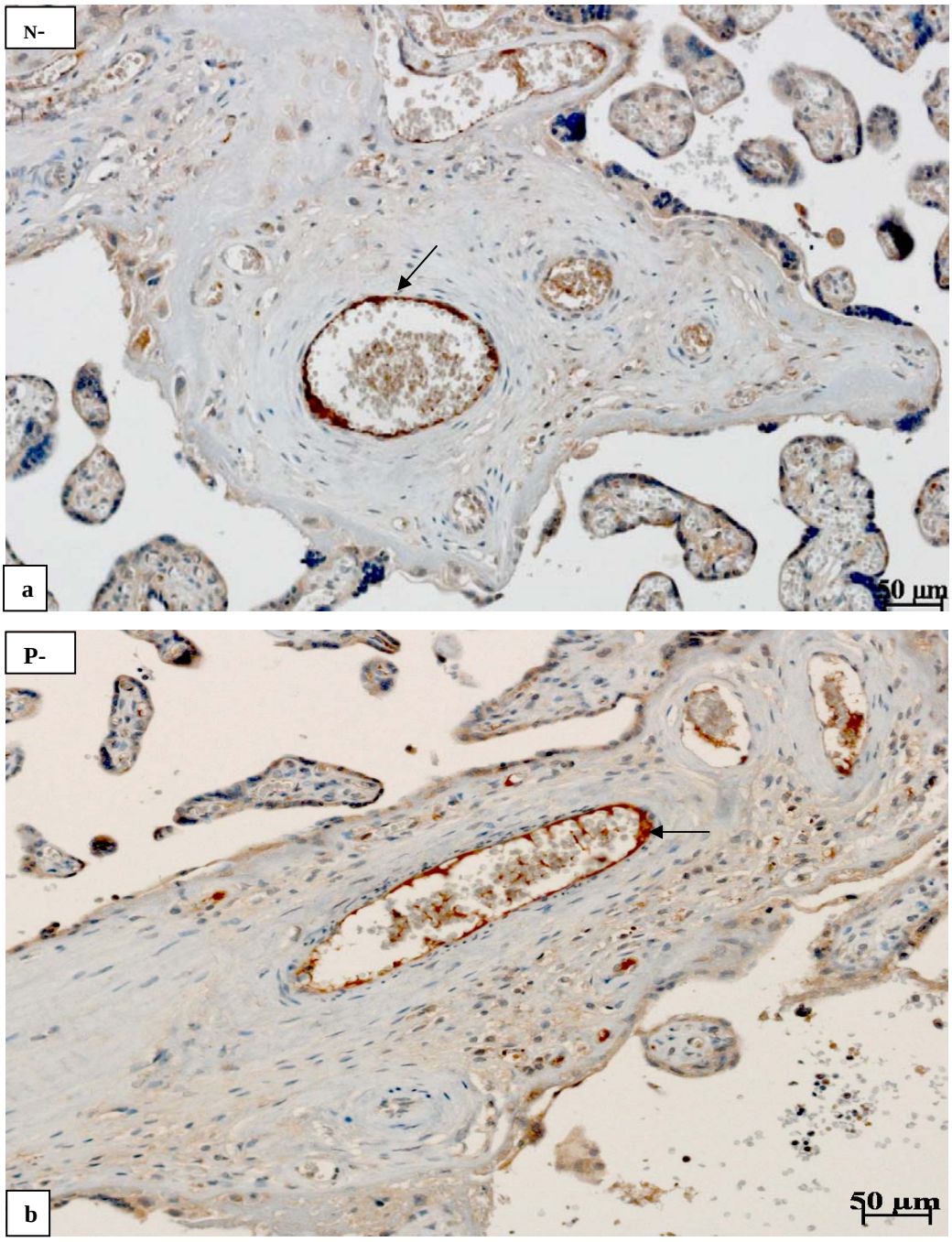

Figure 3.20: Light micrographs depicting immunoreactivity of PlGF within (a-b) stem villi in N- (a) and P- (b) groups. Note endothelial cells (arrows).

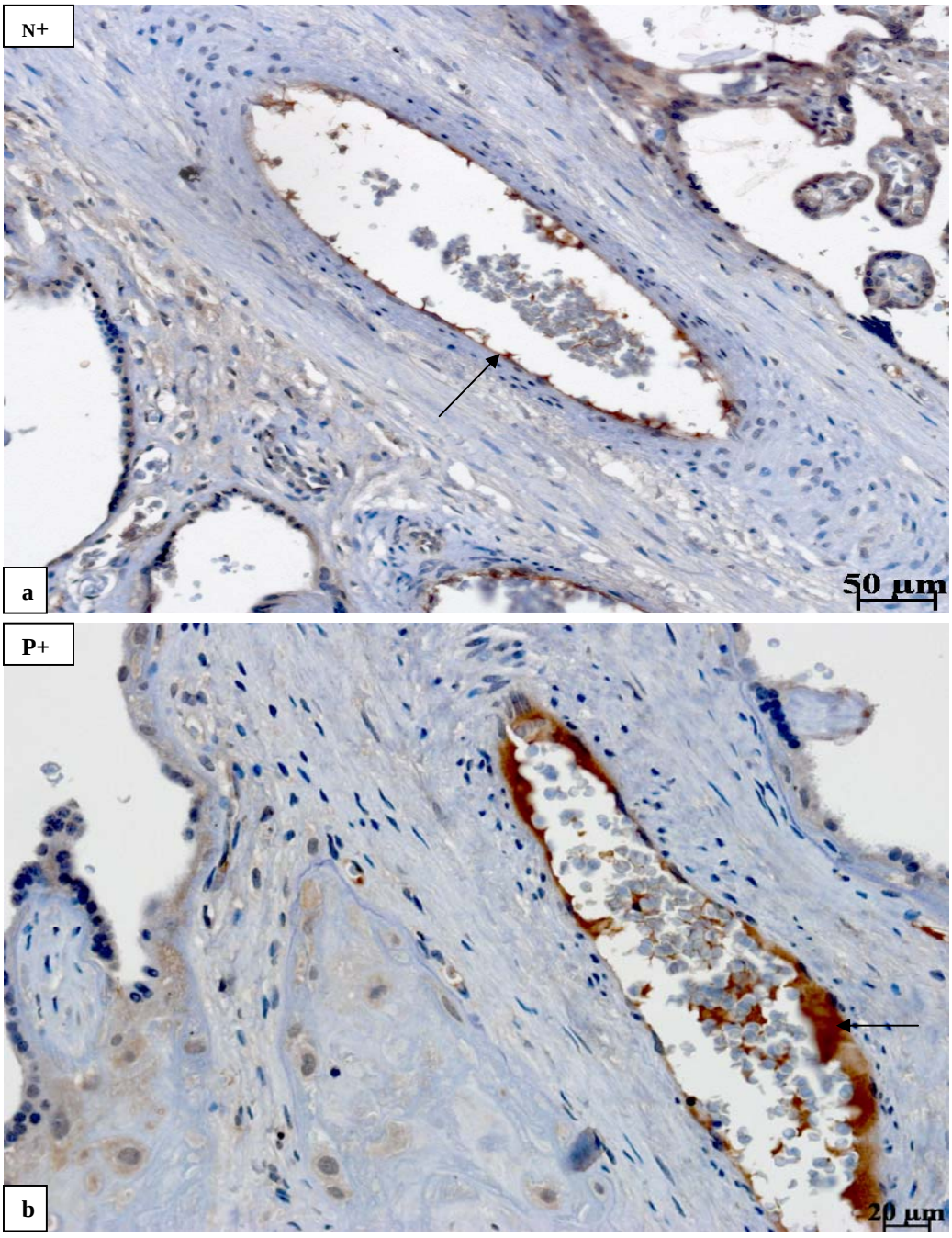

Figure 3.21: Light micrographs depicting immunoreactivity of PlGF within (a-b) stem villi in N+ (a) and P+ (b) groups. Note endothelial cells (arrows).

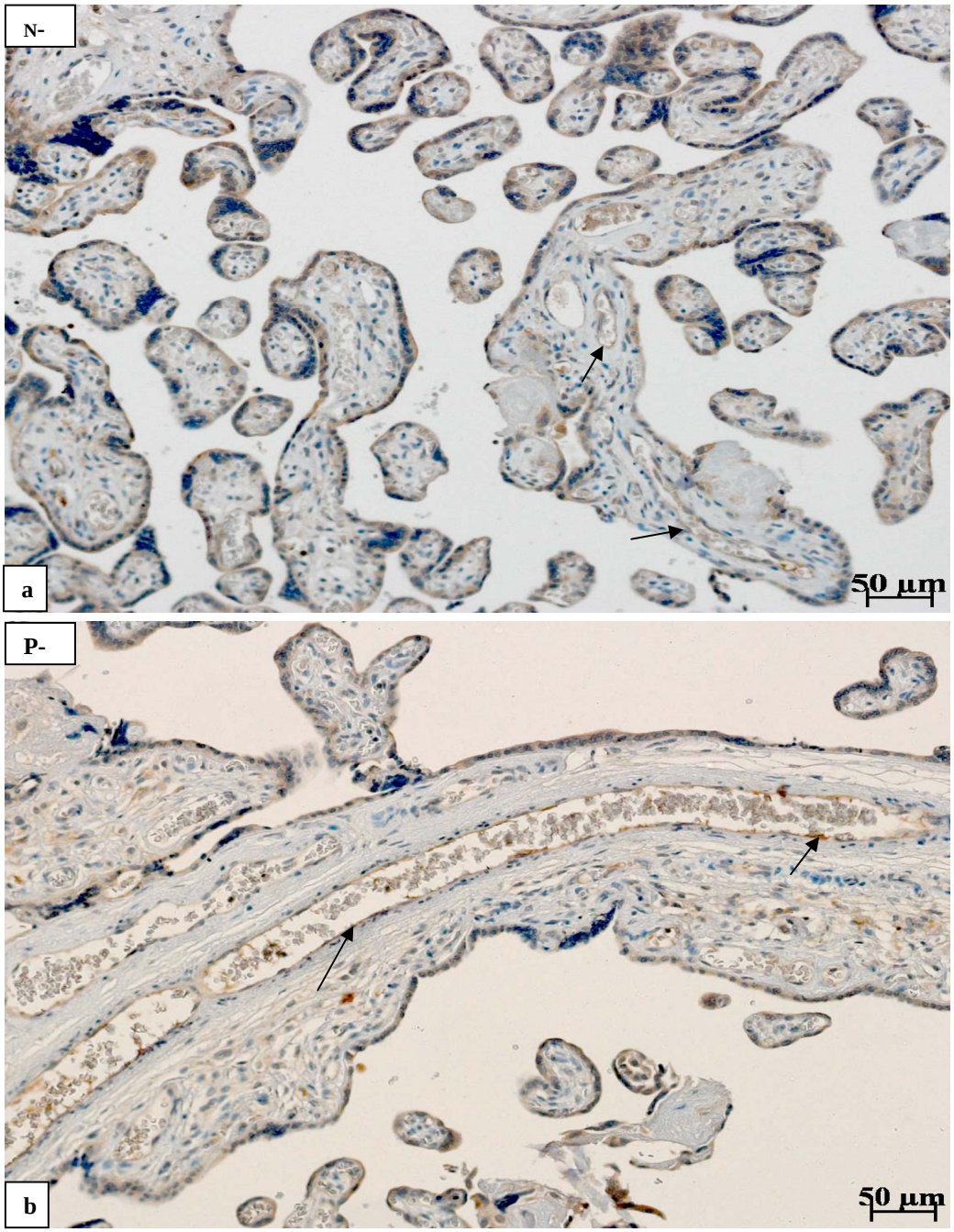

Figure 3.22: Light micrographs depicting immunoreactivity of PlGF within (a-b) exchange villi in N- (a) and P- (b) groups. Note endothelial cells (arrows)

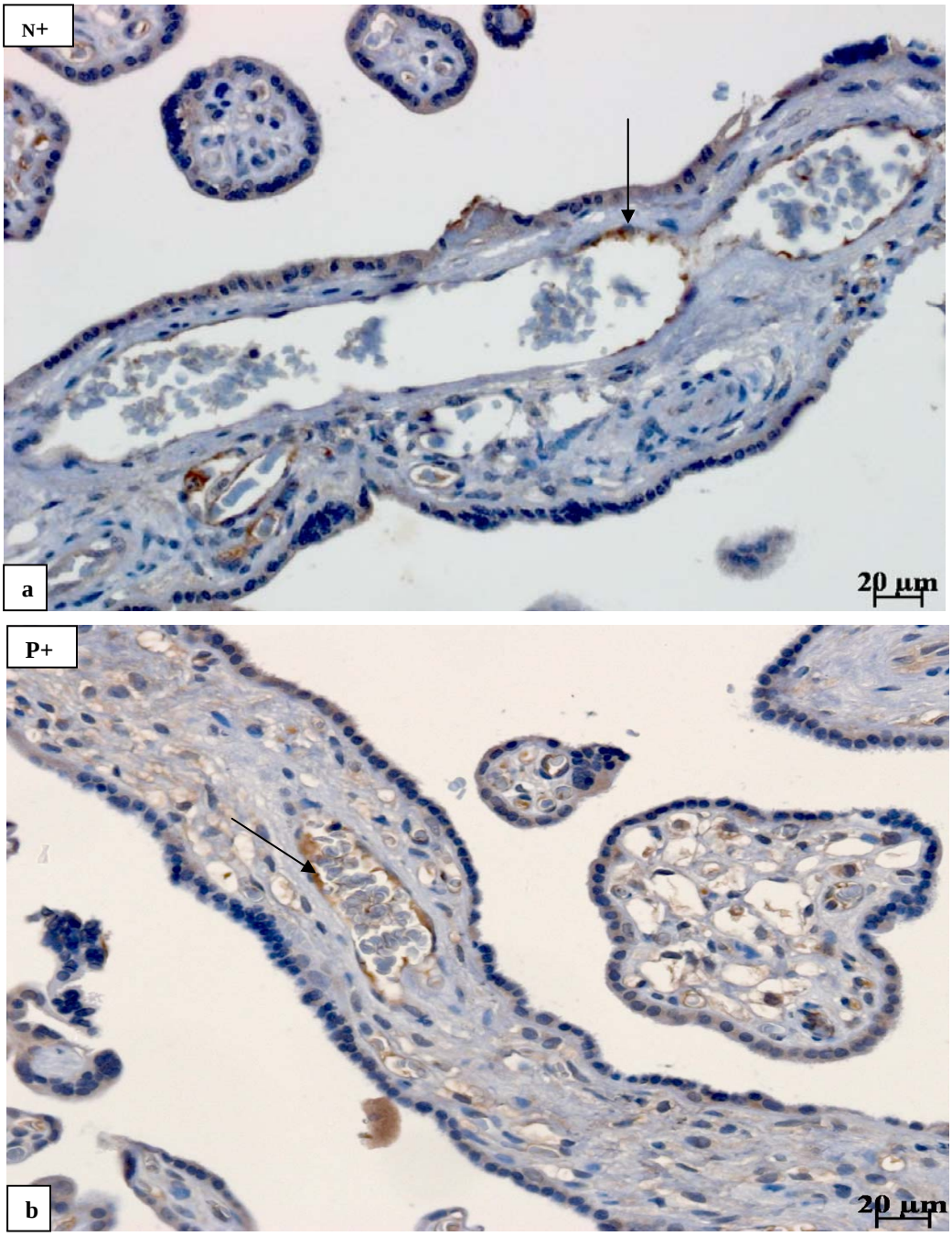

Figure 3.23: Light micrographs depicting immunoreactivity of PlGF within (a-b) exchange villi in N+ (a) and P+ (b) groups. Note endothelial cells (arrows).

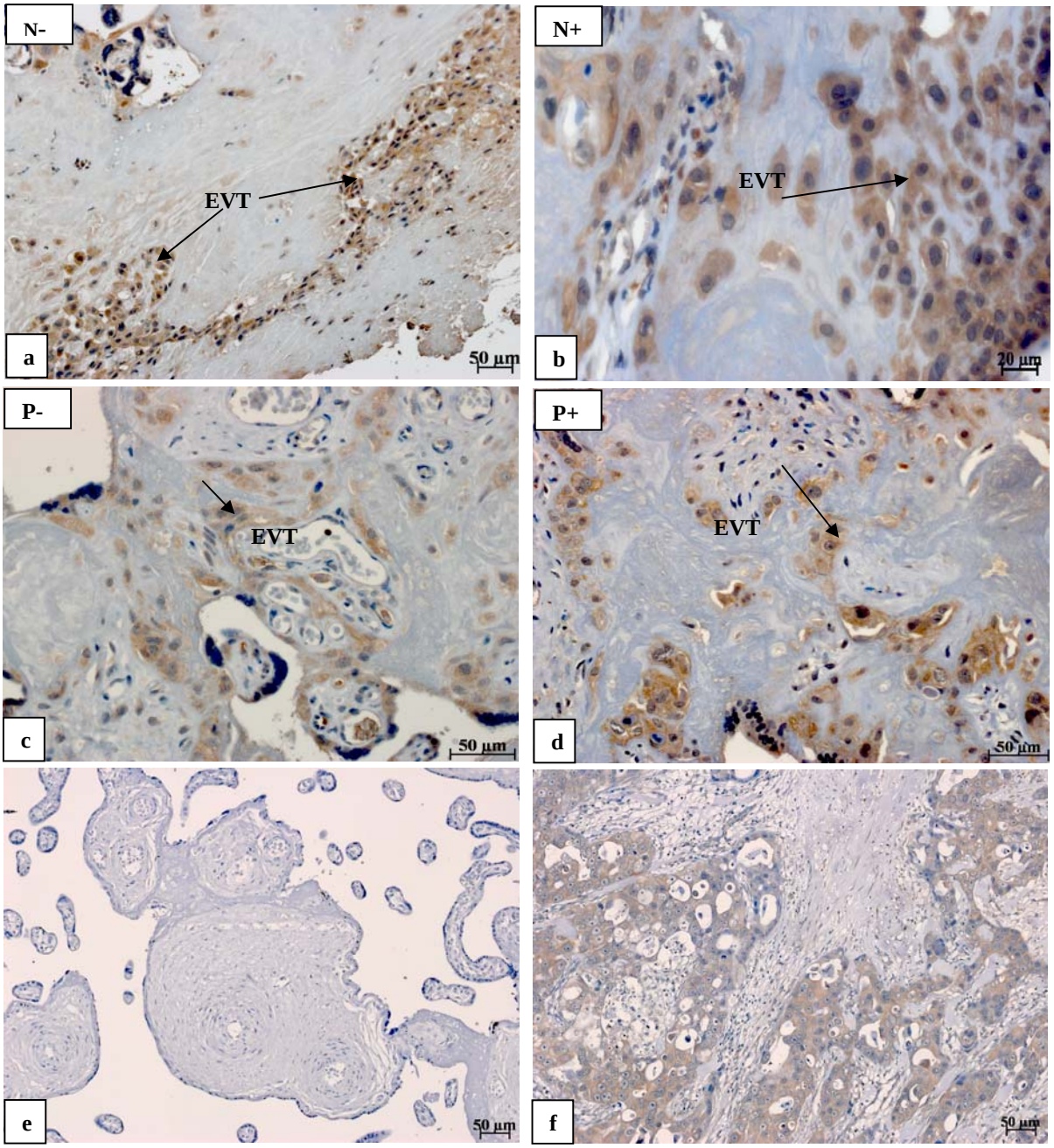

Figure 3.24: Light micrographs depicting immunoreactivity of PlGF within (a-d) extravillous trophoblast cells (EVT, arrows) in N- (a), P- (c), N+ (b) and P+ (d) groups; (e) negative control and (f) positive control.

3.3.3.2 Morphometric image analysis of PlGF immunoexpression within placental tissue

3.3.3.2.1 Two-way Analyses of Variance

i. Exchange villi (intermediate and terminal villi)

The mean area percentage occupied by villi for immunoreactivity of PlGF within the N-, N+, P- and P+ groups was 38.3±3.8%; 45.5±5%; 44.3±8.9% and 35.6±14.8% respectively (**Table 3.6**). Consequently, the mean percentage of the intervillous space was 61.7%; 54.53%; 55.67% and 64.37, respectively.

There was no significant effect of HIV status on PlGF immunoexpression (field area percent of the chromogen) within exchange villi [$F(1,21)=3.359$, $p=0.081$]. However, a significant effect of type of pregnancy (normotensive and pre-eclamptic) on PlGF immunoexpression within exchange villi [$F(1,21)=138.892$, $p<0.001$] was shown. Bonferroni post hoc analyses demonstrated a significant difference between P+ (mean=7.464, 95% CI: 6.245-8.683, $p=0.000$) *vs* N+ (mean=17.687, 95% CI: 16.370-19.004, $p=0.000$), and between P- (mean=9.220, 95% CI: 7.903-10.537, $p=0.000$) vs N- (mean=13.652, 95% CI: 12.336-14.969, $p=0.000$) groups (**Table 3.6; Fig. 3.25**).

The mean intensity of immunoreactivity (chromogen) for PlGF within the N-, N+, P- and P+ groups expressed as a densitometric grey value was 14 (12.7-14.2); 18 (15.6-19.2); 9 (8.3-10.40) and 7.4 (5.6-10) respectively, with a range of 22 (18-28) to 94 (86-105).

Additionally, a significant effect between HIV status and type of pregnancy (normotensive and pre-eclamptics) on PlGF immunoexpression within exchange villi [$F(1,21)=21.682$, $p<0.001$] was noted.

ii. Conducting villi (stem villi)

Morphometric image analysis for immunoreactivity of PlGF is shown (**Table 3.7; Fig. 3.26)**. A significant effect of HIV status on PlGF immunoexpression within stem villi [$F(1,21)=11.241$, $p=0.003$] was noted, indicating lower PlGF immunoreactivity within the HIV positive (mean=8.175, 95% CI: 7.043-9.307) compared to the HIV negative (mean=10.806, 95% CI: 9.631-11.981) groups.

In addition, a significant effect of type of pregnancy (normotensive and pre-eclamptics) on PlGF immunoexpression within stem villi [$F(1,21)=23.771$, $p<0.001$] was noted, with the pre-eclamptics showing lower immunoreactivity (mean=7.577, 95%CI: 6.445-8.710) compared to the normotensive (mean=11.403, 95%CI: 10.228-12.578) groups. Bonferroni post hoc analyses (**Fig. 3.26)** revealed significant differences between the P+ (mean=7.107, 95% CI: 5.568-8.645, $p=0.000$) *vs* N+ groups (mean=9.243, 95%CI: 7.581-10.904, $p=0.000$) and between the N- *vs* N+ ($p=0.000$) and N- *vs* P+ ($p=0.000$) groups.

The mean intensity of PlGF immunoreactivity (**Table 3.7**) among N-, N+, P- and P+ groups expressed as a densitometric grey value was 13 (11.2-16.5); 9 (7.2-10.9); 8 (6.8-9.1) and 7 (6.4-7.8) respectively, with a range of 33(27-37) to 99(92-101).

Moreover, a significant interaction was noted between HIV status and type of pregnancy (normotensive and pre-eclamptics) on PlGF immunoexpression within stem villi [$F(1,21)=4.639$, $p=0.043$].

3.3.3.2.2 Correlation of PlGF immunoexpression between exchange and stem villi

A Pearson correlation coefficient was computed to assess the relationship between exchange and stem villi immunoexpression of PlGF across all study groups. There was no correlation between the exchange and stem villi immunoexpression of PlGF (r=0.397, $p=0.04$, **Fig. 3.27).**

Table 3.6: Morphometric image analysis of PlGF immunoexpression in exchange villi

	Normotensive Pregnant Women		Pre-eclamptic Women		
	HIV Negative (N-)	**HIV Positive (N+)**	**HIV Negative (P-)**	**HIV Positive (P+)**	**P value**
n	36	40	30	24	
Area Frame µm^2	312843.68	312843.68	312843.68	312843.68	
Field Area in villi %	38.3±3.8	45.5±5	44.3±8.9	35.6±14.8	*p*=0.04
Immunoreactivity %	13.65±0.92	17.68±1.83	9.2±1.12	7.46±1.96	*p*<0.001
Intensity of immunoreactivity (median + interquartile range)					
Mean	63(62-71)	52(46-56)	79(70-87)	51(41-59)	
Min	36(31-41)	22(18-28)	53(44-59)	33(28-40)	
Max	82(80-88)	78(69-80)	94(86-105)	65(53-74)	

Significance:* p<0.05; n=130; parametric-data (mean ± SD)
Densitometry (grayscale 0-255)

Table 3.7: Morphometric image analysis of PlGF immunoexpression in stem villi

	Normotensive Pregnant Women		Pre-eclamptic Women		
	HIV Negative (N-)	**HIV Positive (N+)**	**HIV Negative (P-)**	**HIV Positive (P+)**	**P value**
n	36	40	30	24	
Immunoreactivity %	13.56±2.86	9.24±2.36	8.04±1.11	7.1±0.94	p=0.043
Intensity of immunoreactivity (median + interquartile range)					
Mean	67(61-78)	56(48-58)	82(77-85)	67(44-76)	
Min	35(30-51)	33(27-37)	58(51-61)	48(31-57)	
Max	90(83-96)	71(69-74)	99(92-101)	82(56-95)	

Significance:* p<0.05; n=130; parametric-data (mean ± SD)
Densitometry (grayscale 0-255)

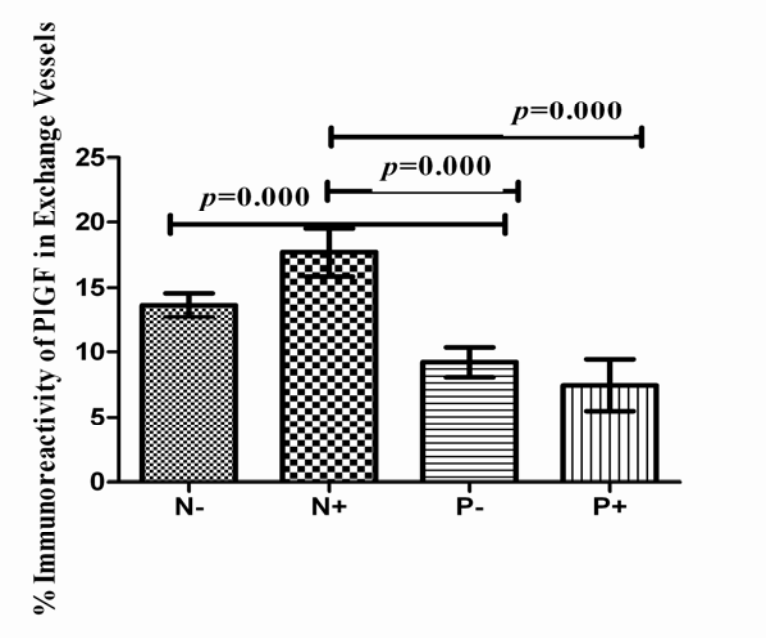

Figure 3.25: Percentage immunoreactivity of PlGF in exchange villi (mean ±SD) within HIV positive pre-eclamptic (P+); HIV negative pre-eclamptic (P-); HIV negative normotensive pregnant (N-) and HIV positive pregnant women (N+).

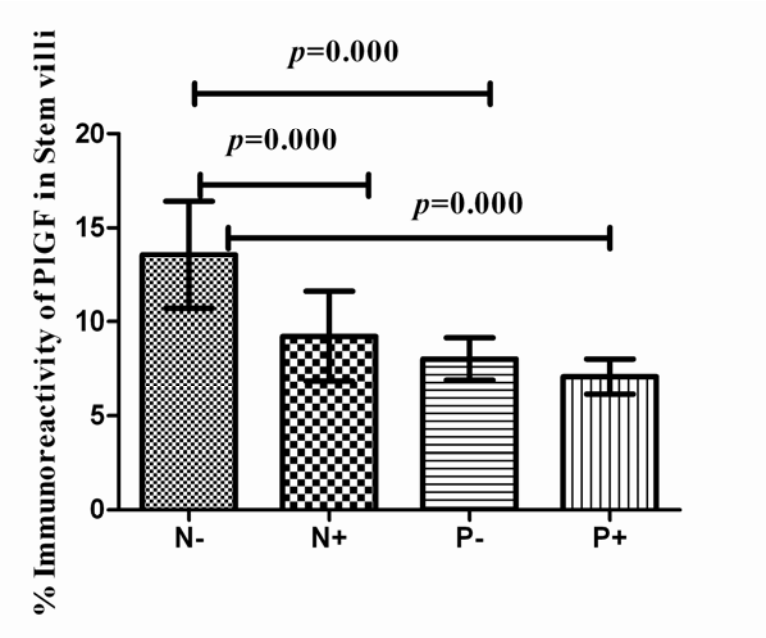

Figure 3.26: Percentage immunoreactivity of PlGF in stem villi (mean ±SD) within HIV positive pre-eclamptic (P+); HIV negative pre-eclamptic (P-); HIV negative normotensive pregnant (N-) and HIV positive pregnant women (N+).

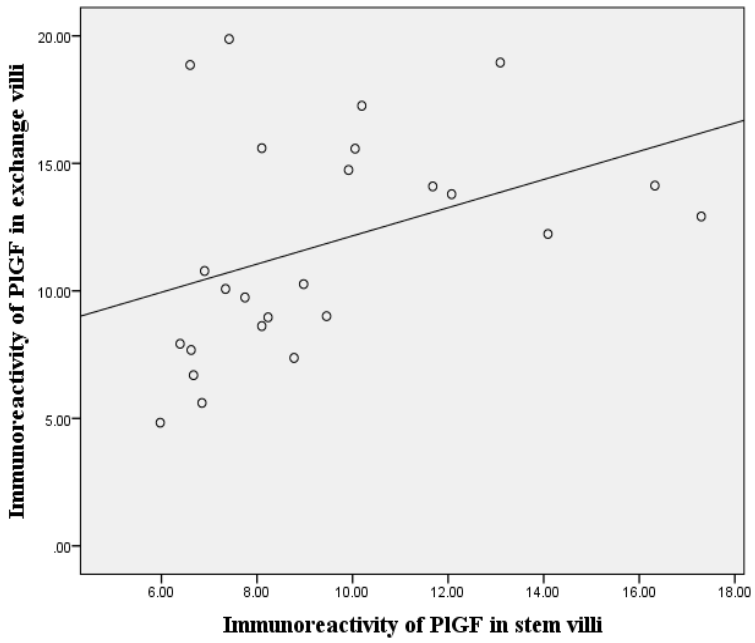

Figure 3.27: Correlation analyses of PlGF immunoexpression between exchange and stem villi across study groups

3.3.4 Vascular endothelial growth factor (VEGF)

3.3.4.1 Immunolocalisation

Immunoreactivity of VEGF within placental villi across all study groups (N-, N+, P- and P+) is shown (**Figs. 3.28-3.32**). The syncytiotrophoblast layer of placental villi showed weak to moderate immunoreactivity across all groups (**Figs. 3.28-3.31**). Immunoreactivity of VEGF in endothelial cells within stem villi varied from moderate in the normotensive group (N- and N+; **Figs. 3.28a; 3.29a**) to a low immunoprecipitation within the pre-eclamptic group (P- and P+; **Figs. 3.28b; 3.29b**).

Evaluation of syncytiotrophoblasts within exchange villi demonstrated low VEGF immunoreactivity across all groups (**Fig. 3.30-3.31**). Similarly, the endothelial cells lining the dilated fetal blood vessels within the exchange villi showed mild VEGF immunostaining across all groups. Mild VEGF immunostaining was also noted within the extravillous trophoblast cell populations (**Fig. 3.32a-d**).

The negative control in which the primary antibody was replaced with PBS was observed as being non-reactive (**Fig. 3.32e**). The positive control (breast carcinoma) was immunopositive for VEGF (**Fig. 3.32f**).

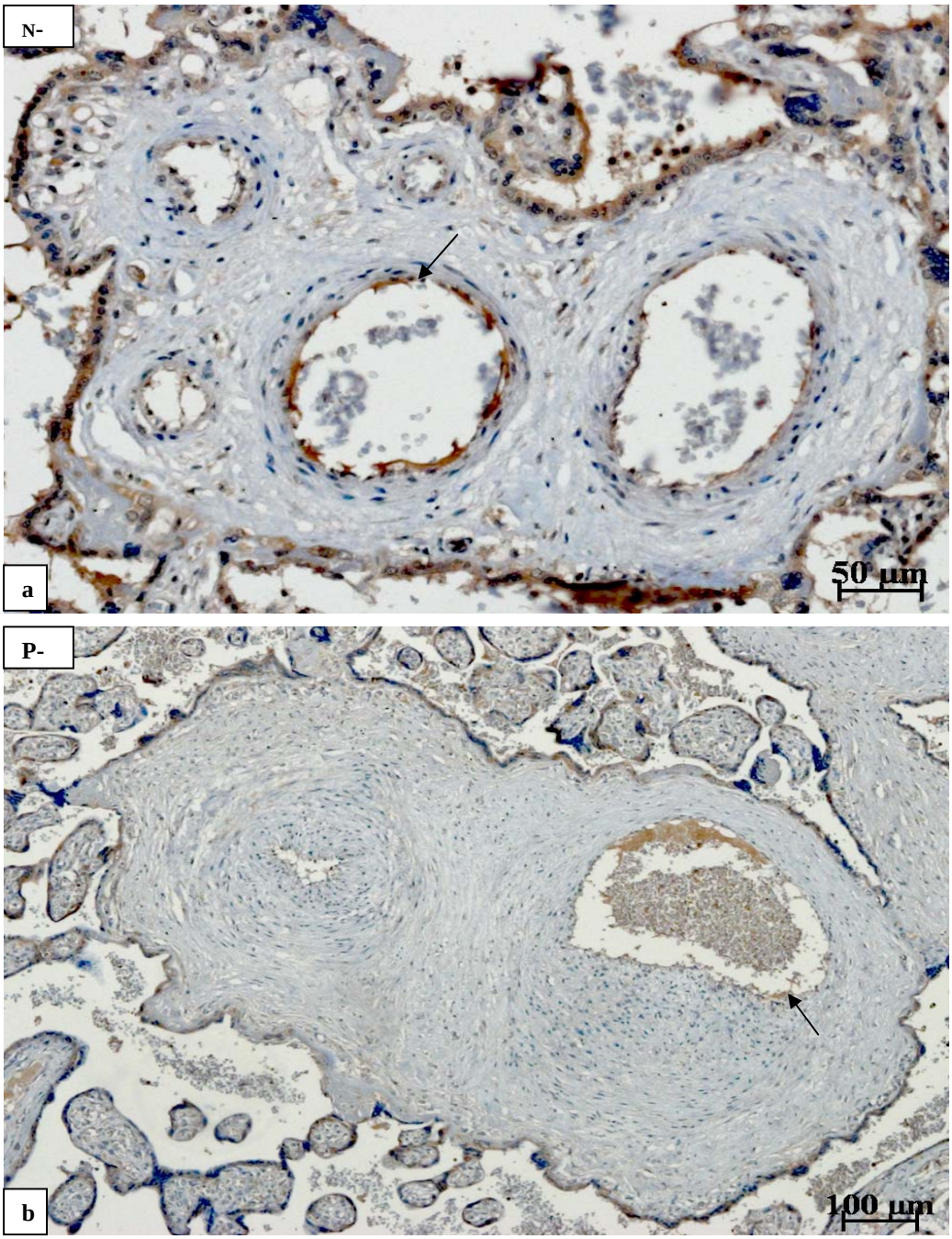

Figure 3.28: Light micrographs depicting immunoreactivity of VEGF within (a-b) stem villi in N-(a) and P- (b) groups. Note endothelial cells (arrows).

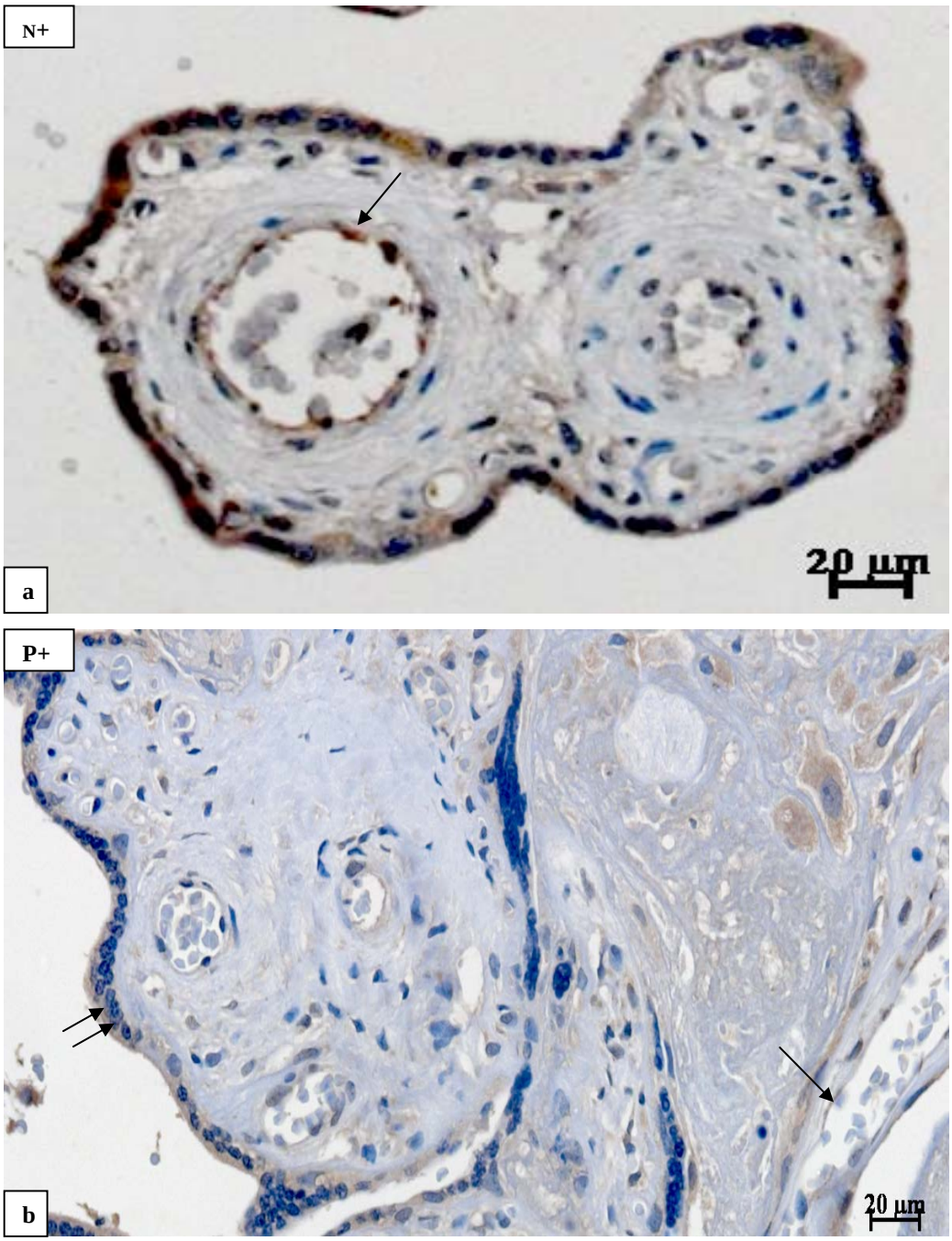

Figure 3.29: Light micrographs depicting immunoreactivity of VEGF within (a-b) stem villi within N+ (a) and P+ (b) groups. Note endothelial cells (arrows) and syncytium (double arrow).

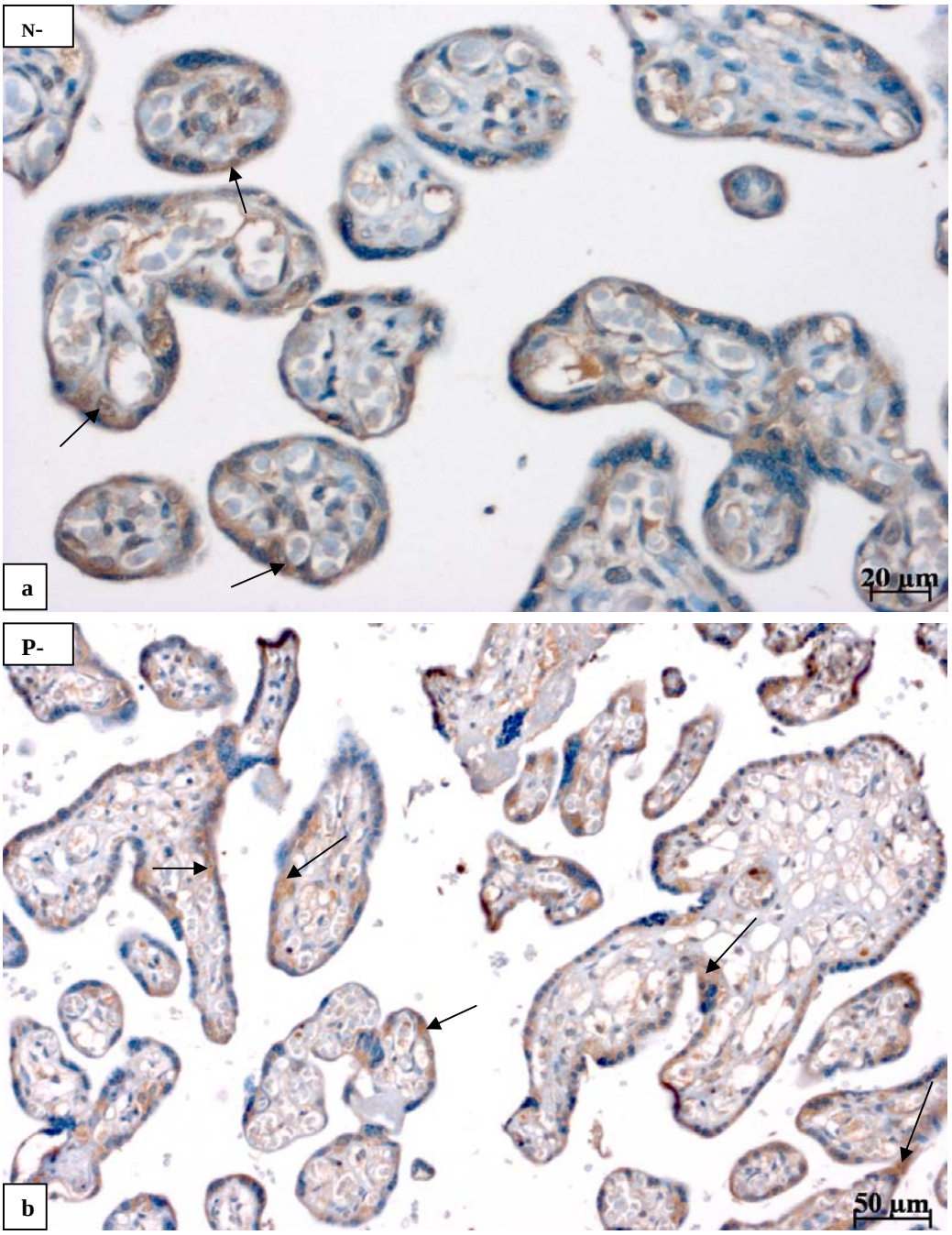

Figure 3.30: Light micrographs depicting immunoreactivity (arrows) of VEGF within (a-b) exchange villi in N-(a) and P- (b) groups.

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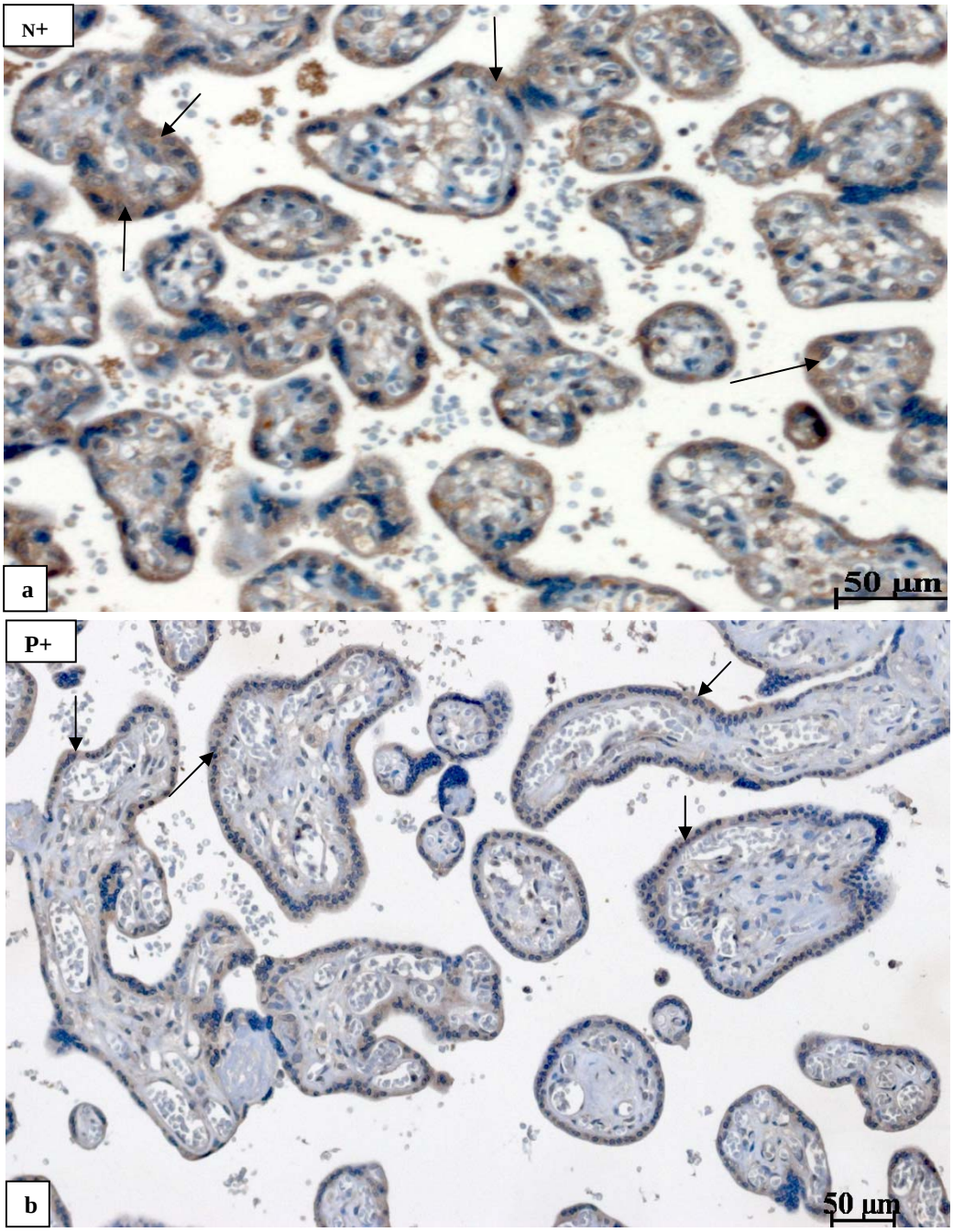

Figure 3.31: Light micrographs depicting immunoreactivity (arrows) of VEGF within (a-b) exchange villi within N+ (a) and P+ (b) groups.

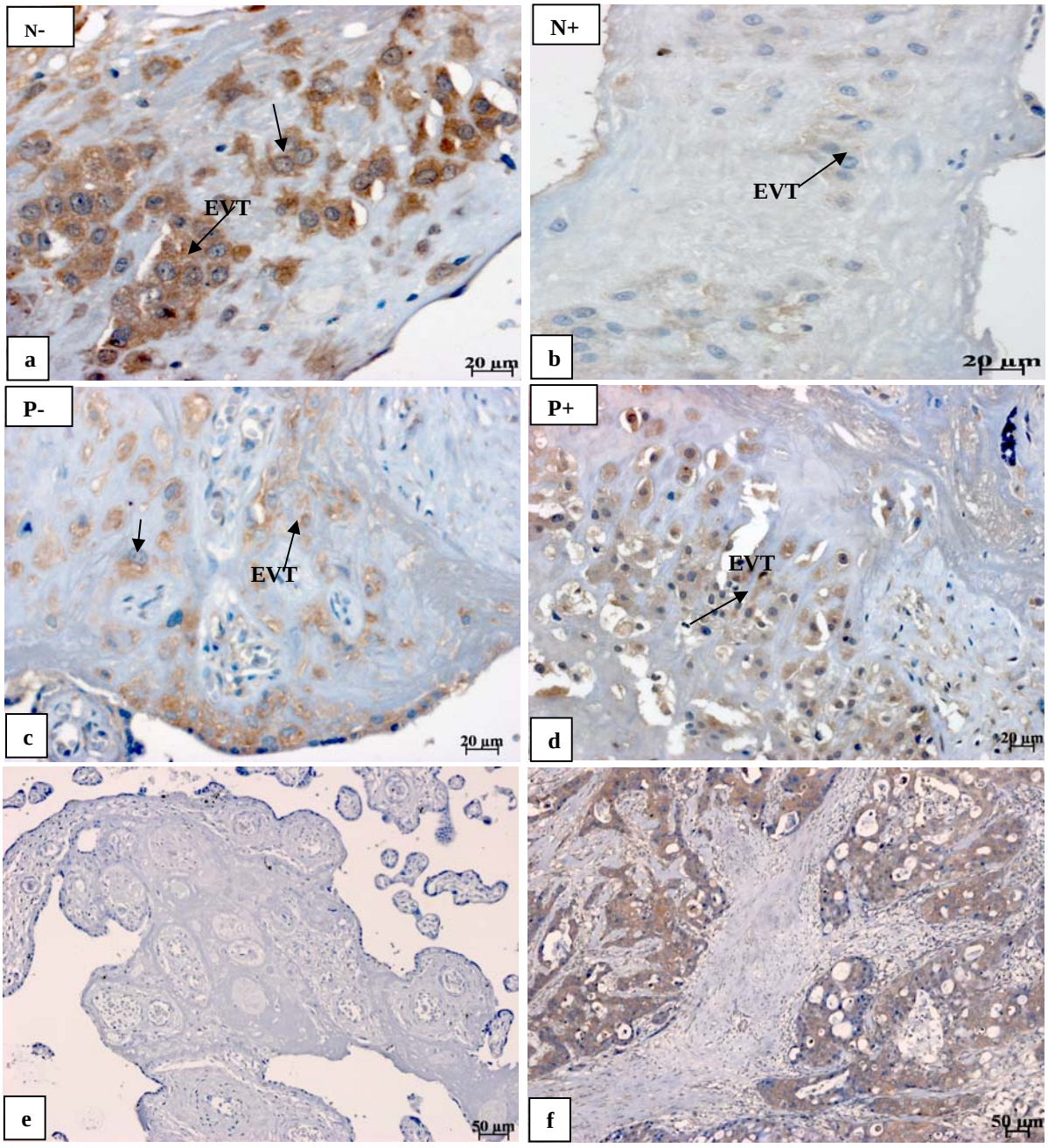

Figure 3.32: Light micrographs depicting immunoreactivity of VEGF within (a-d) extravillous trophoblast cells (EVT arrows) within N- (a), P- (c), N+ (b) and P+ (d) groups; (e) negative control and (f) positive control.

3.3.4.2 Morphometric image analysis of VEGF within placental tissue

3.3.4.2.1 Two-way Analyses of Variance

i. Exchange villi (intermediate and terminal villi)

The mean area percentage occupied by villi for immunoreactivity of VEGF within the N-, N+, P- and P+ groups was 33.5±10.8%; 36.9±9%; 37.4±8.4% and 41±4.4%, respectively (**Table 3.8**). Hence, the mean percentage intervillous space was 66.5%, 63.14%, 62.63% and 58.83%, respectively.

There was a significant effect of HIV status on the immunoexpression (field area percent of chromogen) of VEGF in exchange villi [$F(1,20)=9.86$, $p=0.005$]. A higher field area percent immunoreactivity of VEGF was noted between the HIV positive (mean=8.48, 95% CI: 7.45-9.50) compared with the HIV negative (mean=6.29, 95% CI: 5.27-7.32) groups. In addition, a significant effect of type of pregnancy (normotensive and pre-eclamptic) on VEGF immunoexpression within exchange villi [$F(1,20)=15.71$, $p=0.001$] was noted. Bonferroni post hoc analyses revealed a significant difference between N- *vs* P- ($p=0.000$), P- *vs* N+ ($p=0.000$) and P+ *vs* P- ($p=0.000$) groups (**Table 3.8; Fig. 3.33)**.

The mean intensity of VEGF immunoreactivity (chromogen) within the N-, N+, P- and P+ groups expressed as a densitometric grey value was 8(7-9); 10(7-11); 5(3.4-5) and 8(6-9), respectively [(range: 25(19-32)-72(60-82)].

Finally, no interaction was noted between HIV status and type of pregnancy (normotensive and pre-eclamptic) of VEGF immunoexpression in exchange villi [$F(1,20)=3.16$, $p=0.09$].

ii. Conducting villi (stem villi)

There was no significant effect of HIV status on the immunoexpression of VEGF on stem villi [$F(1,20)=0.83$, $p=0.374$]. However, a significant effect of type of pregnancy (normotensive and pre-eclamptics) on VEGF immunoexpression within stem villi [$F(1,20)=5.10$, $p=0.035$] was noted, with the N- showing higher VEGF immunoexpression (mean=10.16, 95% CI: 8.61-11.72) compared to P- (mean=5.93, 95% CI: 4.38-7.49) groups. Notably, within the HIV positive groups, a high immunoexpression of VEGF was noted among P+ (mean=7.80, 95% CI: 6.24-9.35) compared to N+ (mean=6.94, 95% CI: 5.38-8.50, $p=0.093$) groups. Bonferroni post hoc analyses demonstrated a significant effect between the N- *vs* P- ($p=0.000$) and the N- vs N+ ($p=0.005$) groups (**Table 3.9; Fig. 3.34)**.

The mean intensity of VEGF immunoreactivity among the N-, N+, P- and P+ groups expressed as a densitometric grey value was 10(9.2-11); 6(5-9.4); 5(4.5-7.2) and 8(5.9-9.6) respectively, with a range of 30(23-33) to 85(77-89).

In addition, a significant effect was noted between HIV status and type of pregnancy (normotensive and pre-eclamptic) of VEGF immunoexpression in stem villi [$F(1,20)=11.59$, $p=0.003$].

3.3.4.2.2 Correlation of VEGF immunoexpression between exchange and stem villi

A Pearson correlation coefficient was computed to assess the relationship between exchange and stem villi immunoexpression of VEGF across all study groups. There was no correlation between the exchange and stem villi immunoexpression of VEGF (r=0.147, *p*=0.49).

Table 3.8: Morphometric image analysis of VEGF immunoreactivity in exchange villi

	Normotensive Pregnant women		Pre-eclamptic women		
	HIV Negative (N-)	**HIV Positive (N+)**	**HIV Negative (P-)**	**HIV Positive (P+)**	**p value**
n	36	40	30	24	
Area Frame μm^2	312843.68	312843.68	312843.68	312843.68	
Field Area of villi %	33.5±10.8	36.9±9.4	37.4±8.4	41±4.4	*p*=0.63
Immunoreactivity %	8.29(±1.53)	9.23(±2.58)	4.3(±0.9)	7.72(±1.31)	*p*=0.001
Intensity of immunoreactivity(median + interquartile range)					
Mean	49(41-61)	47(42-55)	49(40-50)	55(48-59)	
Min	25(19-32)	27(23-30)	31(24-32)	36(30-41)	
Max	72(60-82)	63(57-72)	63(53-65)	68(61-73)	

Significance:*p<0.05; n=130; parametric-data (mean ± SD)

Densitometry (grayscale 0-255)

Table 3.9: Morphometric image analysis of VEGF immunoreactivity in stem villi

	Normotensive Pregnant women		Pre-eclamptic women		
	HIV Negative (N-)	**HIV Positive (N+)**	**HIV Negative (P-)**	**HIV Positive (P+)**	**p value**
n	36	40	30	24	
Immunoreactivity%	10.16(±0.92)	6.94(±2.24)	5.94(±2.01)	7.79(±1.85)	*p*=0.003
Intensity of immunoreactivity (median + interquartile range)					
Mean	62(46-76)	59(53-69)	50(47-62)	70(64-71)	
Min	30(23-33)	35(29-41)	32(28-43)	50(46-51)	
Max	82(62-105)	74(67-94)	66(60-76)	85(77-89)	

Significance:*p<0.05; n=130; parametric-data (mean ± SD)

Densitometry (grayscale 0-255)

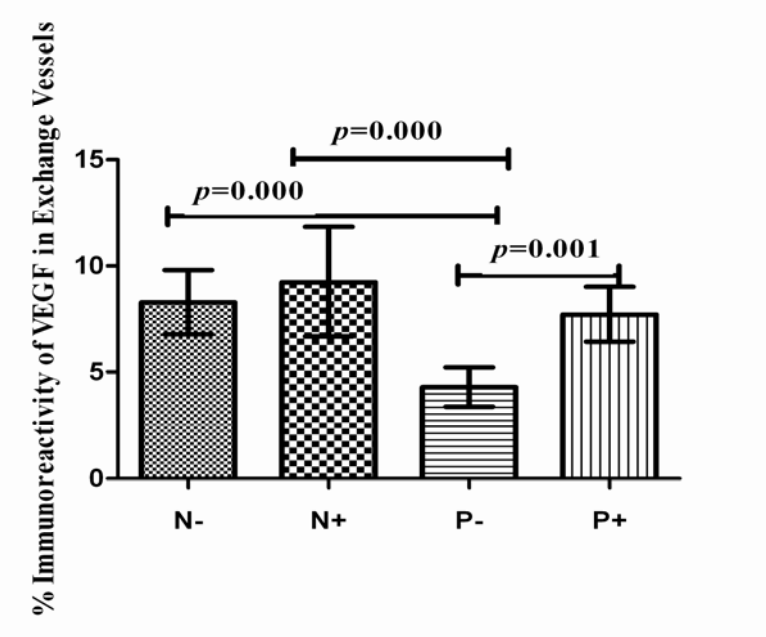

Figure 3.33: Percentage immunoreactivity of VEGF in exchange vessels (mean ± SD) within HIV positive pre-eclamptic (P+); HIV negative pre-eclamptic (P-); HIV negative normotensive pregnant (N-) and HIV positive pregnant women (N+).

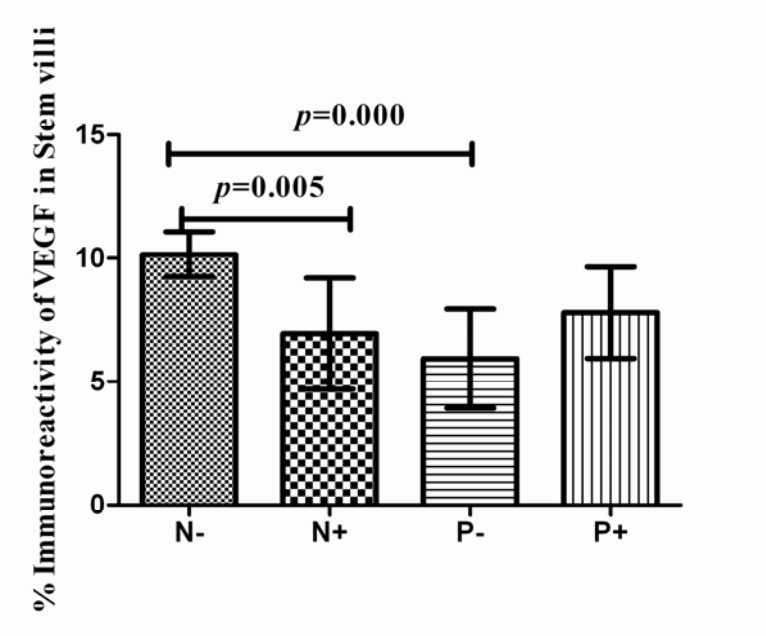

Figure 3.34: Percentage immunoreactivity of VEGF in stem villi (mean ± SD) within HIV positive pre-eclamptic (P+); HIV negative pre-eclamptic (P-); HIV negative normotensive pregnant (N-) and HIV positive pregnant women (N+).

3.4 Immunoelectron Microscopy (IEM)

3.4.1..Qualitative assessment of placental villi across all groups

Ultra-structural examination of the placental villi of all groups conformed to the IHC findings (**3.3**). All villi displayed syncytiotrophoblasts, intermittent cytotrophoblasts and fetal capillaries within stroma (**Fig. 3.35a-c**). Syncytiotrophoblasts displayed a dark cytoplasm. They were multinucleate with prominent nucleolus and peripherally distributed heterochromatin. The cytotrophoblasts were separated from the stroma by a basement membrane. Microvilli projections were observed on the apical plasmalemma of the syncytiotrophoblasts (**Fig. 3.35a-c**). Cytotrophoblasts (**Fig. 3.35b-c**) were observed beneath the syncytium. They displayed a relatively undifferentiated pale cytoplasm with large nuclei that had a predominance of euchromatin. The villi stroma was occupied by loose connective tissue with occasional fibroblasts and Hofbauer cells.

Accumulation of syncytial nuclei as observed in syncytial knots (**Fig. 3.36a-b; d**) and syncytial bridges (**Fig. 3.36c-d**) were noted across all groups. The nuclei of these knots and bridges displayed pronounced heterochromatin distribution. Subcellularly, well developed pronounced endoplasmic reticulum systems were observed traversing the syncytial cytoplasm (**Fig. 3.38a-c**). Intracytoplasmic vesicles were evident along the luminal intervillous space throughout all groups in the exchange villi (**Fig. 3.38a**).

Terminal villi exhibited a high degree of fetal capillarisation (**Fig. 3.37a-b**). Occasionally, fetal capillaries bulged against the syncytiotrophoblast layer. In this regard, the syncytial membrane was reduced to a thin vasculosyncytial membrane (**Fig. 3.37a**).

Intermediate villi displayed narrow capillaries and dilated sinusoids (**Fig. 3.37c-d**). A thin syncytiotrophoblastic cover (**Fig. 3.37d**) was evident. Fetal capillaries were lined by endothelial cells displaying the typical architecture (**Fig. 3.39a-d**). Stroma of villi contained loose connective tissue interspersed with cellular types and fibroblasts were noted. Mesenchymal cells with electron dense nuclei were also noted (**Fig. 3.38d**).

Evidence of both intravillous (**Figs. 3.36a; 3.38a**) and perivillous (**Fig. 3.36b**) fibrin deposition was noted across all groups. Qualitative assessment indicated a predominance of dilated pools of cisternal type ER in the HIV positive groups (**Fig. 3.38c**).

Due to pre-selection of region of interests from toluidine blue, stem villi was excluded across all groups.

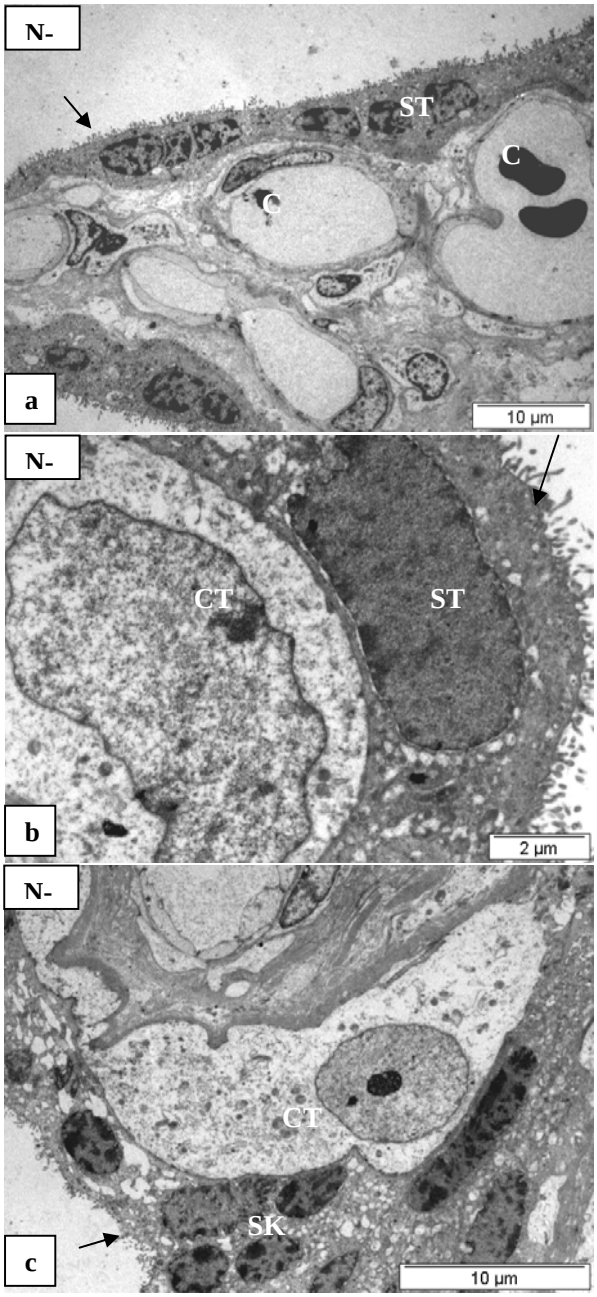

Figure 3.35: Transmission electron micrographs illustrating (a-c) syncytiotrophoblast (ST) and (b-c) cytotrophoblast (CT) in N- groups. Note fetal capillaries (C), microvilli (arrow) and syncytial knots (SK).

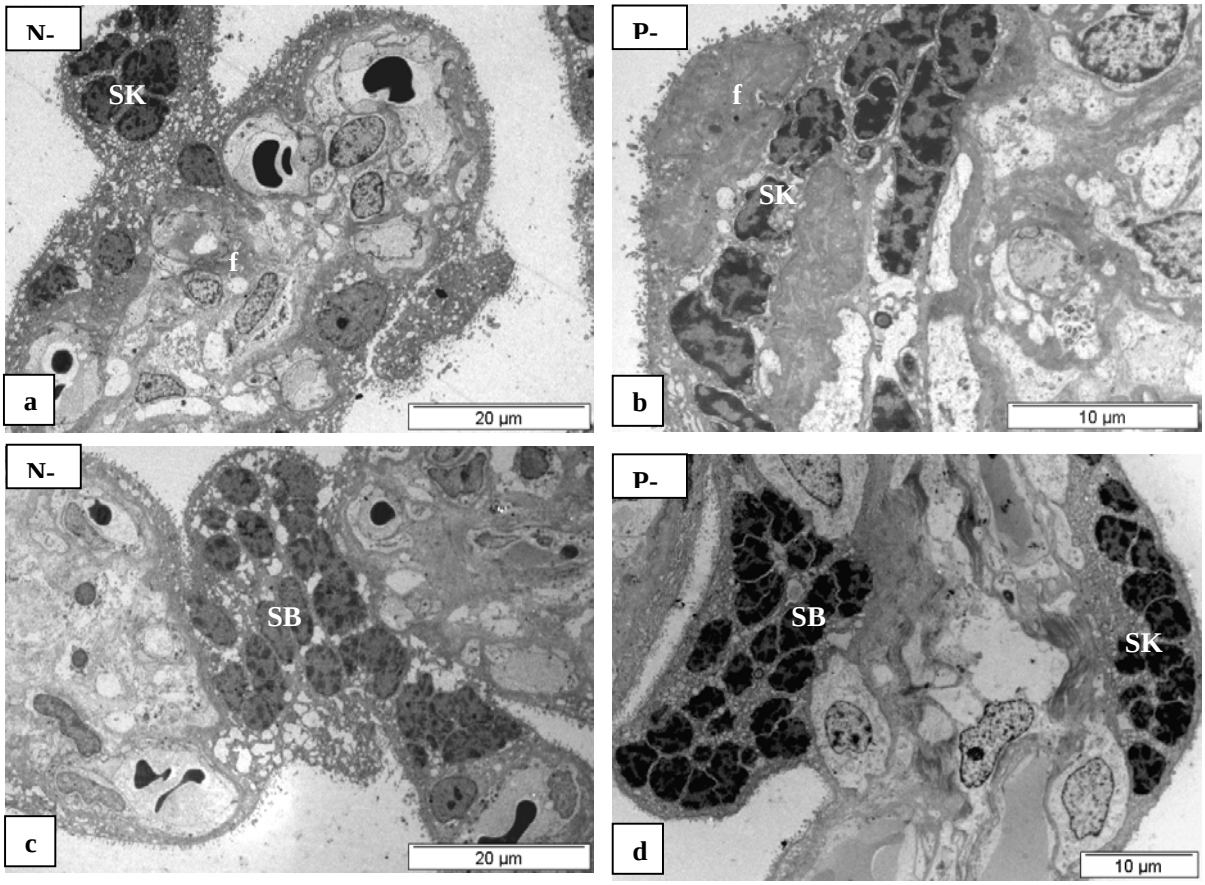

Figure 3.36: Transmission electron micrographs illustrating accumulation of syncytial nuclei within (a,b,d) syncytial knots (SK) and (c-d) syncytial bridges (SB) in N- (a,c) and P- (b,d) groups. Note fibrin deposition (f) in syncytial knot

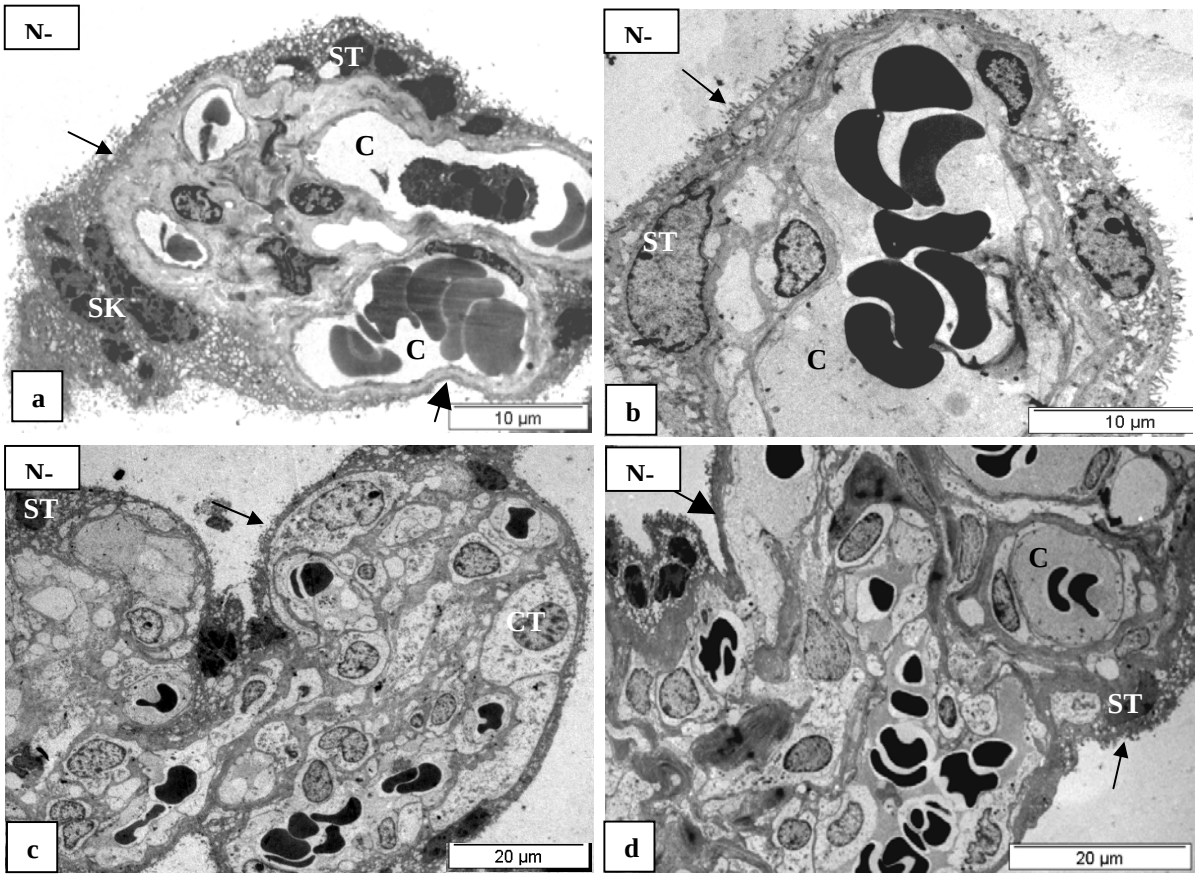

Figure 3.37: Transmission electron micrographs illustrating terminal villi (a-b) and intermediate villi (c-d) in N- groups. Note syncytiotrophoblast (ST), cytotrophoblast (CT), fetal capillaries (C), microvilli (arrows), syncytial knot (SK) and thin vasculosyncytial membrane (arrows).

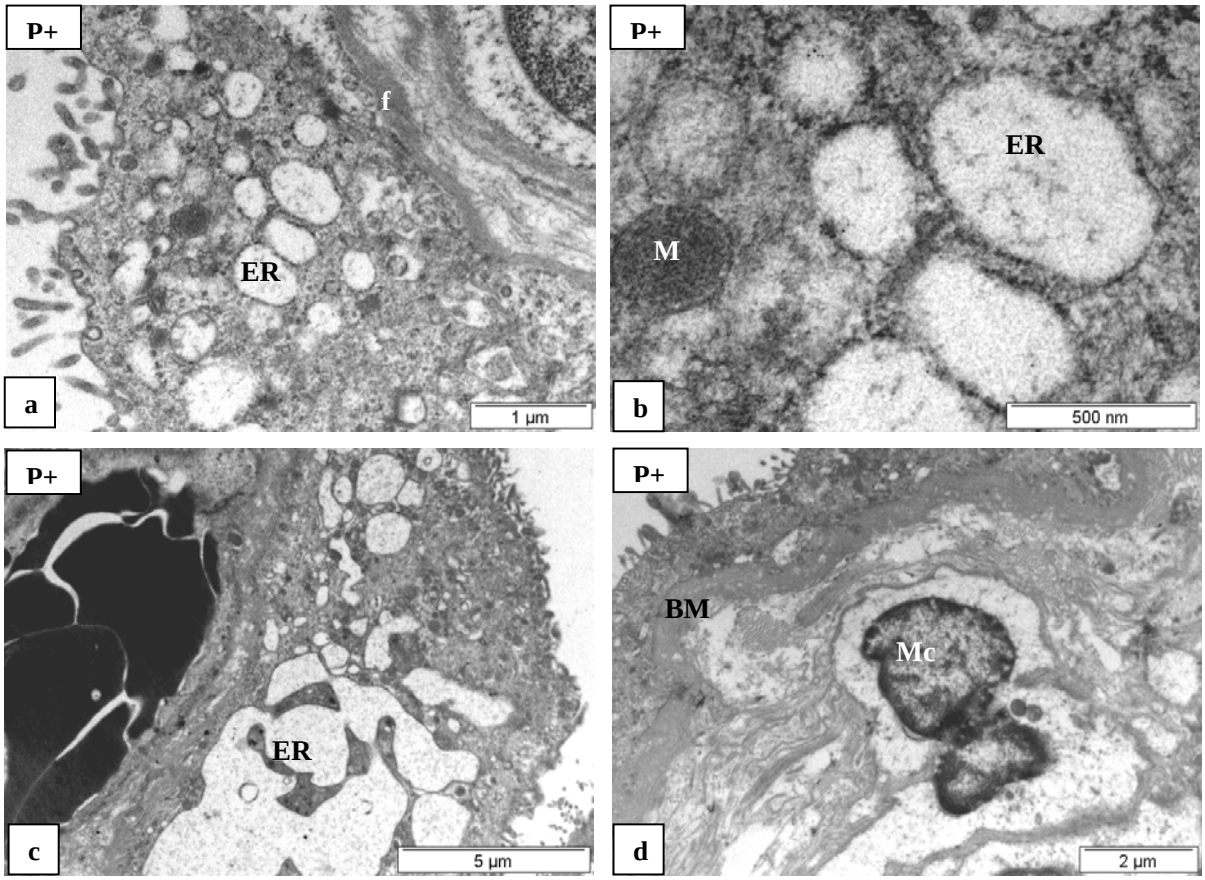

Figure 3.38: Electron micrographs depicting (a-c) pools of cisternal type endoplasmic reticulum (ER) in syncytiotrophoblasts and (d) mesenchymal cells (Mc) in P+ groups. Note mitochondria (M) and thickened basement membrane (BM).

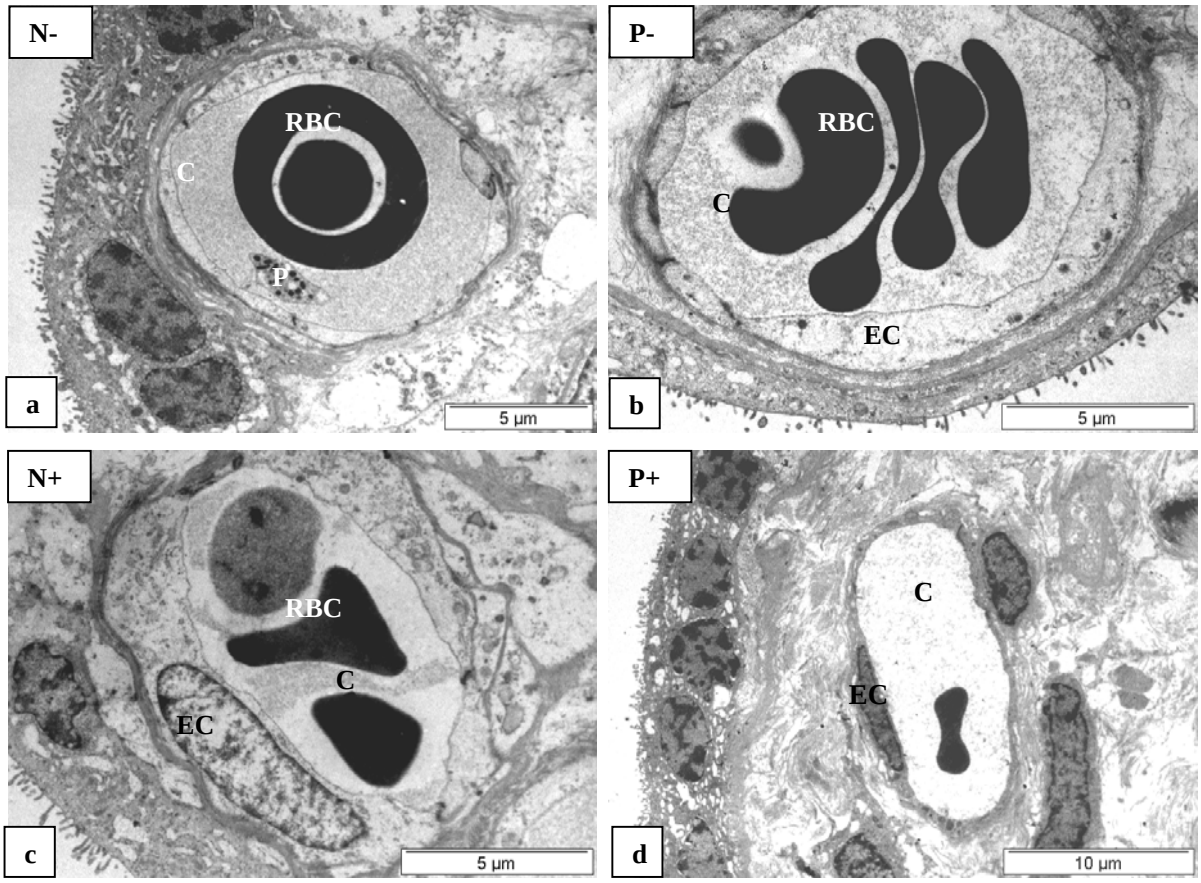

Figure 3.39: Electron micrographs depicting (a-d) endothelial cells (EC) within fetal vessels (C) in N- (a), P- (b), N+ (c) and P+ (d) groups. Note. red blood cells (RBC) and platelet (P).

3.4.2 Immunoelectron localisation of antibody expression

3.4.2.1 Immunoelectron localisation of sVEGF-R1/Flt-1 in placental tissue

Ultra-structural evaluation for sFlt-1 revealed 10nm gold particles immunolocalised within the cyto- and syncytiotrophoblast cell populations across the N- (**Fig. 3.40a-b**) and P- (**Fig. 3.41a-b**) groups, and the N+ (**Fig. 3.42a-b**) and P+ (**Fig. 3.43a-b**) groups. This anti-angiogenic Flt-1 molecule was occasionally immunolocalised on the apical and basal surface of the syncytial membrane (**Fig. 3.46a-b; 3.47a**).

Gold particles were also observed within cytoplasm of the syncytiotrophoblasts (**Fig. 3.40a-b; 3.45a-b**). Subcellular gold particles were localised predominantly within the ER and mitochondria (**Fig. 3.44a-b-3.45a-b**). Occasionally, they were observed in the perinuclear region of both the syncytio and cytotrophoblasts.

Additionally, gold particles were noted in all endothelial cells across the N- (**Fig. 3.48a-b**) and P- (**Fig. 3.49a-b**), and the N+ (**Fig. 3.50a-b**) and P+ (**Fig. 3.51a-b**) groups. An absence of immunoreactivity was observed in control tissue (**Fig. 3.52a-b**).

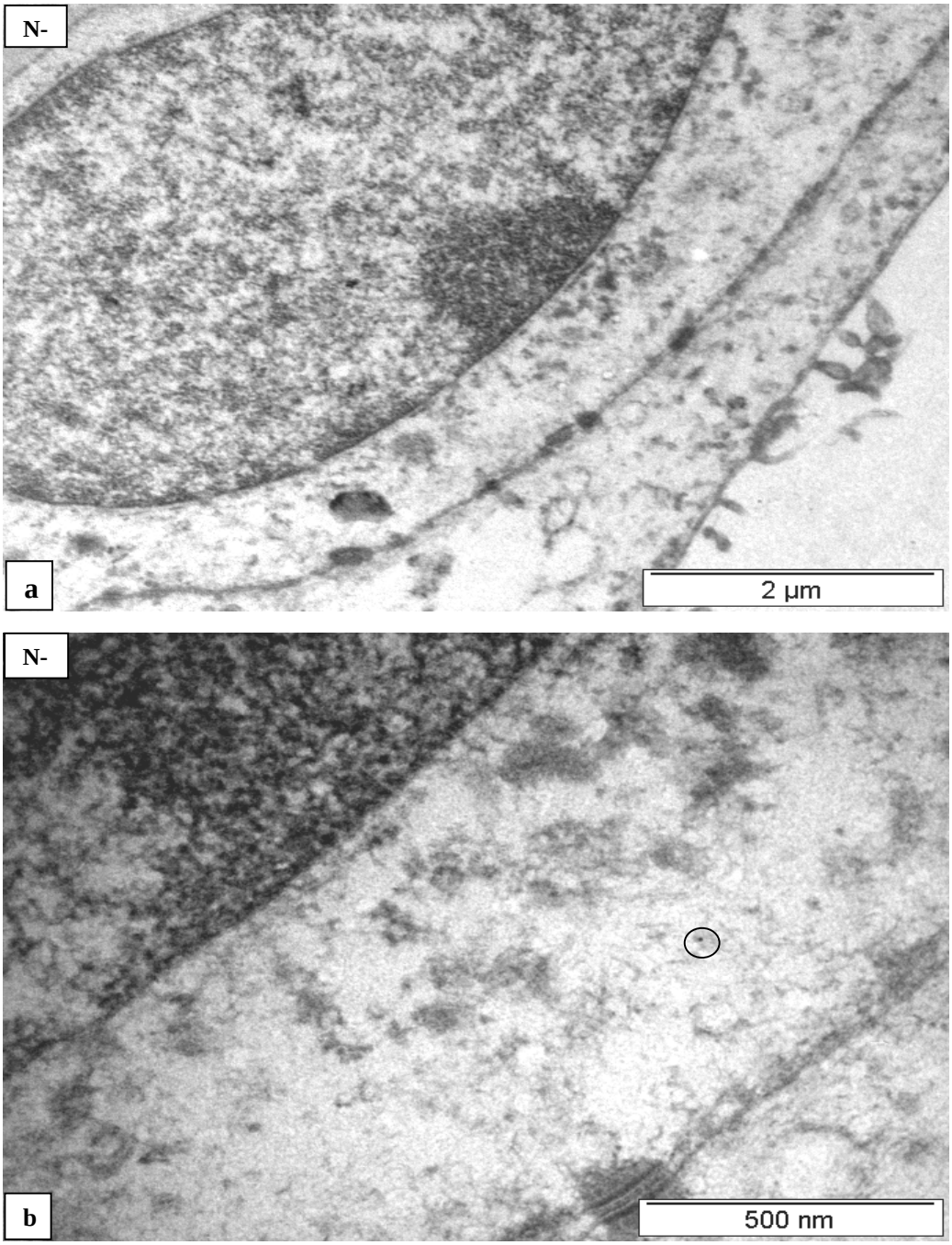

Figure 3.40: Electron micrographs depicting subcellular sFlt-1 immunolocalisation in the N- (circle) group within (a-b) syncytiotrophoblast (ST). Note nuclei (N).

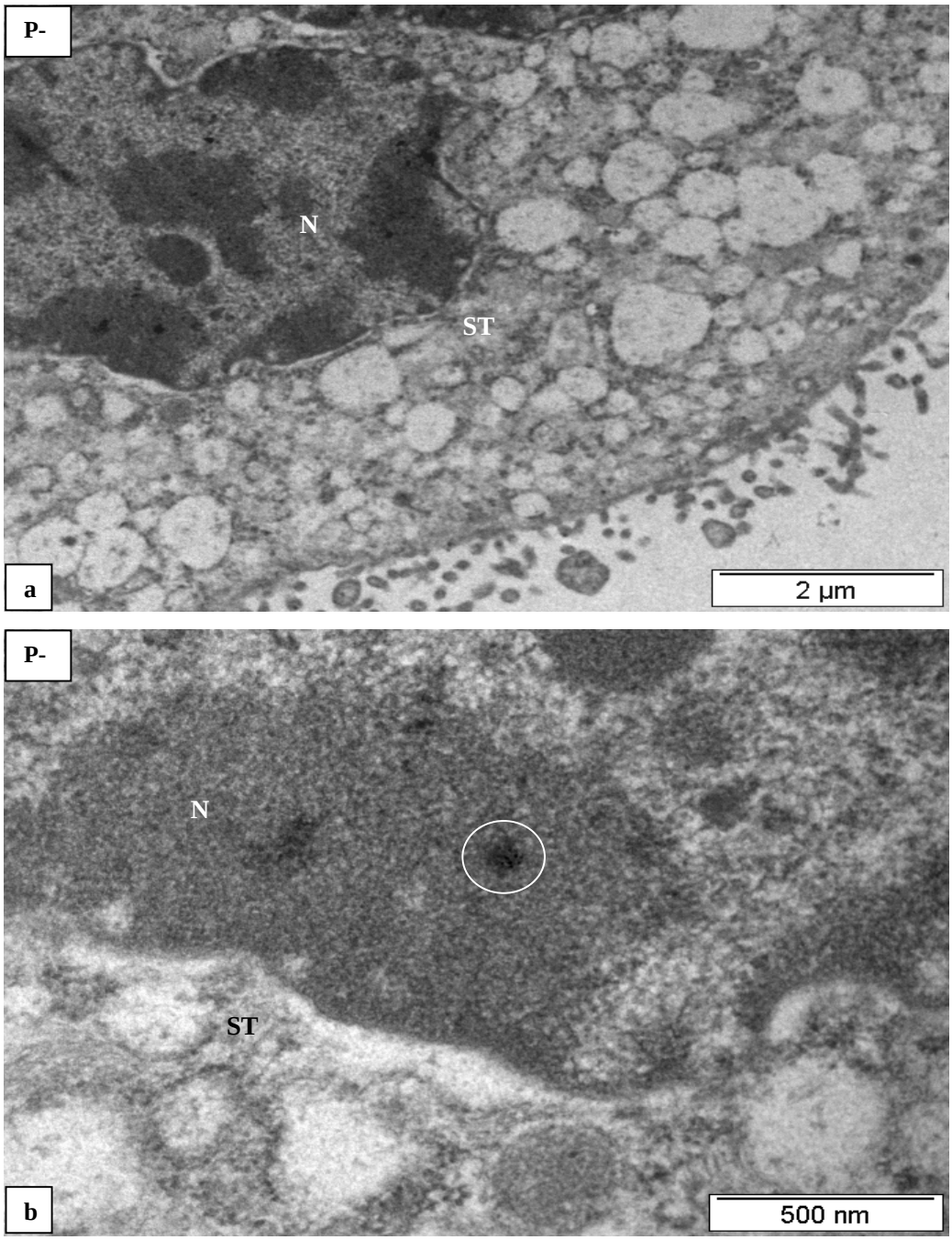

Figure 3.41: Electron micrographs depicting subcellular sFlt-1 immunolocalisation in the P- (circle) group within (a-b) syncytiotrophoblast (ST). Note nuclei (N).

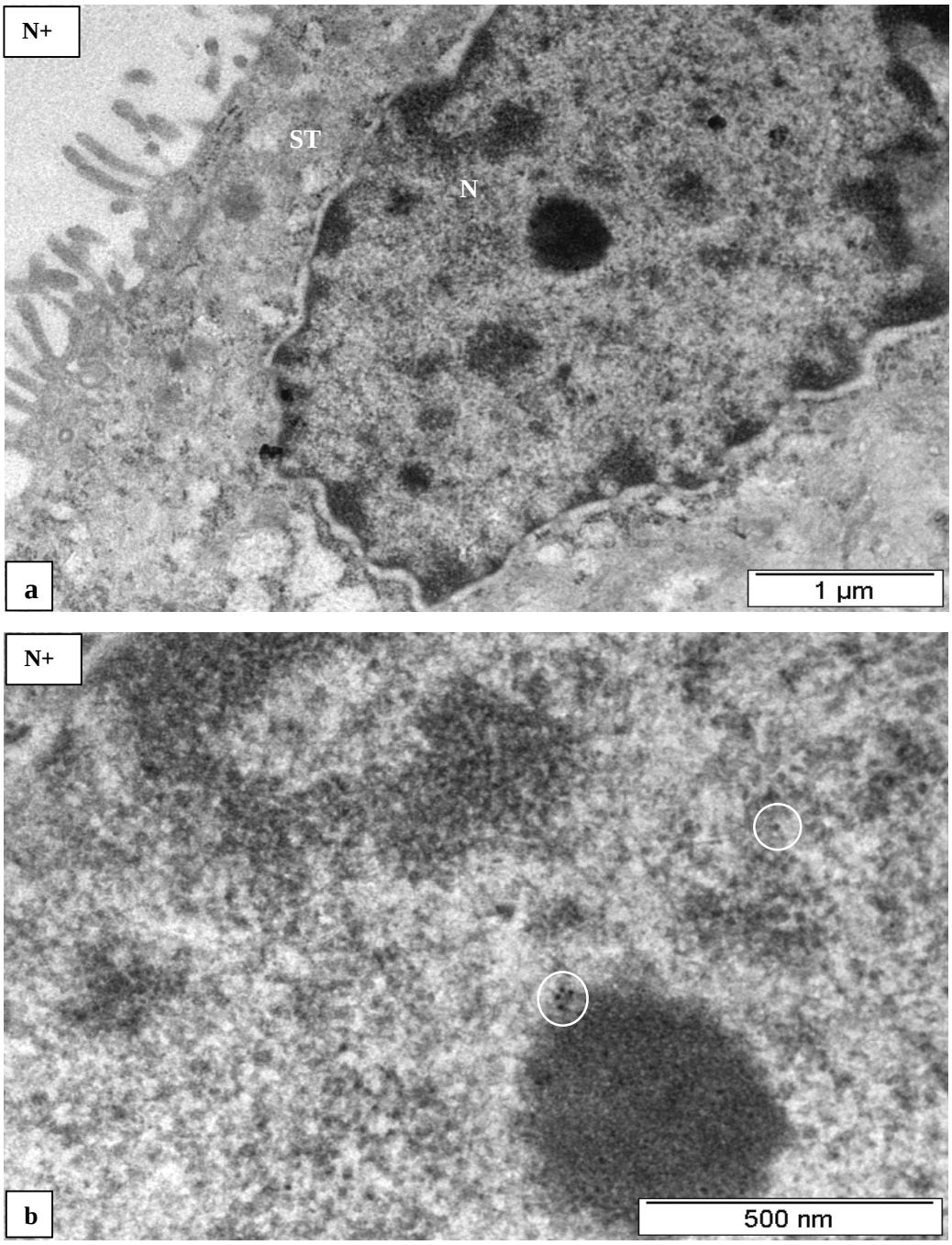

Figure 3.42: Electron micrographs depicting sFlt-1 immunolocalisation across N+ (circle) group within (a-b) syncytiotrophoblast (ST). Note nuclei (N).

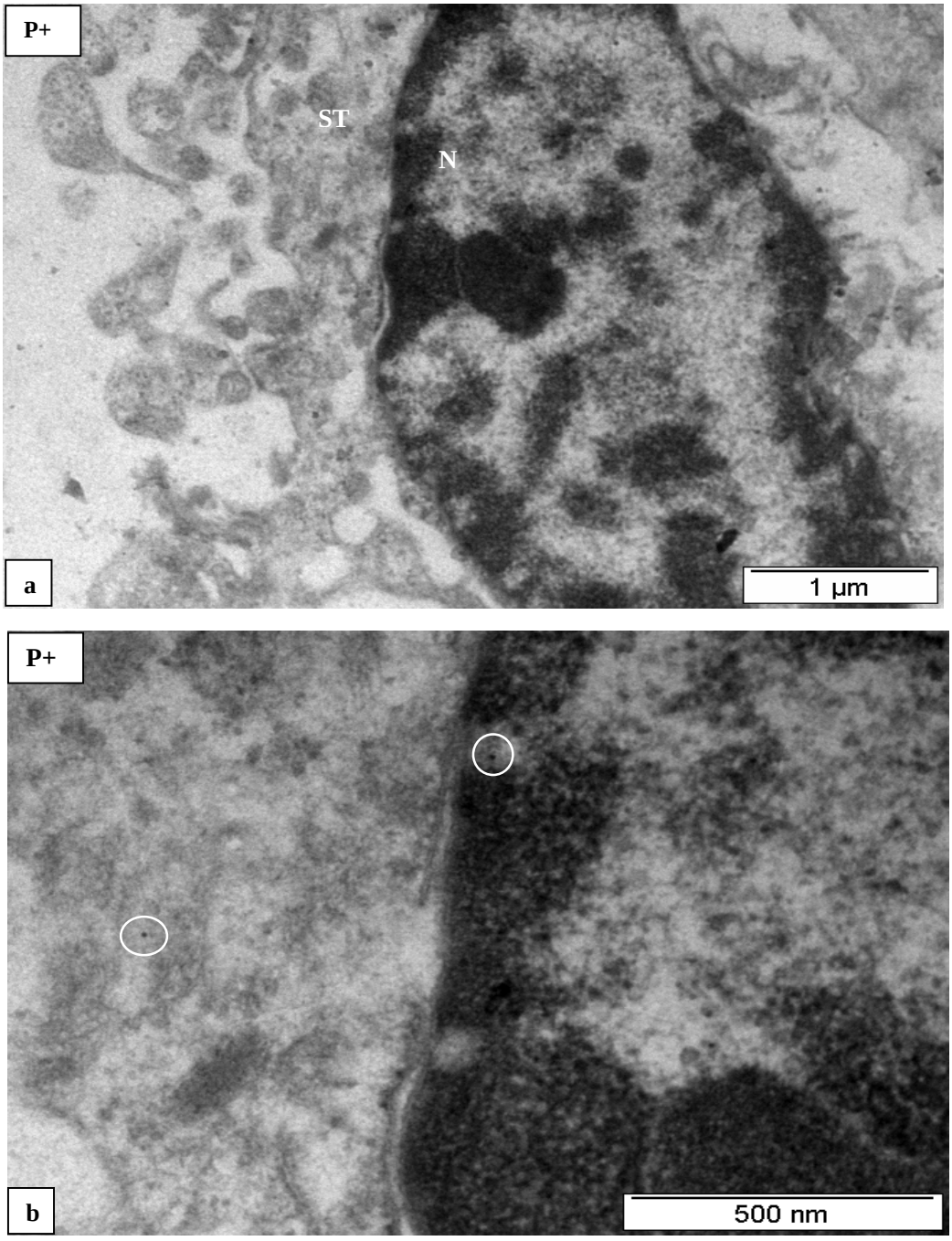

Figure 3.43: Electron micrographs depicting sFlt-1 immunolocalisation across P+ (circle) group within (a-b) syncytiotrophoblast (ST). Note nuclei (N).

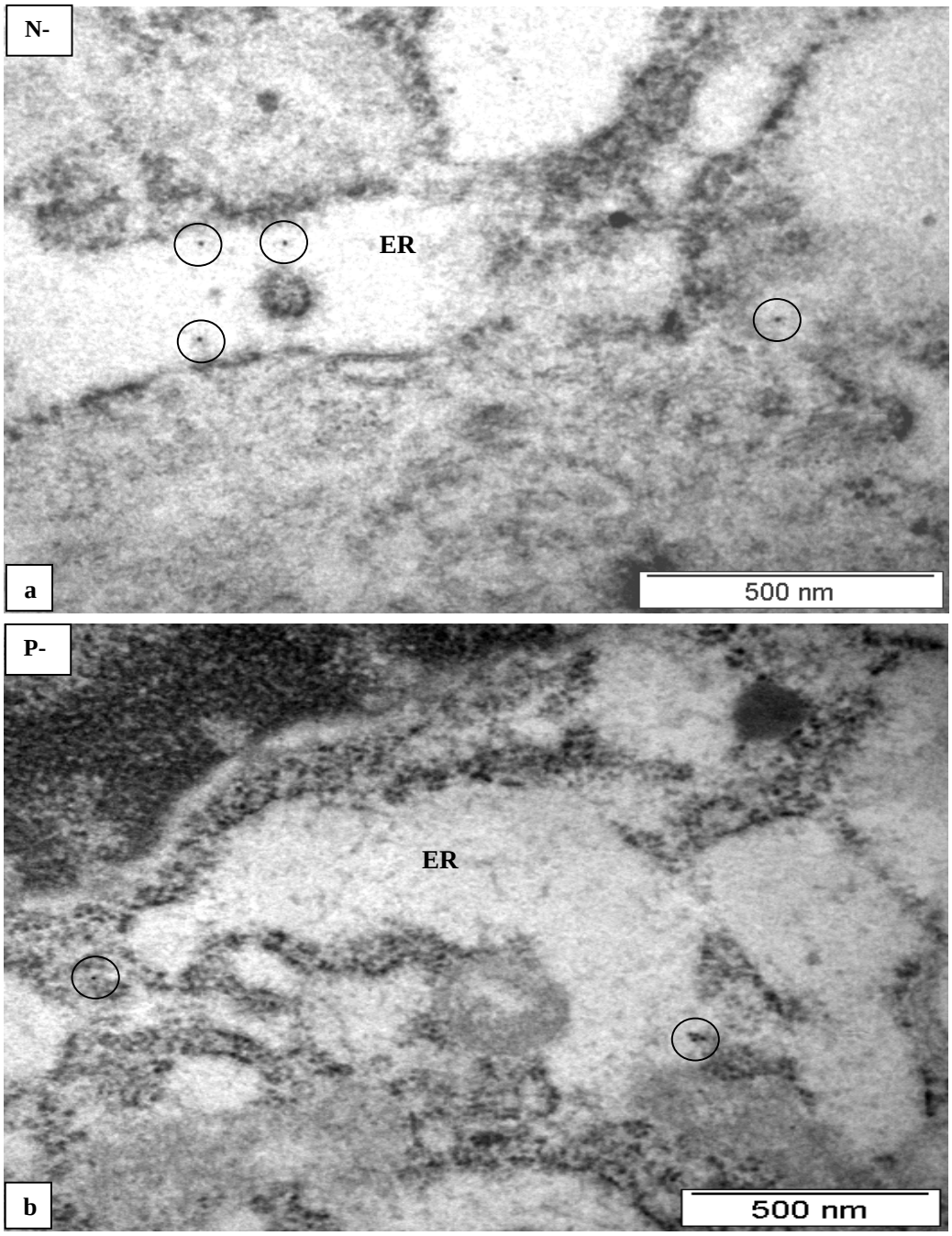

Figure 3.44: Electron micrographs depicting sFlt-1 immunolocalisation across N- and P-groups (circle) within (a-b) endoplasmic reticulum (ER) of syncytiotrophoblast.

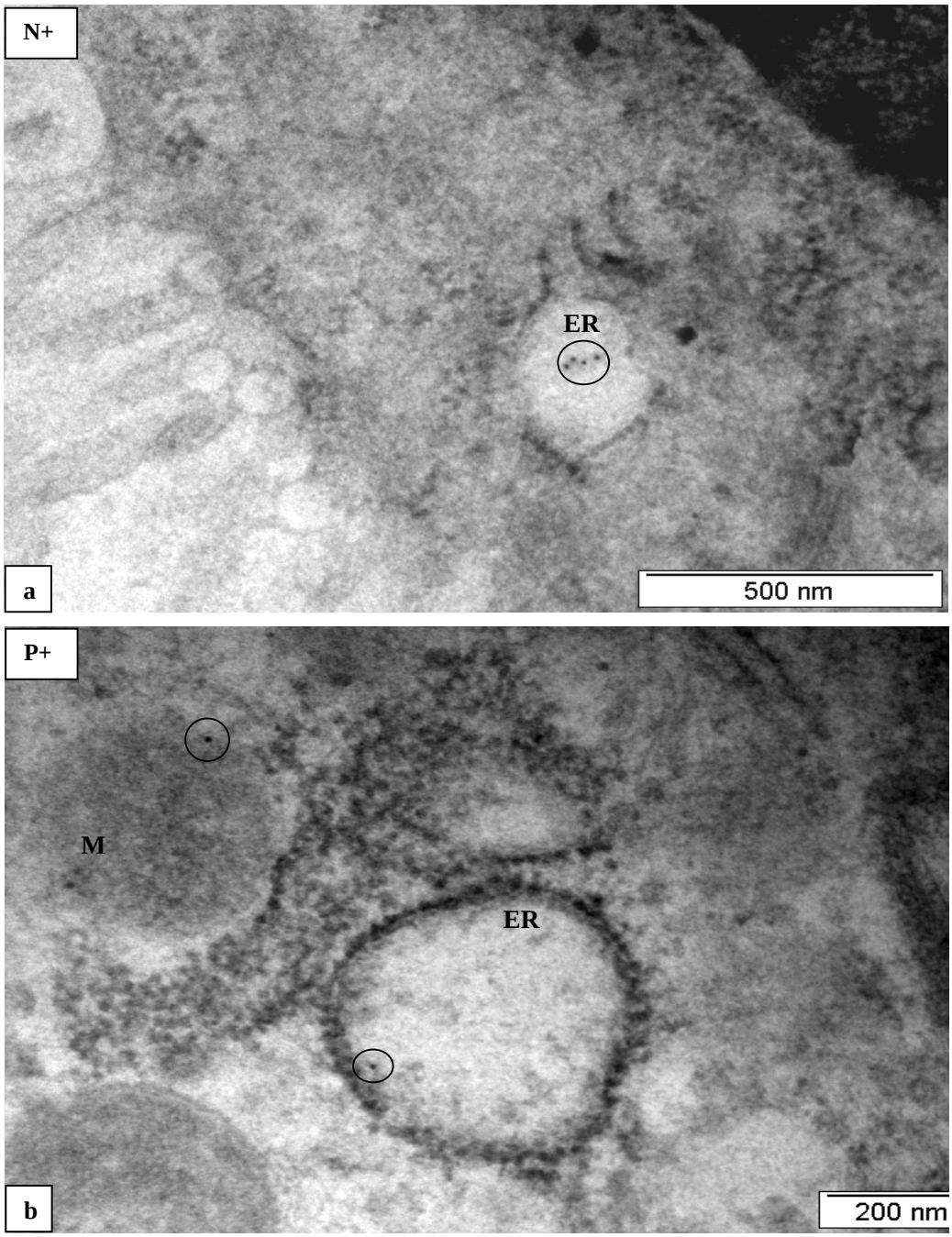

Figure 3.45: Electron micrographs depicting sFlt-1 immunolocalisation across N+ and P+ groups (circle) within (a-b) endoplasmic reticulum (ER) and (d) mitochondria (M) of syncytiotrophoblast.

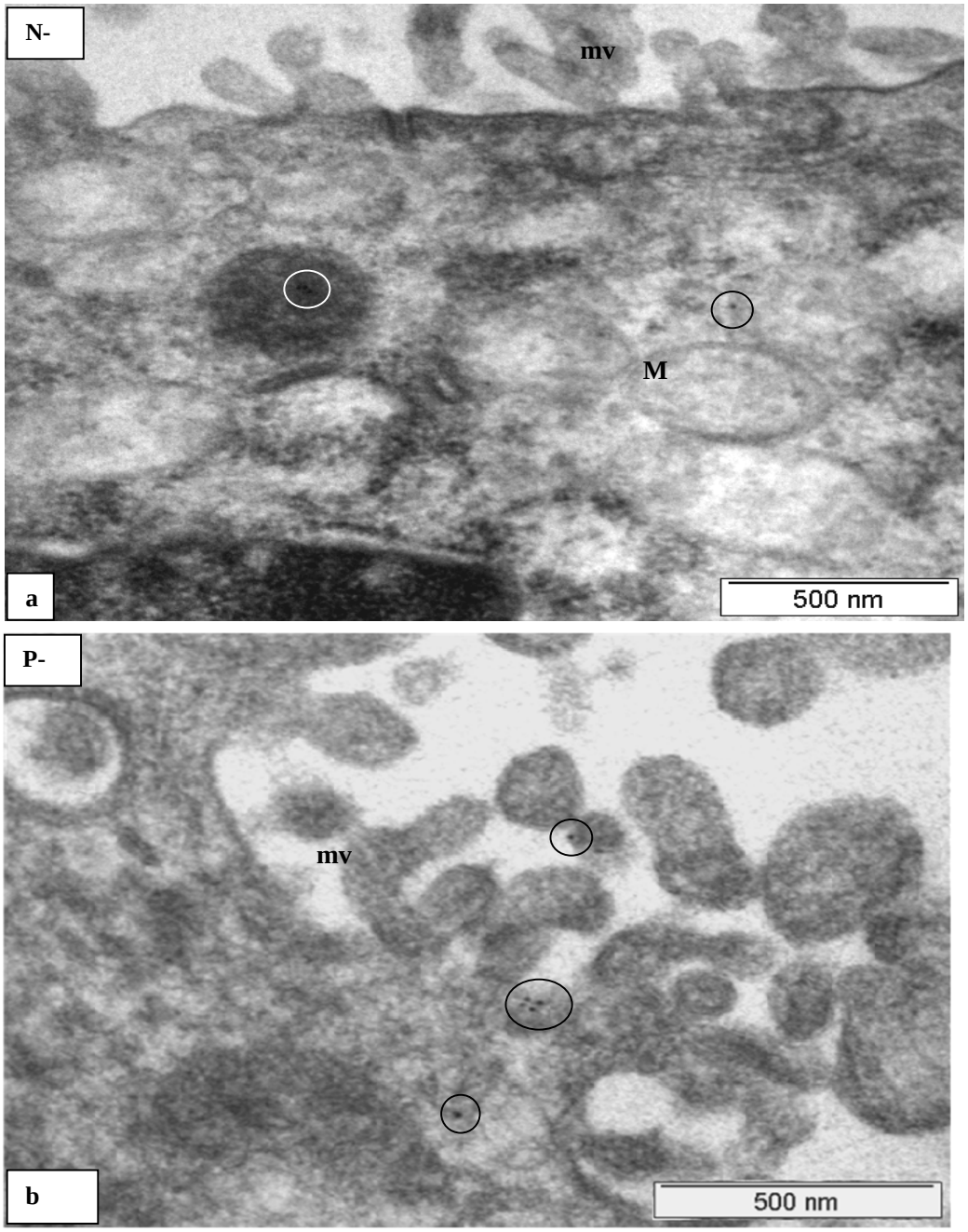

Figure 3.46: Electron micrographs depicting sFlt-1 immunolocalisation across N- and P- (circle) within (a-b) microvilli (mv) and (a) mitochondria (M) of syncytiotrophoblast.

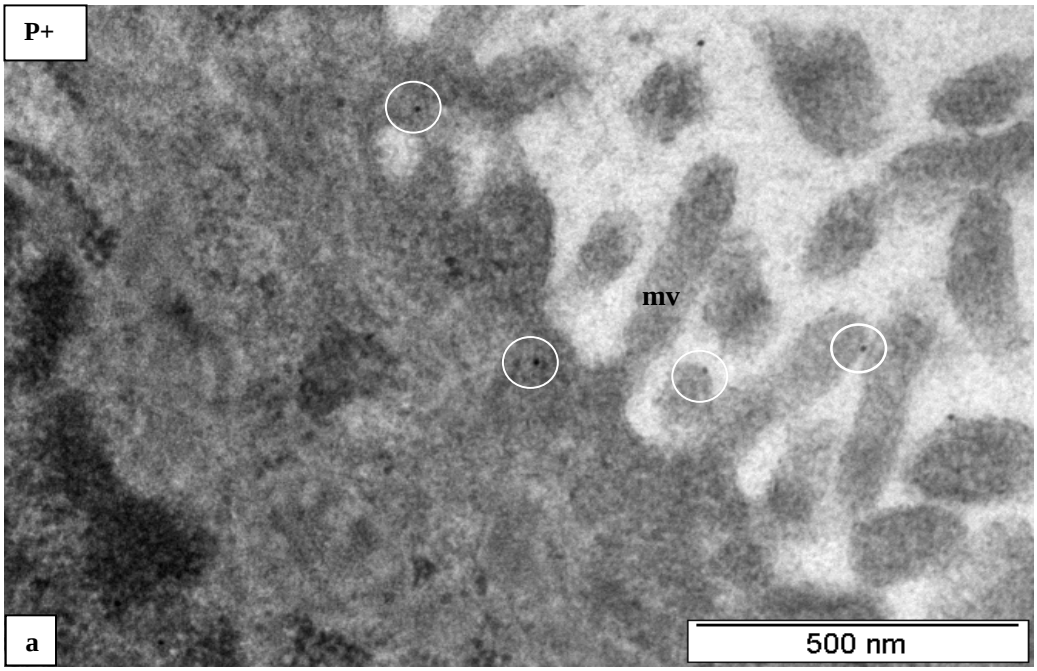

Figure 3.47: Electron micrographs depicting sFlt-1 immunolocalisation across P+ (circle) group within (a) microvilli (mv) of syncytiotrophoblast.

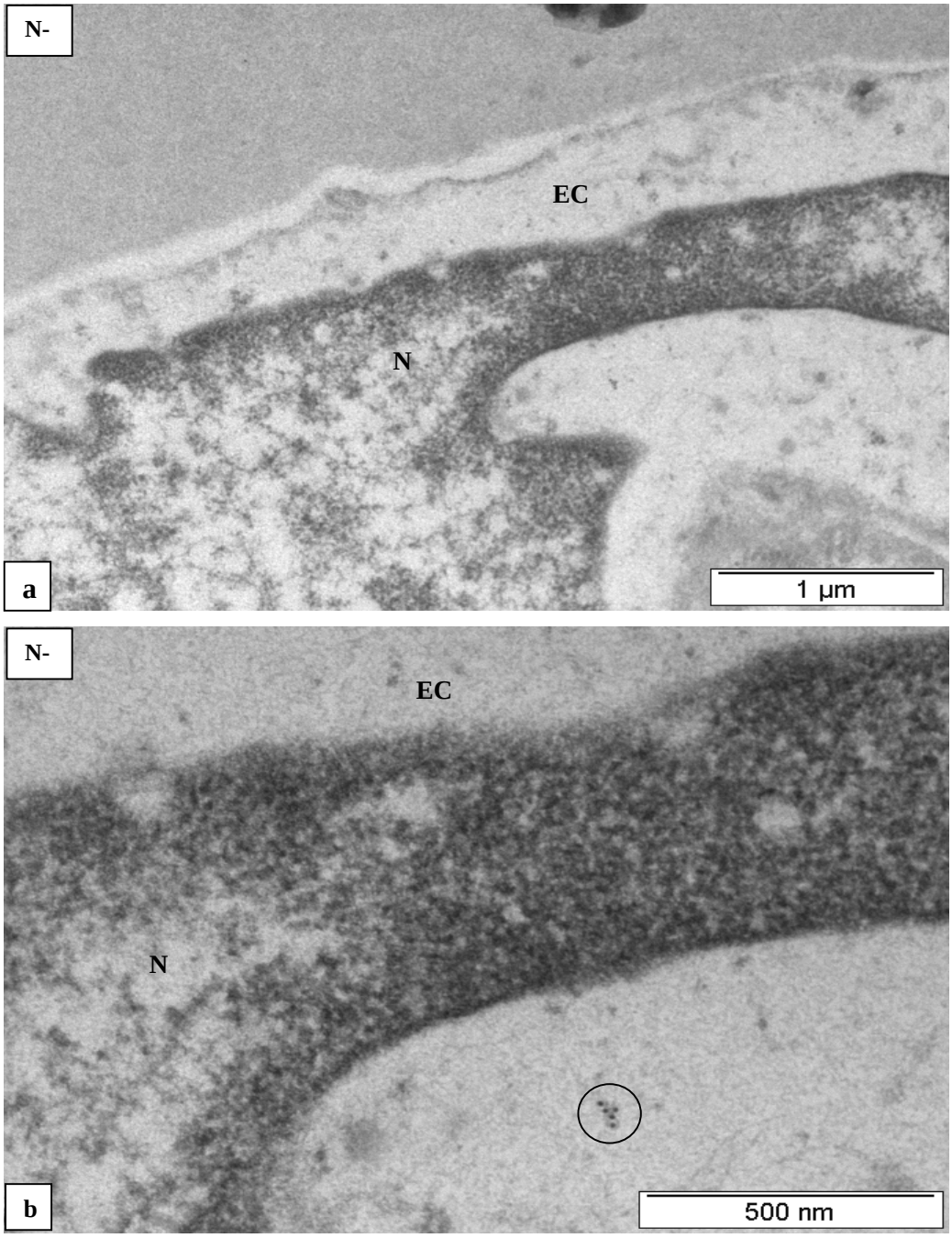

Figure 3.48: Electron micrographs depicting sFlt-1 immunolocalisation in the N- group (circle) within (a-b) endothelial cells (EC).

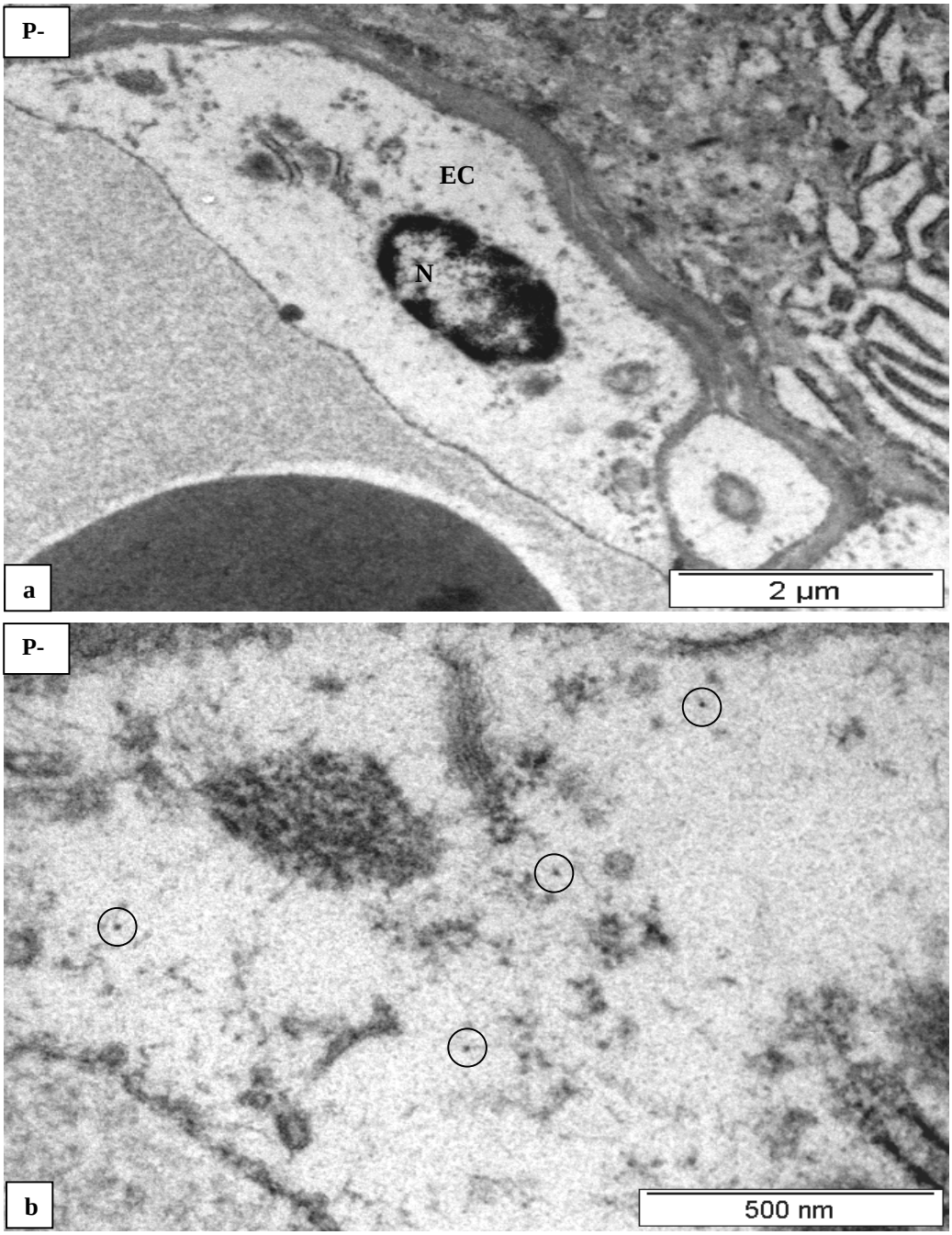

Figure 3.49: Electron micrographs depicting sFlt-1 immunolocalisation in the P- group (circle) within (a-b) endothelial cells (EC).

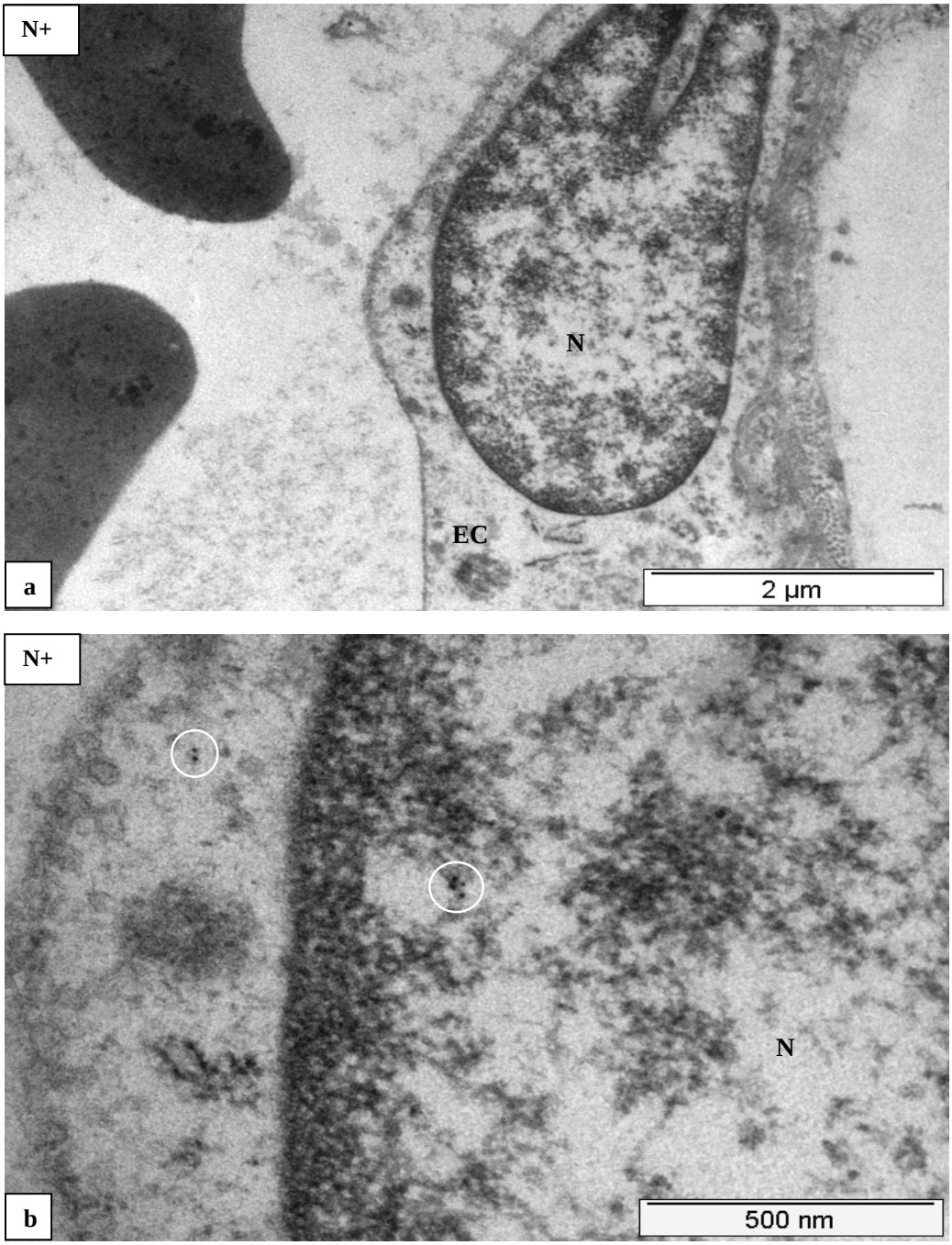

Figure 3.50: Electron micrographs depicting sFlt-1 immunolocalisation in the N+ group (circle) within (a-b) endothelial cells (EC).

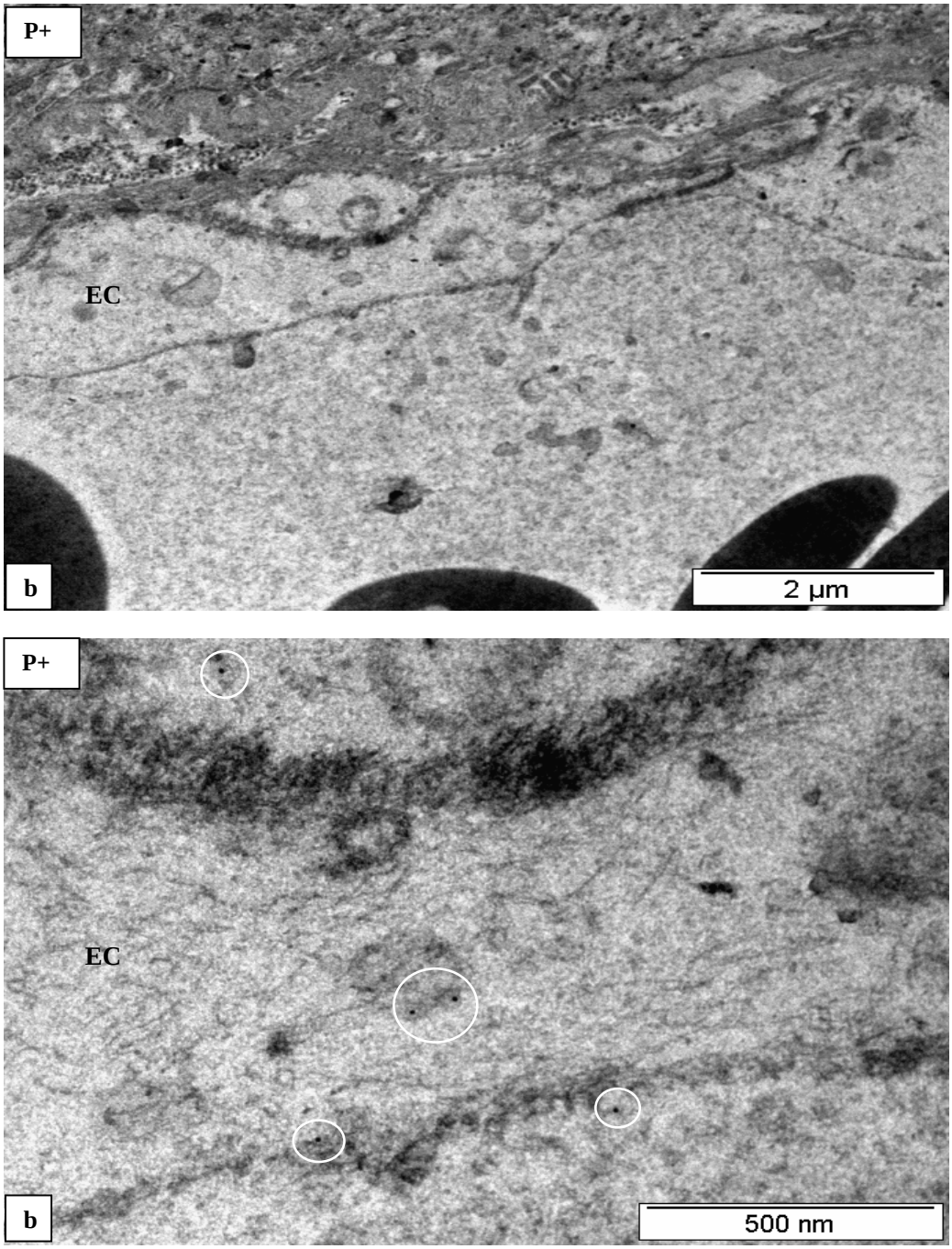

Figure 3.51: Electron micrographs depicting sFlt-1 immunolocalisation in the P+ group (circle) within (a-b) endothelial cells (EC).

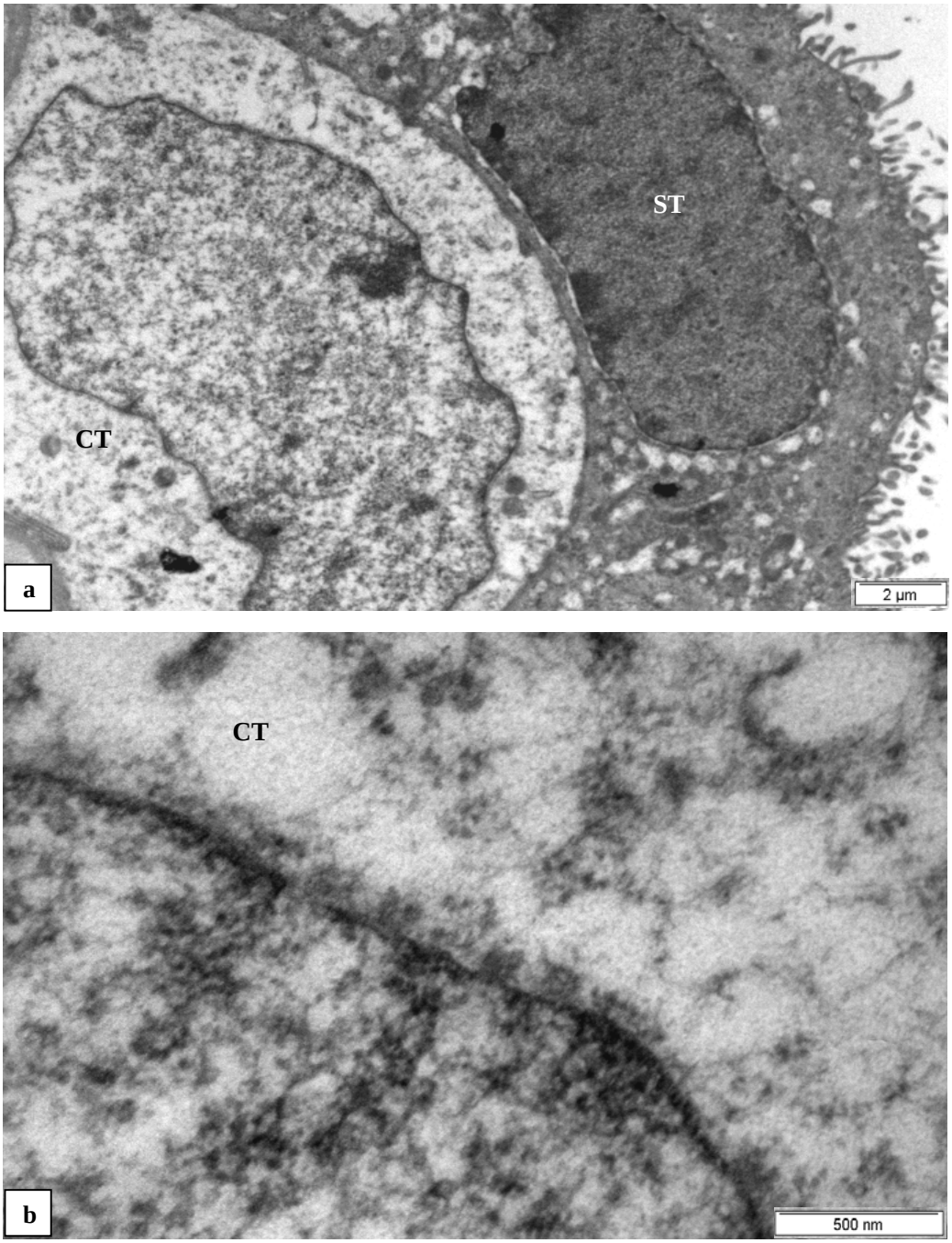

Figure 3.52: Electron micrographs depicting (a-b) the absence of gold particles in control tissue

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3.4.2.2 Immunoelectron localisation of sEng in placental tissue

Endoglin was immunolocalised within the cyto- and syncytiotrophoblast cell populations across the N- (**Fig. 53a-b**) and P- (**Fig. 3.54a-b**) and N+ (**Fig. 55a-b**) and P+ (**Fig. 3.56a-b**) groups. Subcellular immuno-localization for all regions was predominantly within the ER and mitochondria.

Intracytoplasmic gold particles were observed in the mitochondria and cytoplasm of endothelial cells across the N- (**Fig. 3.57a-b**) and P- (**Fig. 3.58a-b**) and N+ (**Fig. 3.59a-b**) and P+ (**Fig. 3.60a-b**) groups.

Additionally, a method control revealed the absence of gold particles (**Fig. 3.61a-b**) in all groups.

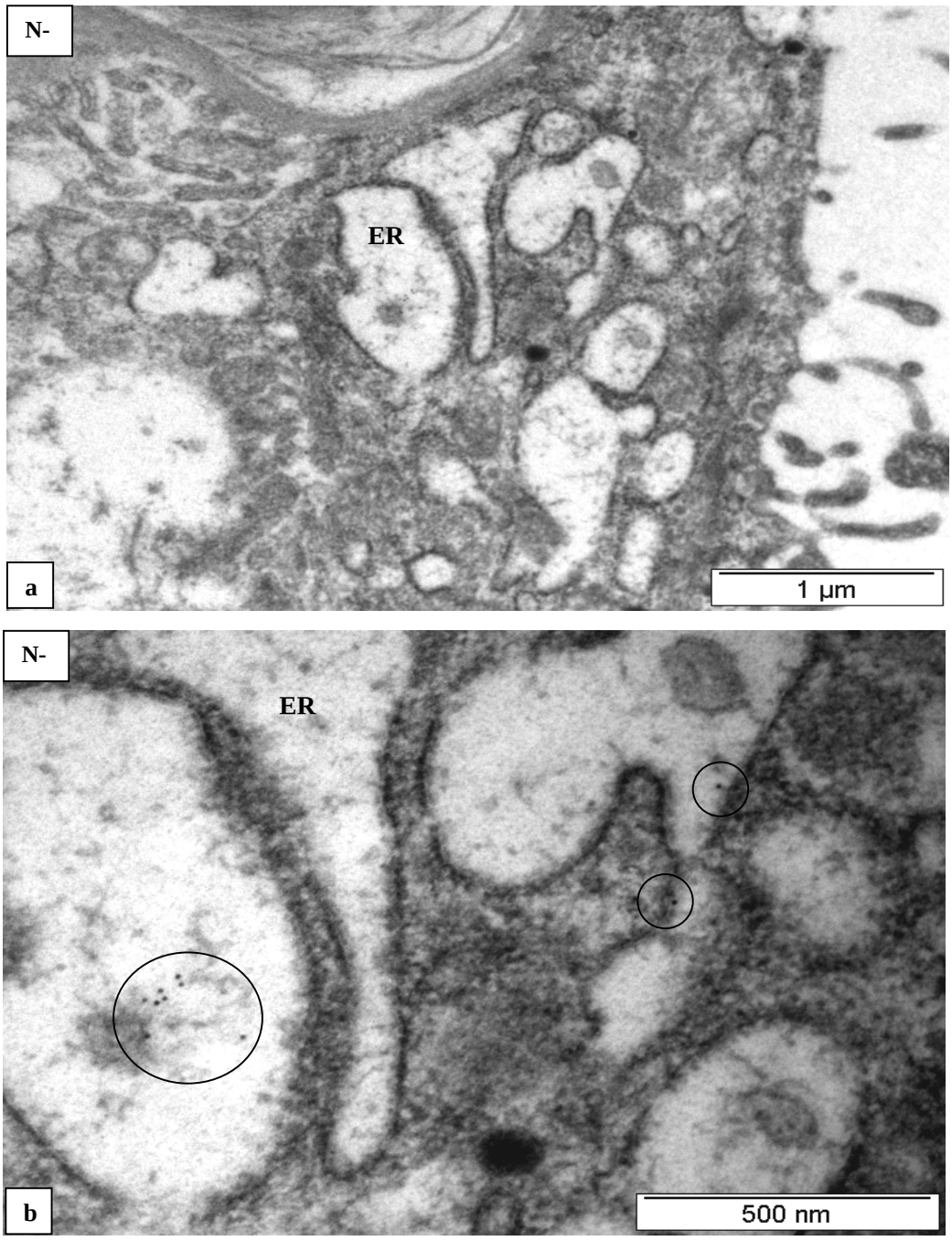


Figure 3.53: Electron micrographs depicting sEng immunolocalisation across N- groups (circle) within (a-b) endoplasmic reticulum (ER) of syncytiotrophoblast (ST).

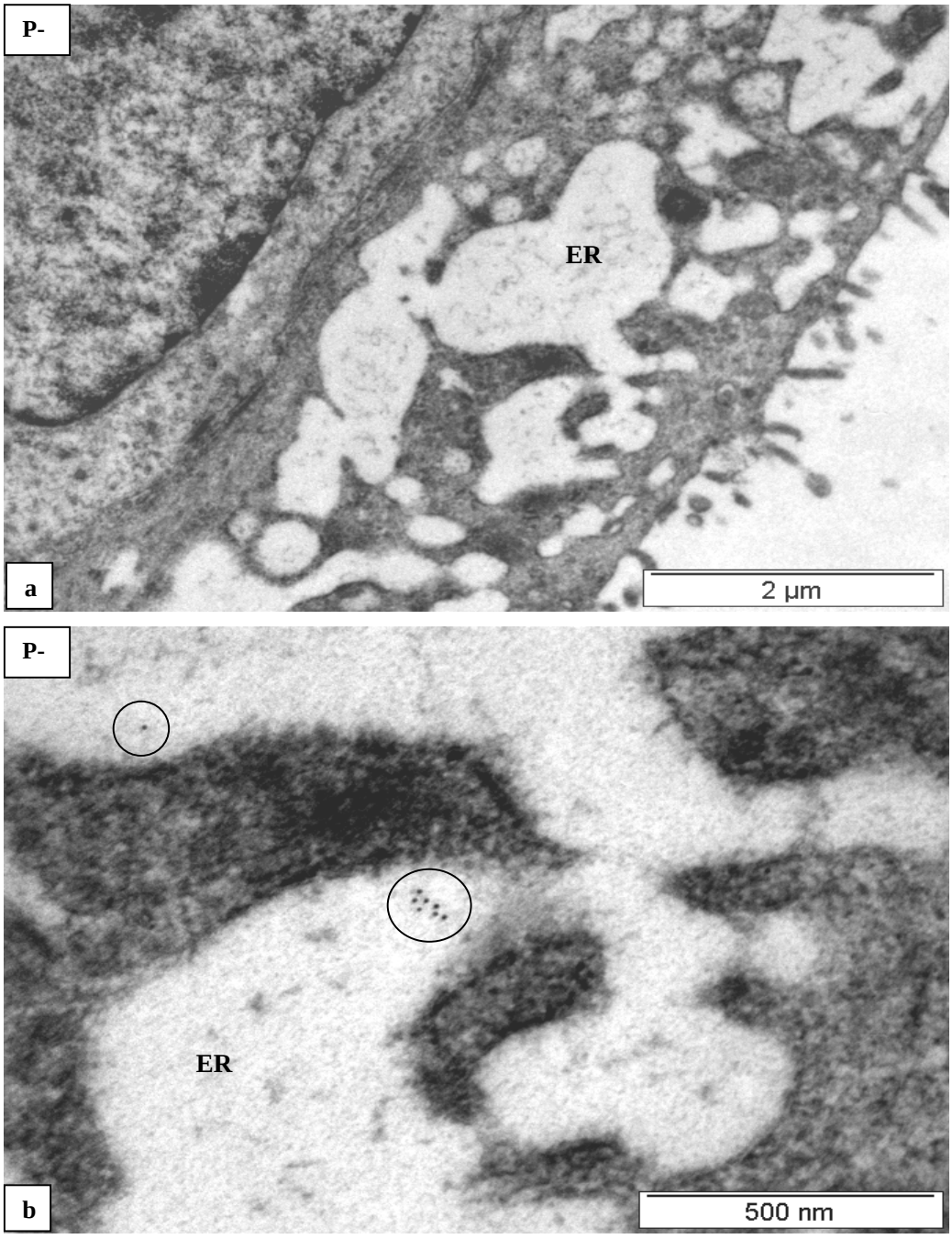


Figure 3.54: Electron micrographs depicting sEng immunolocalisation across P- groups (circle) within (a-b) endoplasmic reticulum (ER) of syncytiotrophoblast (ST).

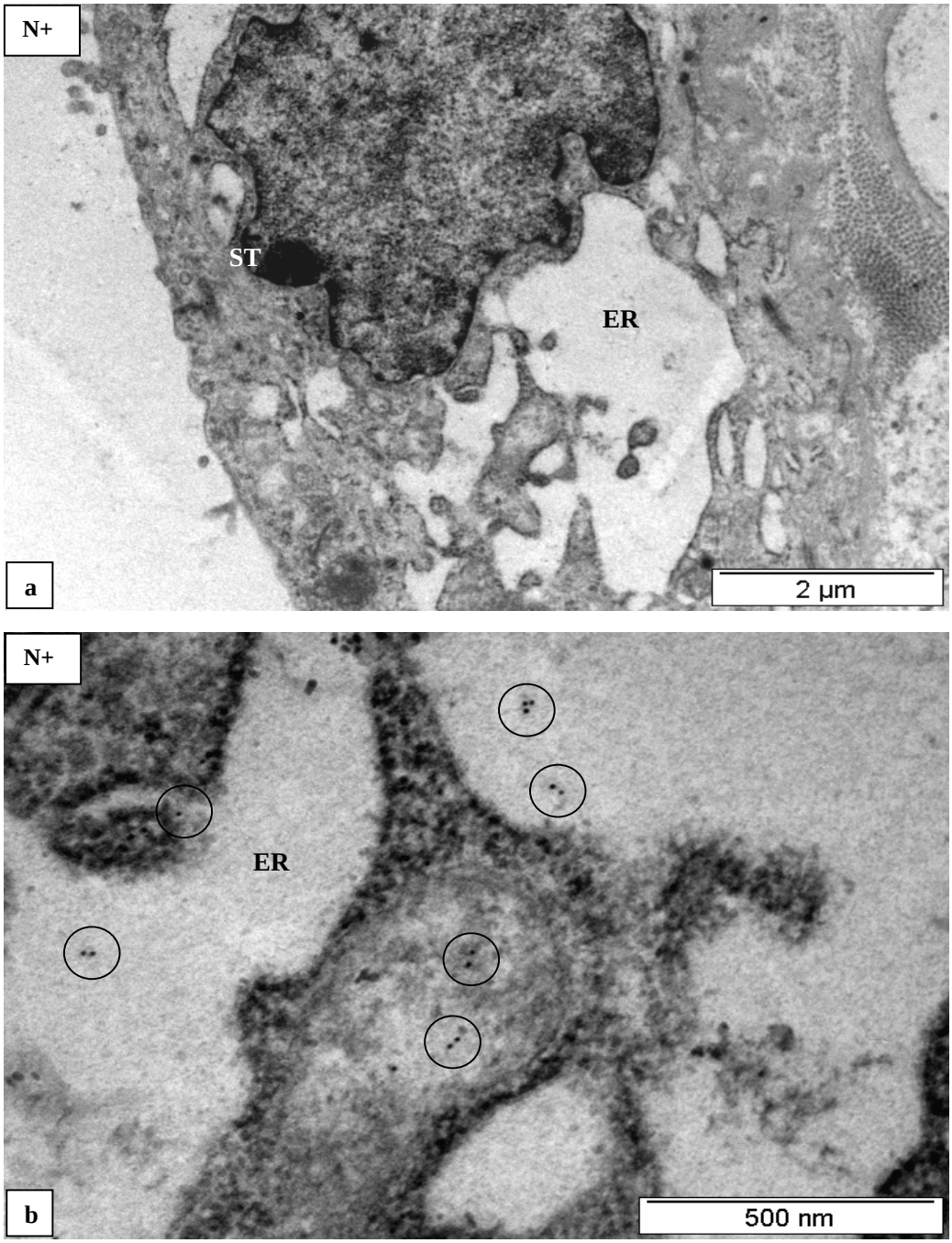


Figure 3.55: Electron micrographs depicting sEng immunolocalisation across N+ groups (circle) within (a-b) endoplasmic reticulum (ER) of syncytiotrophoblast (ST).

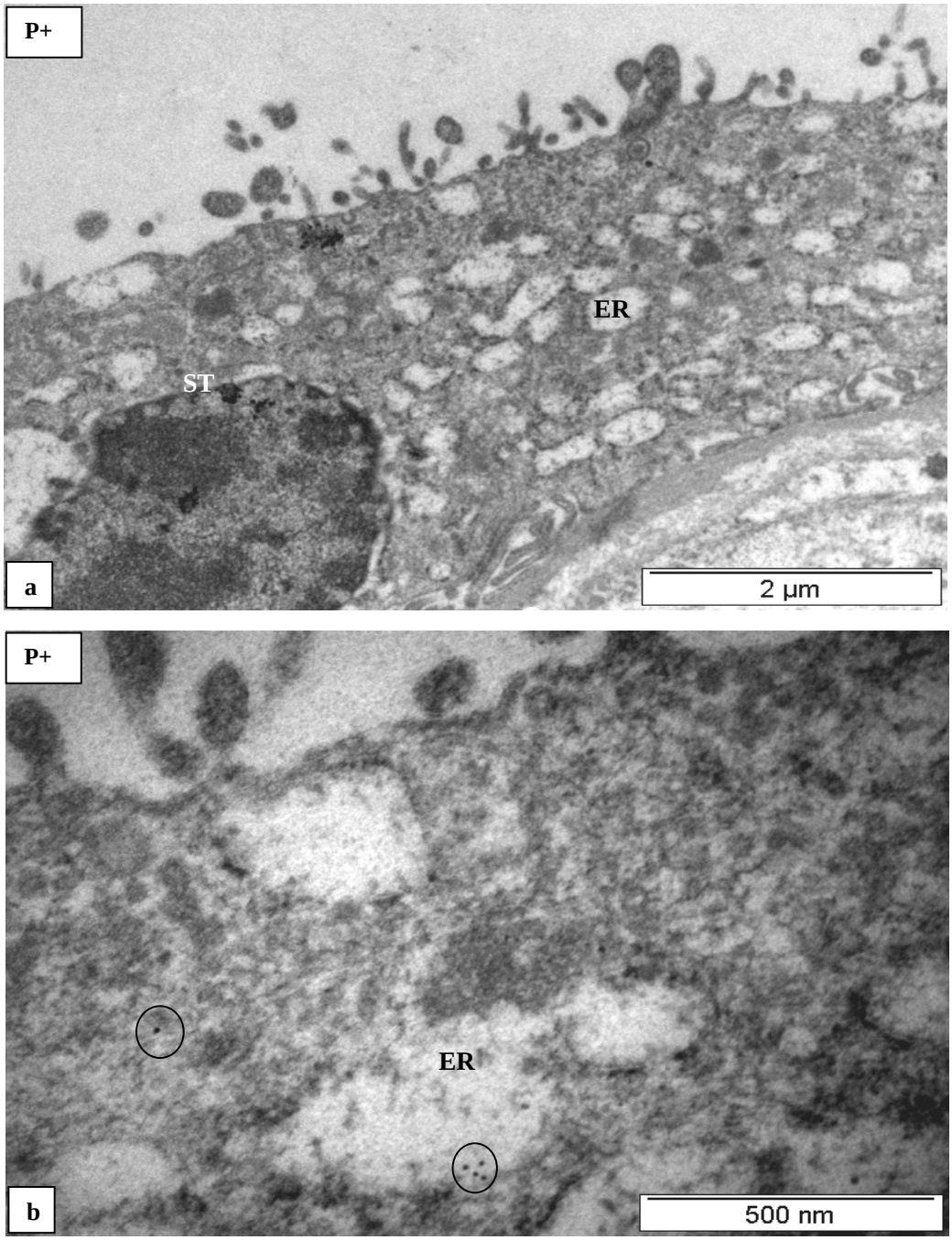


Figure 3.56: Electron micrographs depicting sEng immunolocalisation across P+ groups (circle) within (a-b) endoplasmic reticulum (ER) of syncytiotrophoblast (ST).

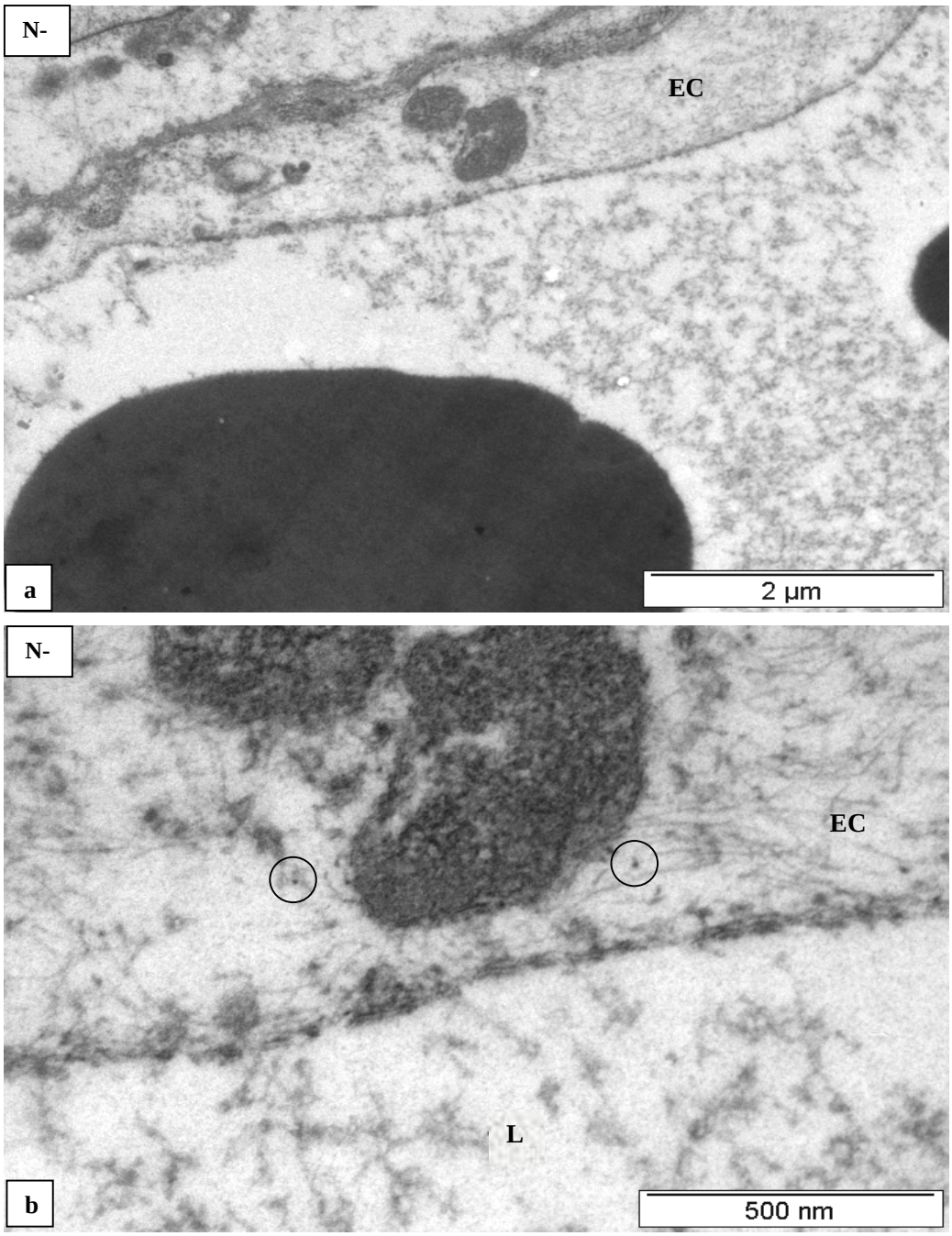


Figure 3.57: Electron micrographs depicting sEng immunolocalisation in the N- group (circle) within (a-b) endothelial cells (EC). Note lumen (L).

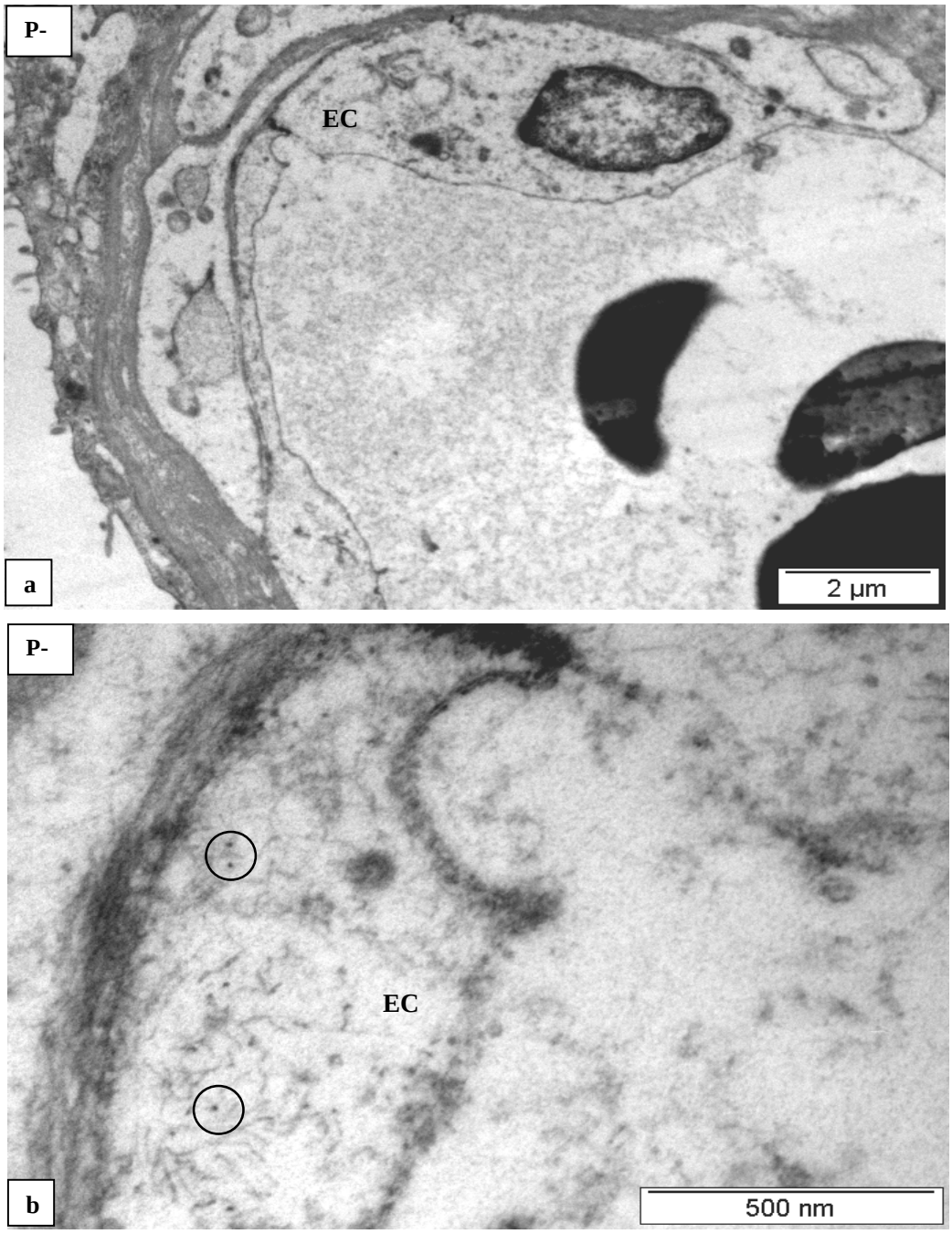


Figure 3.58: Electron micrographs depicting sEng immunolocalisation in the P- groups (circle) within (a-b) endothelial cells (EC).

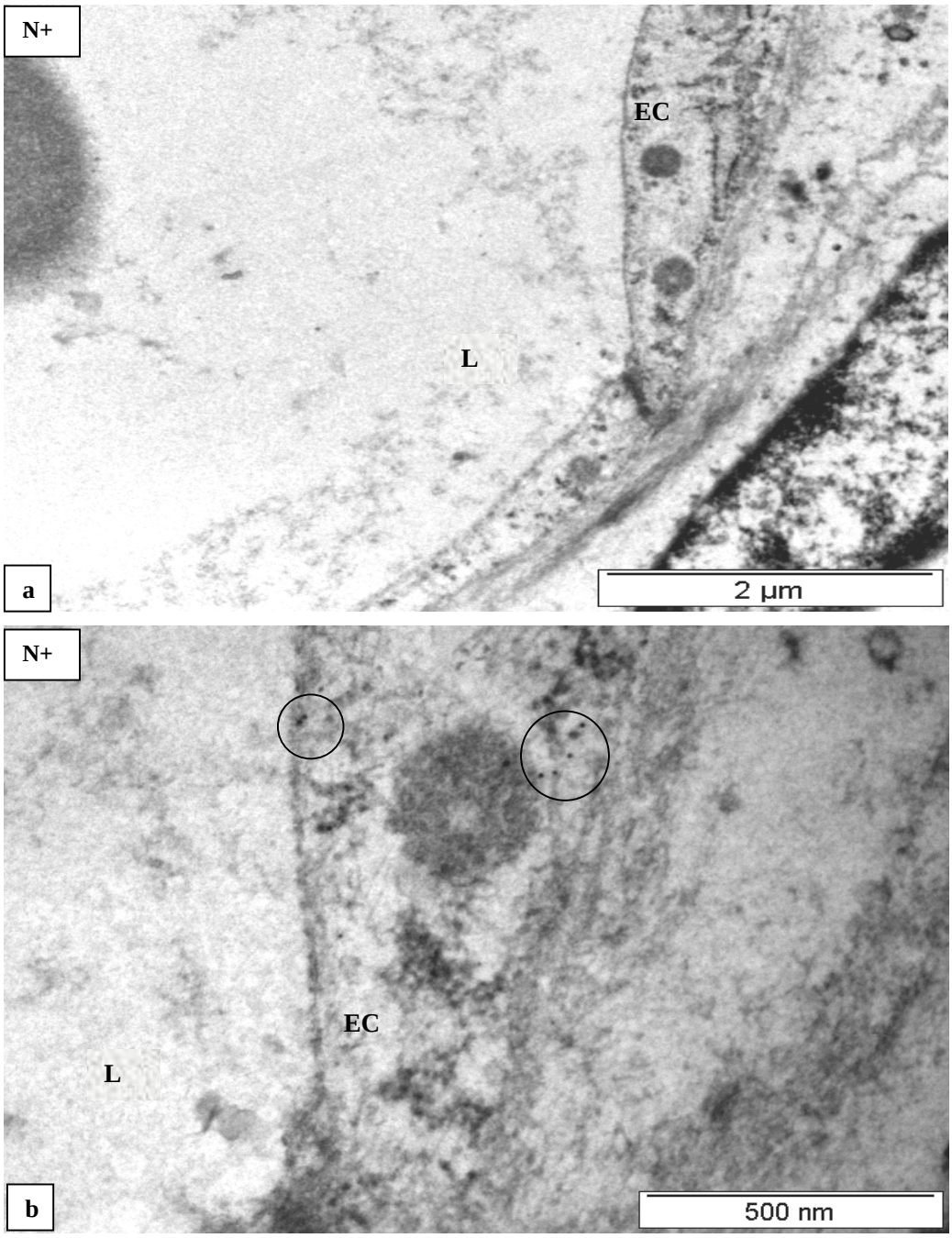


Figure 3.59: Electron micrographs depicting sEng immunolocalisation in the N+ groups (circle) within (a-b) endothelial cells (EC), Note lumen (L).

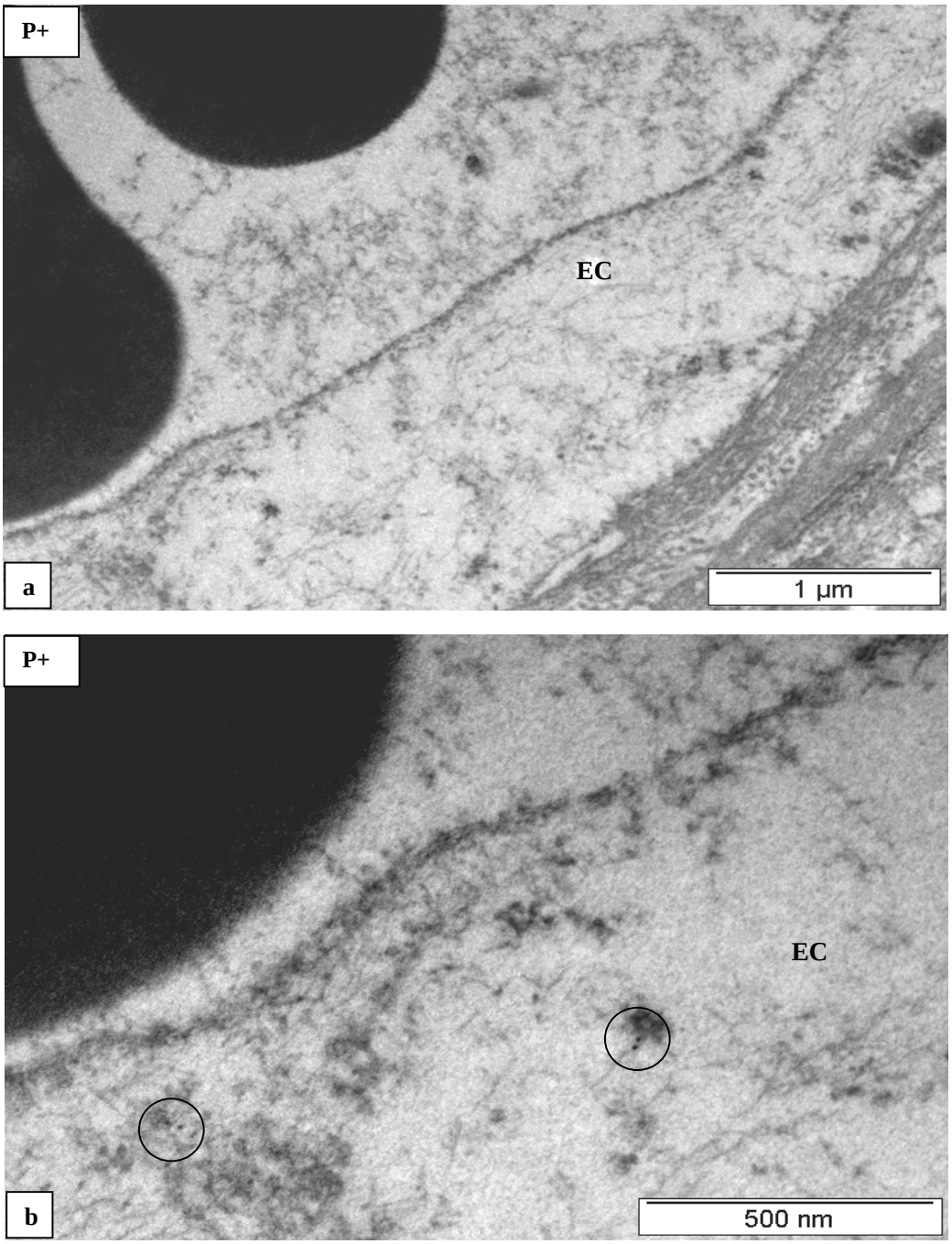


Figure 3.60: Electron micrographs depicting sEng immunolocalisation in the P+ groups (circle) within (a-b) endothelial cells (EC).

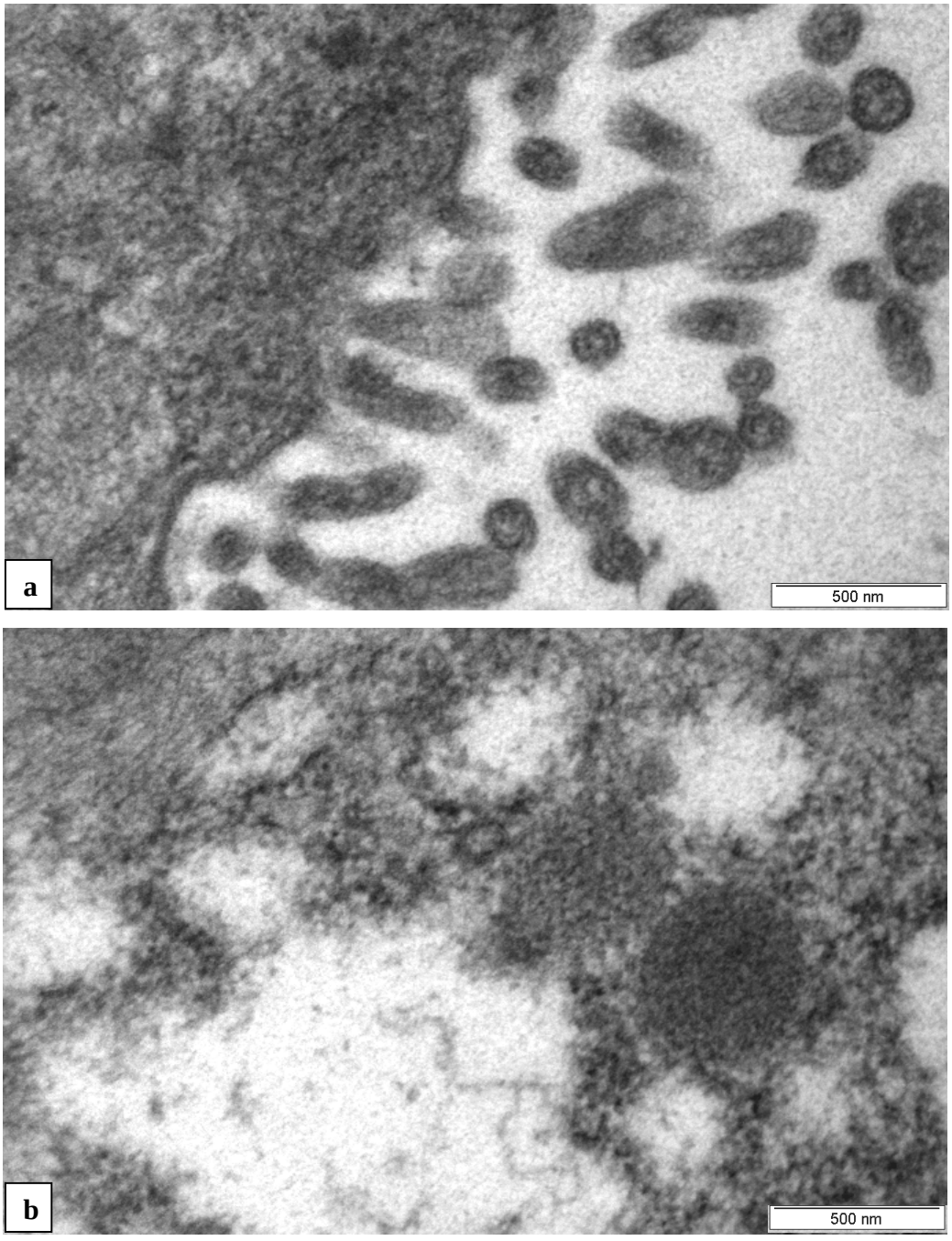


Figure 3.61: Electron micrographs depicting (a-b) the absence of gold particles in control tissue.

3.4.2.3 Immunoelectron localisation of PlGF in placental tissue

Gold particles were immunolocalised within the cyto- and syncytiotrophoblast cell populations across the N- (**Fig.3.62a-b, 3.64a**) and P- (**Fig. 3.62a-b**) and the N+ (**Fig. 3.65a-b**) and P+ (**Fig. 3.63a-b**) groups. This pro-angiogenic factor was observed on the microvilli of the luminal plasmalemma (**Fig. 3.64b**).

Subcellularly, immunogold particles were noted within pools of ER and mitochondria of syncytiotrophoblasts (**Fig. 3.62a-b-3.66-a-b**). Gold particles were also noted within the nucleolemma.

PlGF conjugated to 10nm gold particles were demonstrated within the cytoplasm of endothelial cells, often in close proximity to the basement membrane across the N- (**Fig. 3.67a-b**) and P- (**Fig. 3.84a-b**) and N+ (**Fig. 3.69a-b**) and P+ (**Fig. 3.70a-b**) groups.

Additionally, a method control revealed the absence of gold particles (**Fig. 3.71a-b**) across all groups.

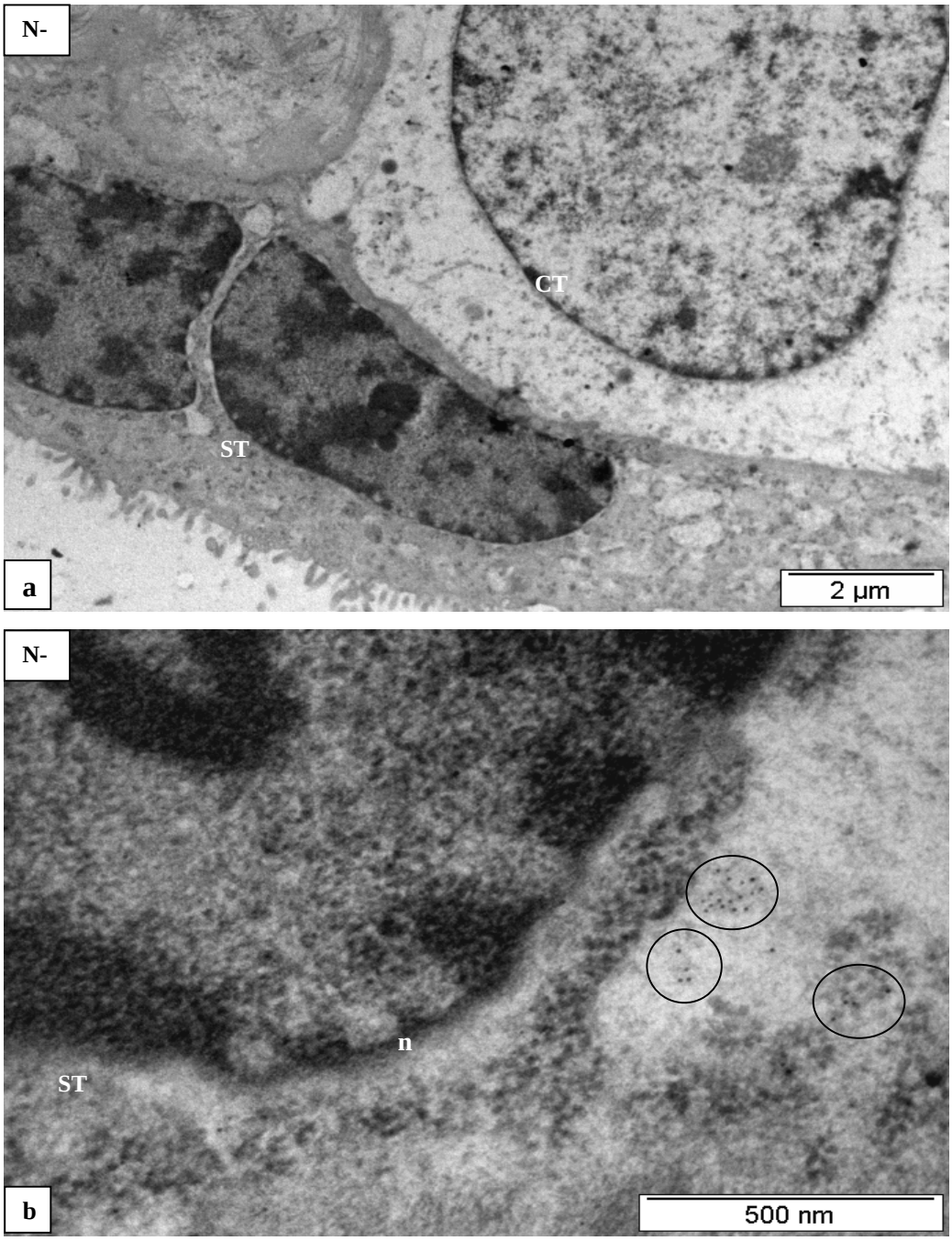


Figure 3.62: Electron micrographs depicting PlGF immunolocalisation in the N- group (circle) within (a-b) endoplasmic reticulum (ER) of syncytiotrophoblast (ST). Note nucleolemma (n).

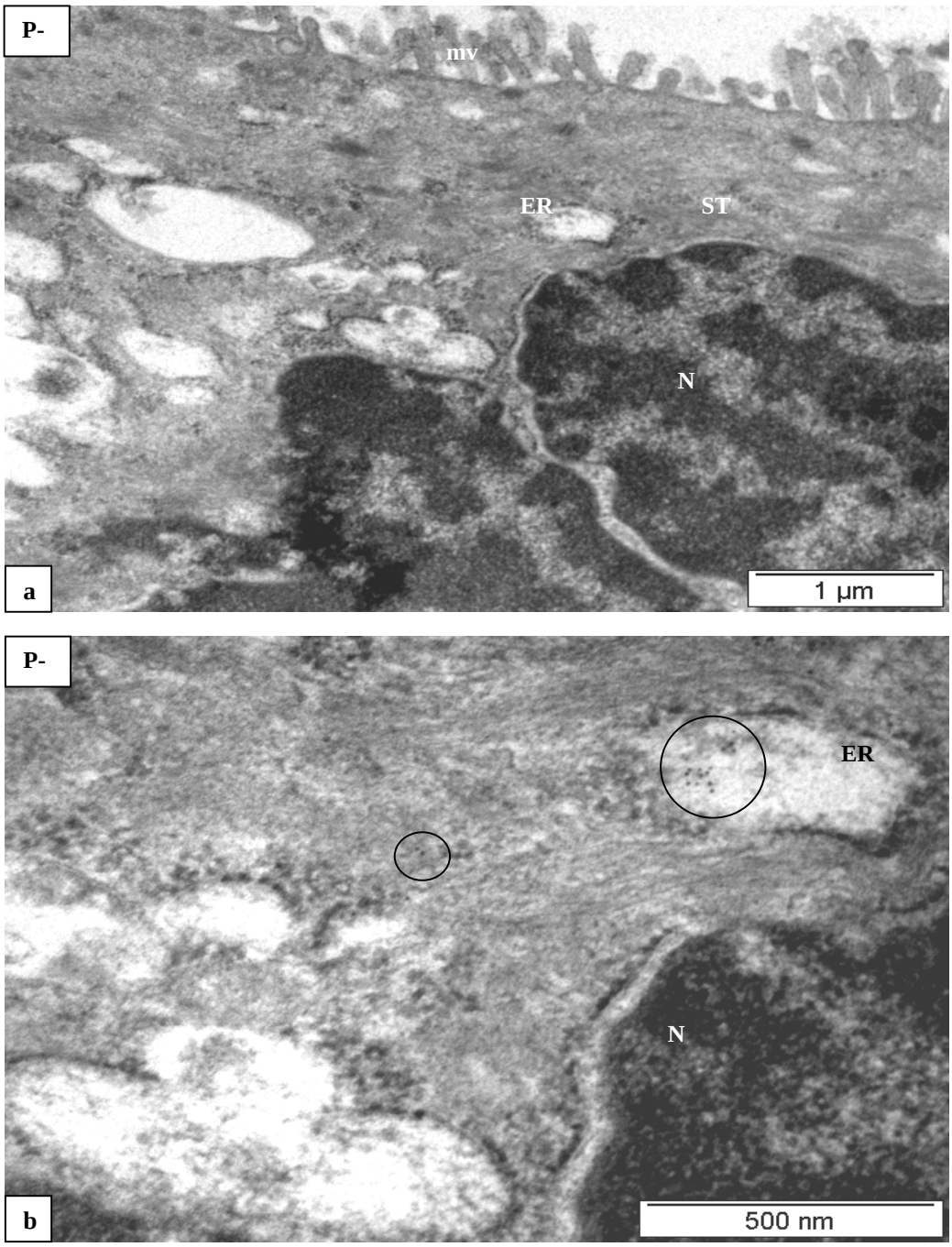


Figure 3.63: Electron micrographs depicting PlGF immunolocalisation across P- groups (circle) within (a-b) endoplasmic reticulum (ER)of syncytiotrophoblast (ST). Note nucleus (N).

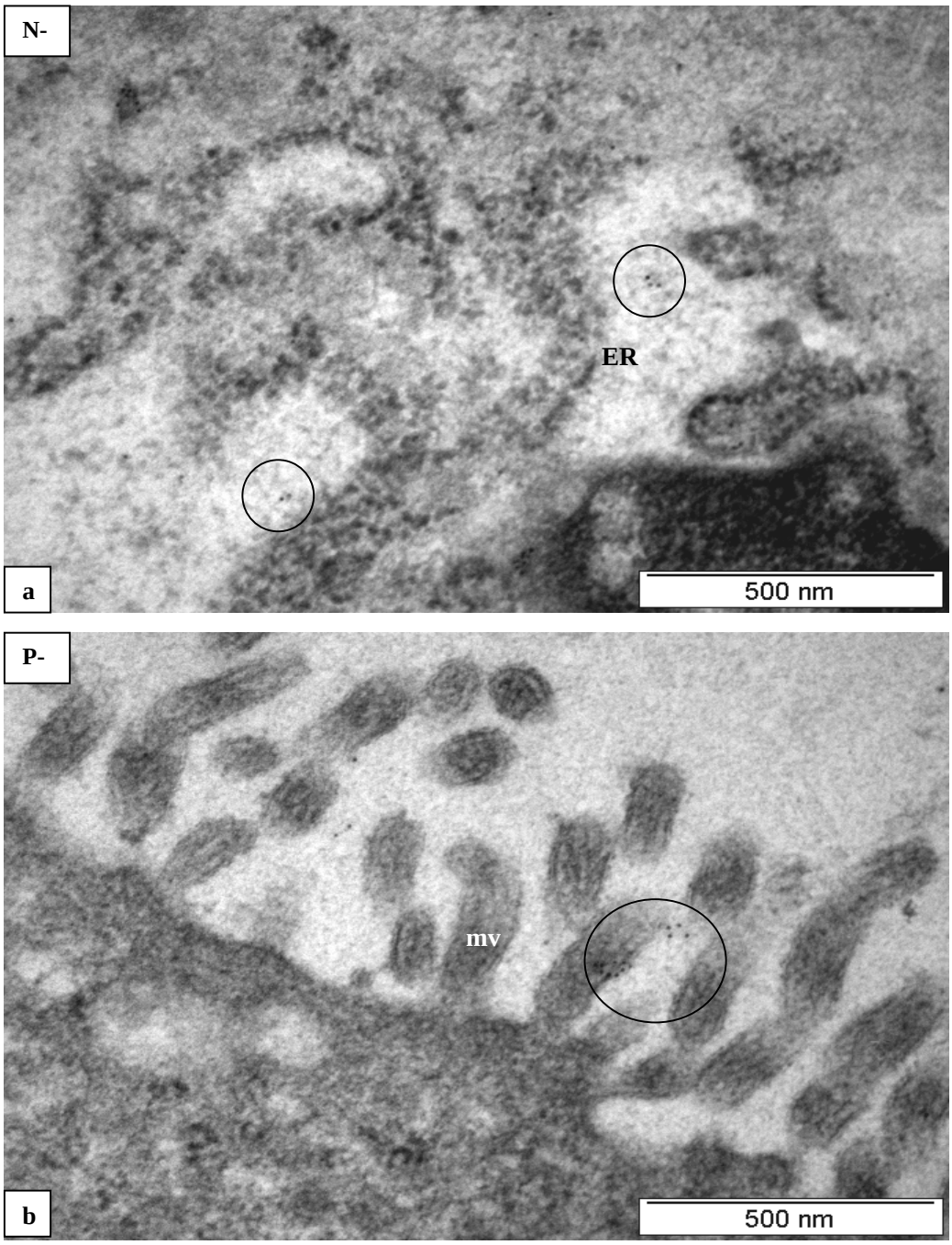


Figure 3.64: Electron micrographs depicting PlGF immunolocalisation across N- and P-groups (circle) within (a-b) endoplasmic reticulum (ER) and microvilli (mv) of syncytiotrophoblast .

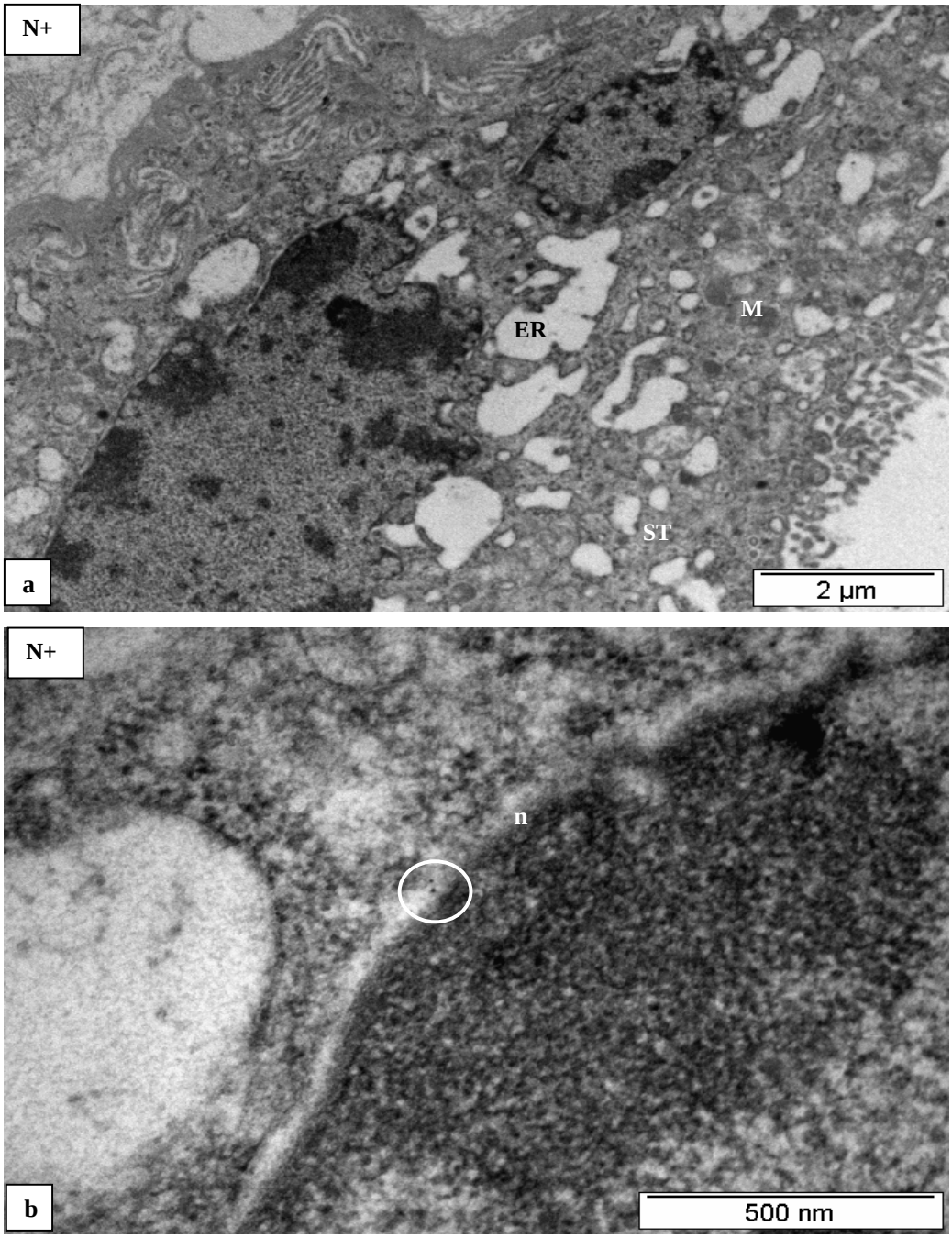


Figure 3.65: Electron micrographs depicting PlGF immunolocalisation in the N+ group (circle) within (a-b) syncytiotrophoblast (ST). Note nucleolemma (n)

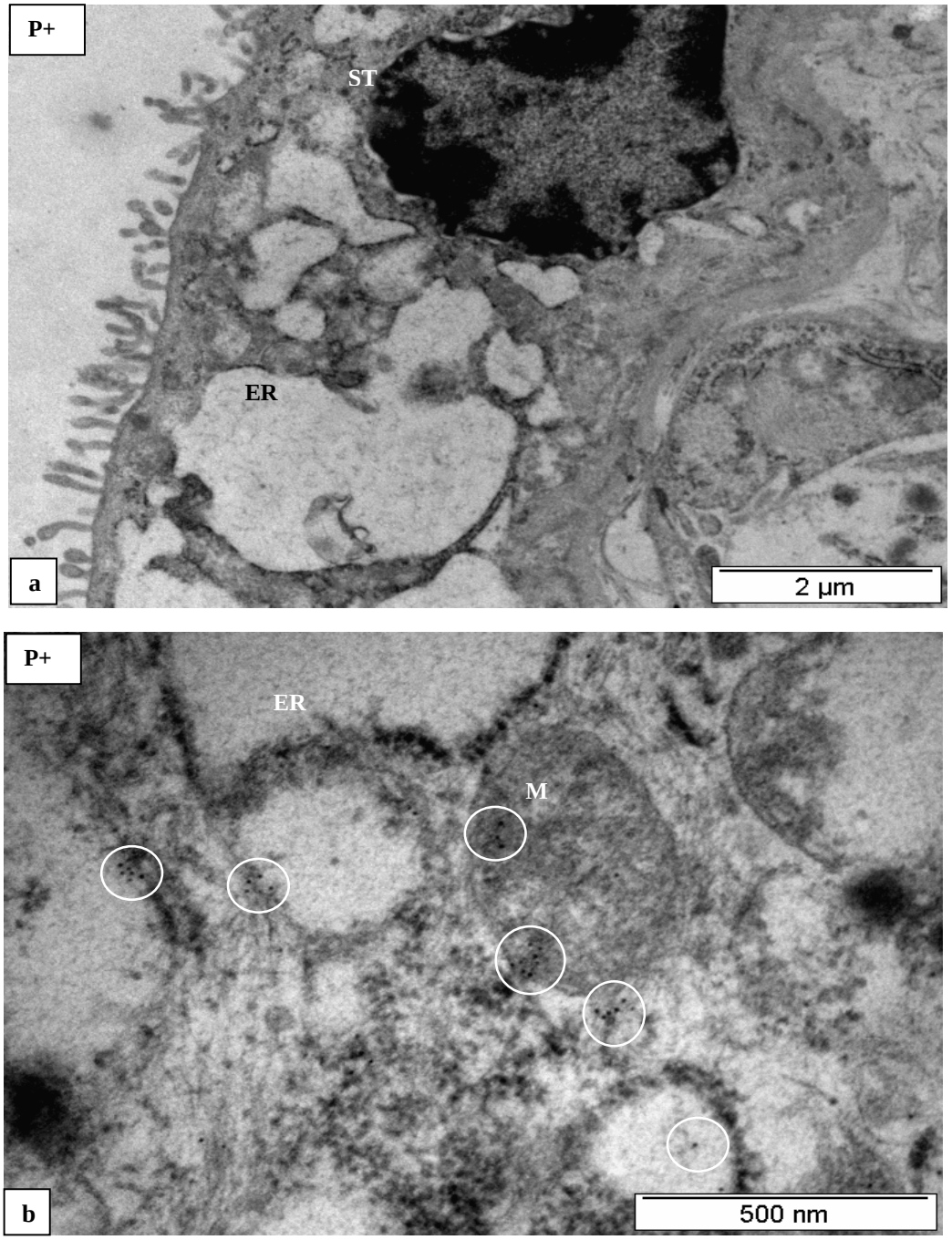


Figure 3.66: Electron micrographs depicting PlGF immunolocalisation in the P+ group (circle) within (a-b) endoplasmic reticulum (ER) and mitochondria (M) of syncytiotrophoblast (ST).

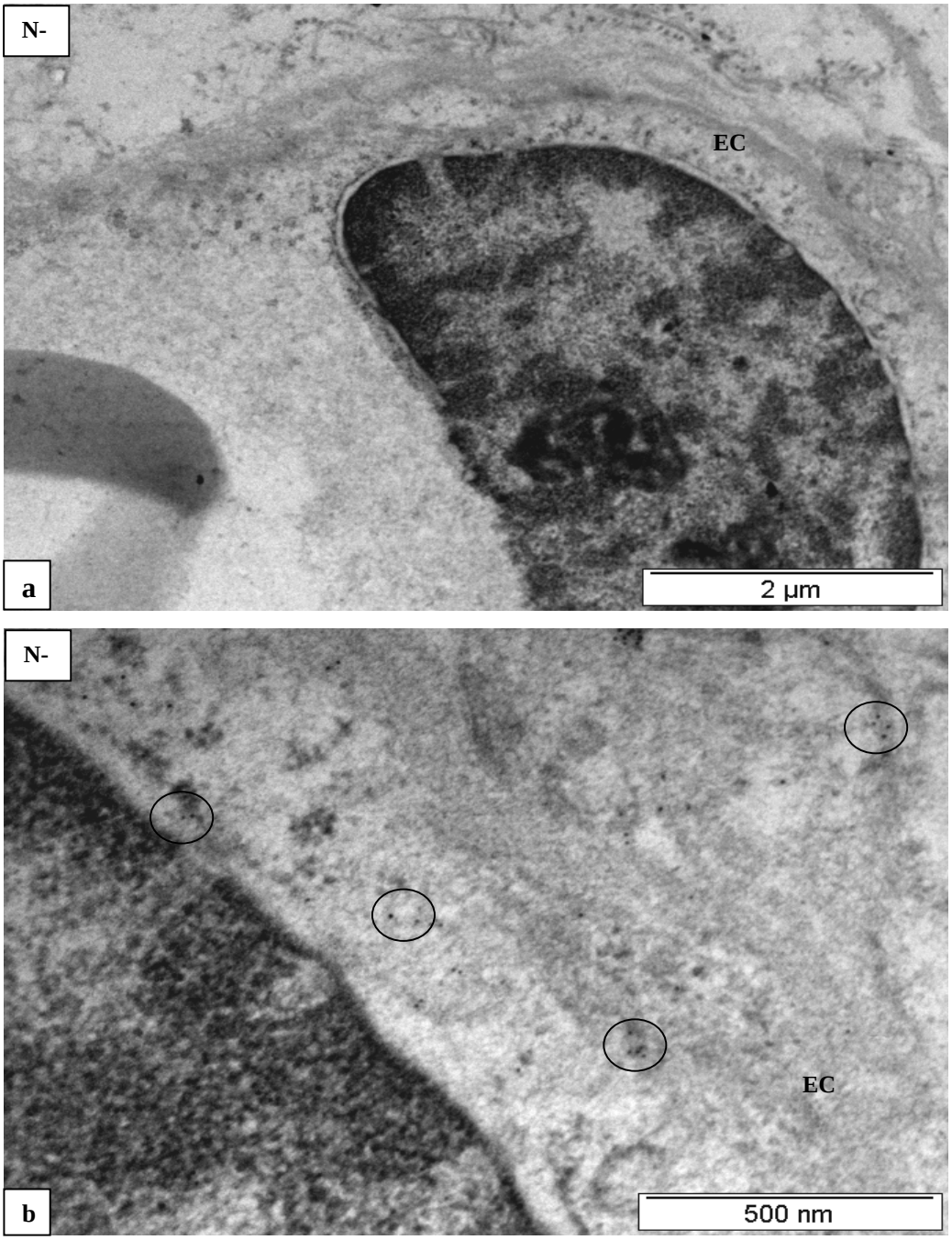


Figure 3.67: Electron micrographs depicting PlGF immunolocalisation across N- groups (circle) within (a-b) endothelial cells (EC).

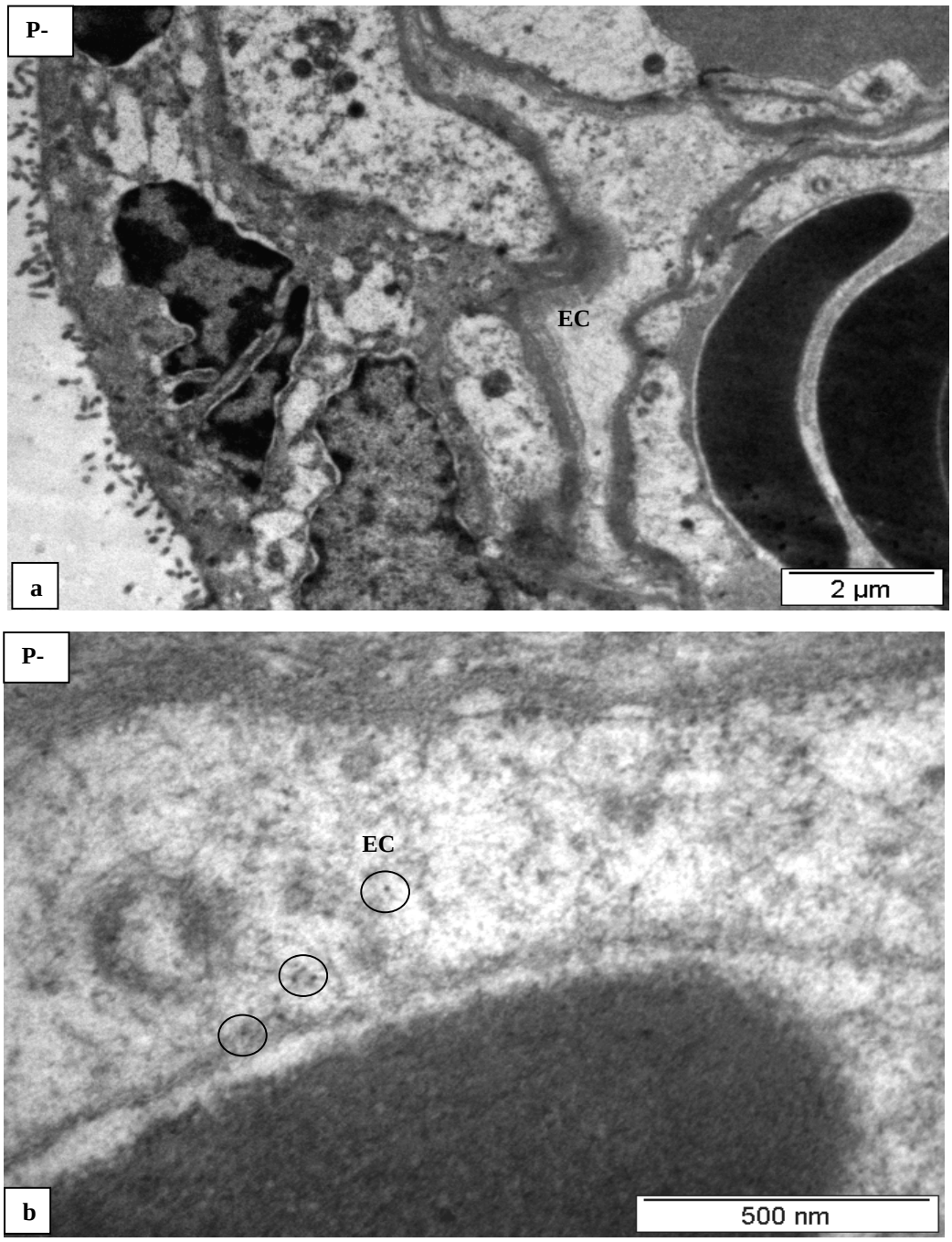


Figure 3.68: Electron micrographs depicting PlGF immunolocalisation in the P- group (circle) within (a-b) endothelial cells (EC).

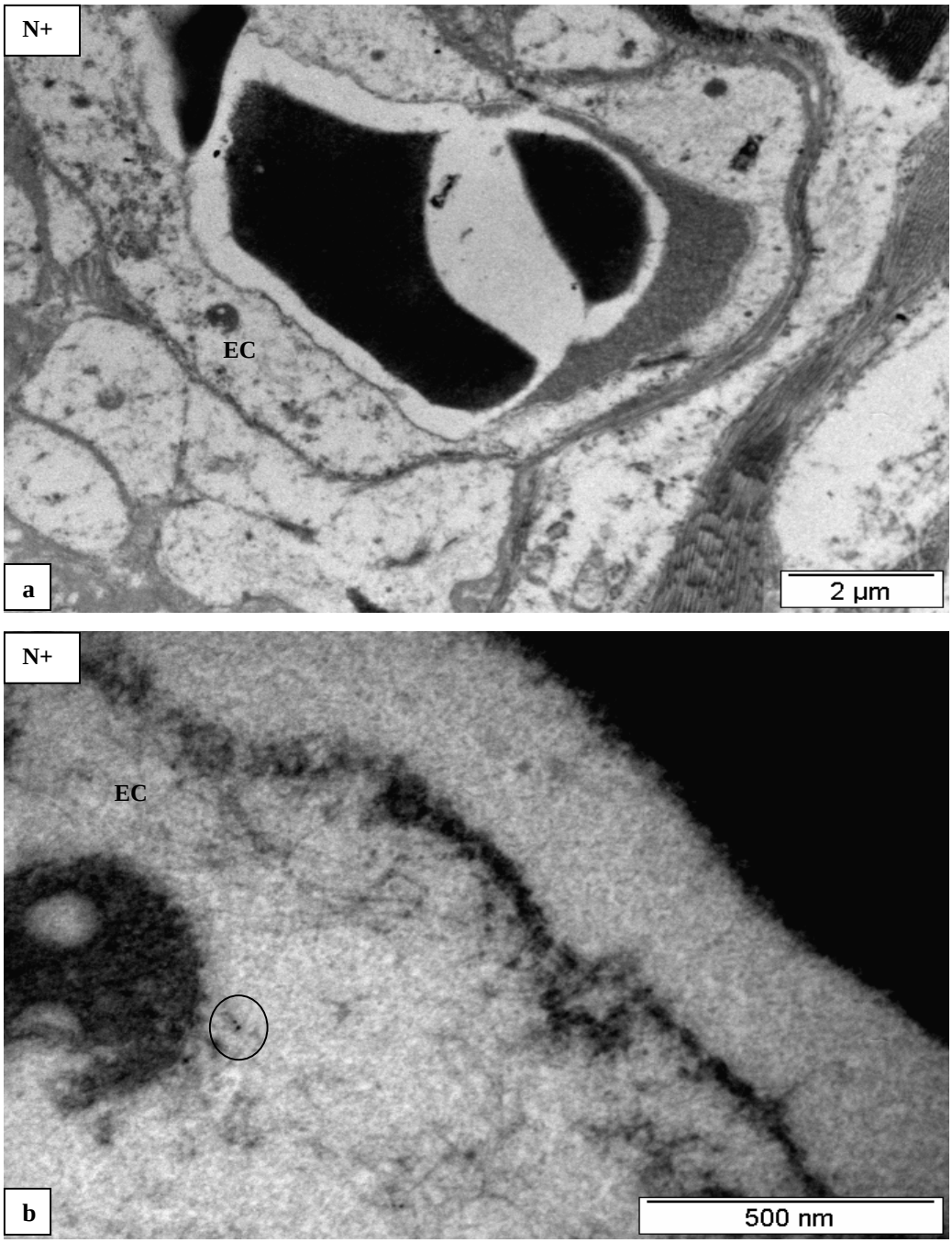


Figure 3.69: Electron micrographs depicting PlGF immunolocalisation in the N+ group (circle) within (a-b) endothelial cells (EC).

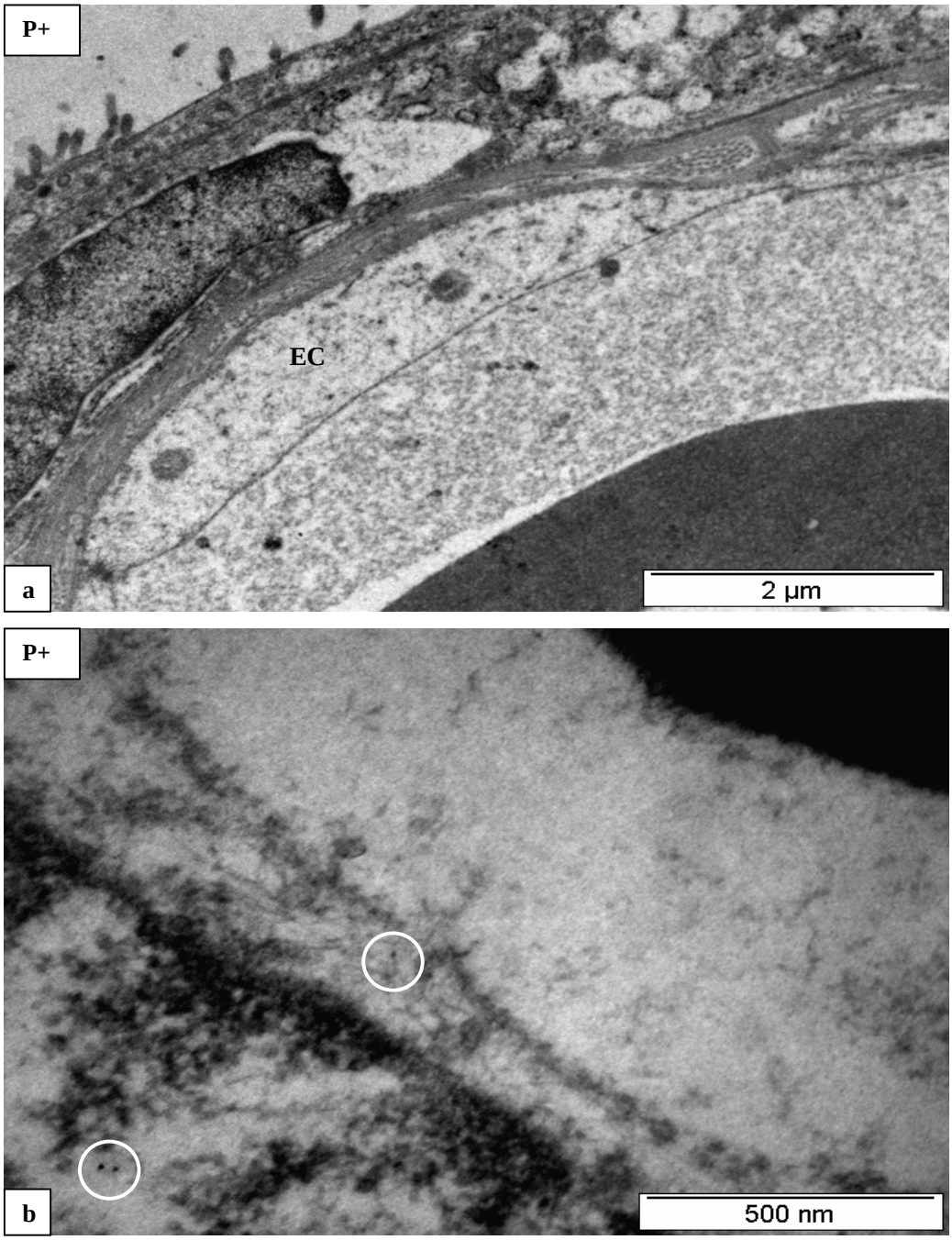


Figure 3.70: Electron micrographs depicting PlGF immunolocalisation in the P+ group (circle) within (a-b) endothelial cells (EC).

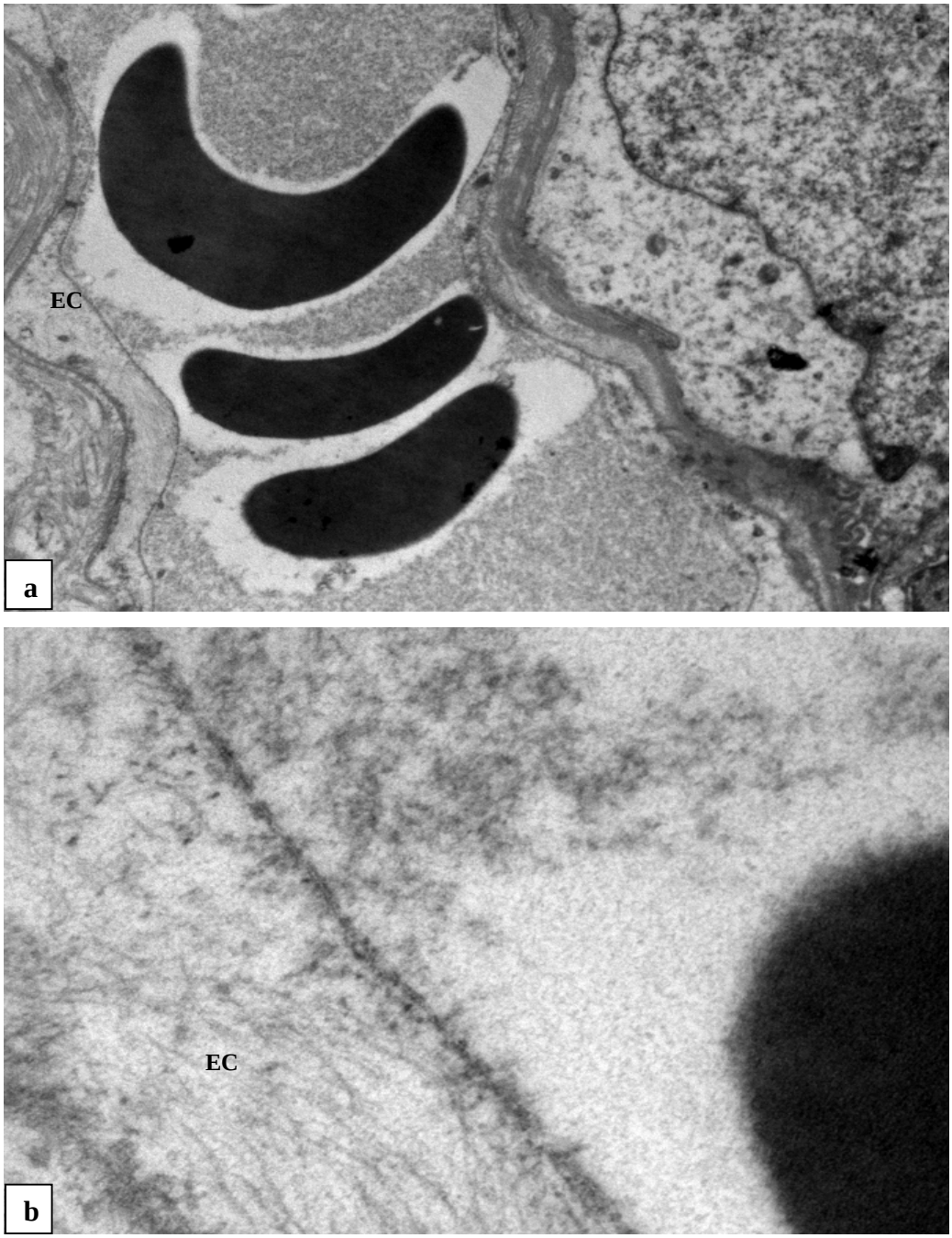


Figure 3.71: Electron micrographs depicting (a-b) the absence of gold particles in control tissue

3.4.2.4 Immunoelectron localisation of VEGF in placental tissue

VEGF conjugated to 10nm gold particles were immunolocalised within the cyto- and syncytiotrophoblast cell populations across the N- (**Fig. 3.72a-b; 3.74a**) and P- (**Fig. 3.72a-b; 3.74b**) and the N+ (**Fig. 3.75a-b**) and P+ (**Fig. 3.76a-b**) groups. Additionally, 10nm gold particles were observed in the perinuclear region of both the syncytio and cytotrophoblasts (**Fig. 3.75a-b**). Subcellularly, they were located within the endoplasmic reticulum and mitochondria of syncytiotrophoblasts (**Fig. 3.76a-b**).

The endothelial cells were immunopositive for VEGF across the N- (**Fig. 3.77a-b**) and P- (**Fig. 3.78a-b**) and the N+ (**Fig. 3.79a-b**) and P+ (**Fig. 3.80a-b**) groups.

Additionally, a method control revealed no gold particles at all (**Fig. 3. 81a**).

Finally, there appeared to be no significant difference in the subcellular immunolocalisation and intensity distribution of both the pro- and anti-angiogenic factors between HIV positive and HIV negative placental villi at term.

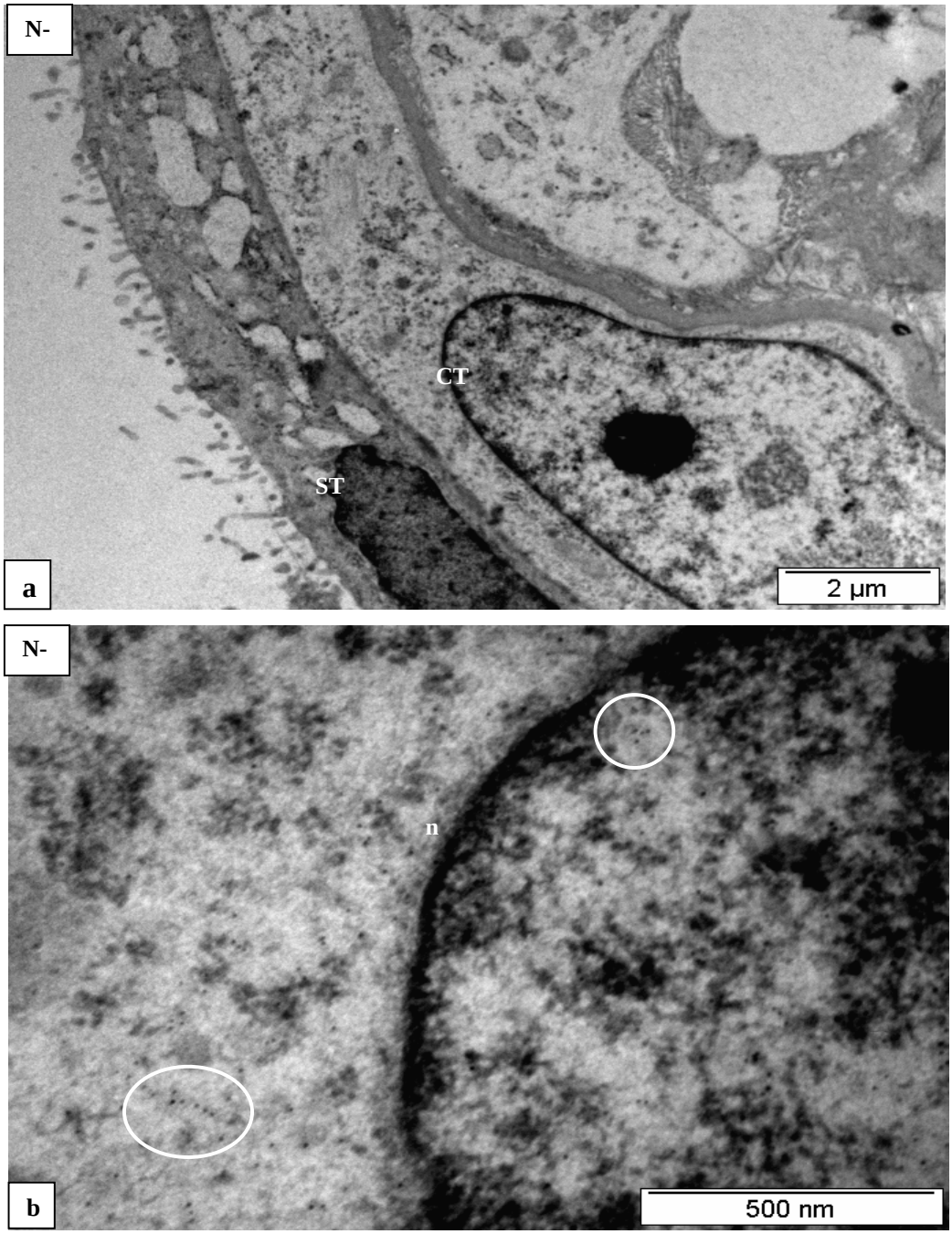


Figure 3.72: Electron micrographs depicting VEGF immunolocalisation (circle) in the N- group within (a-b) cytotrophoblast (CT). Note nucleolemma (n).

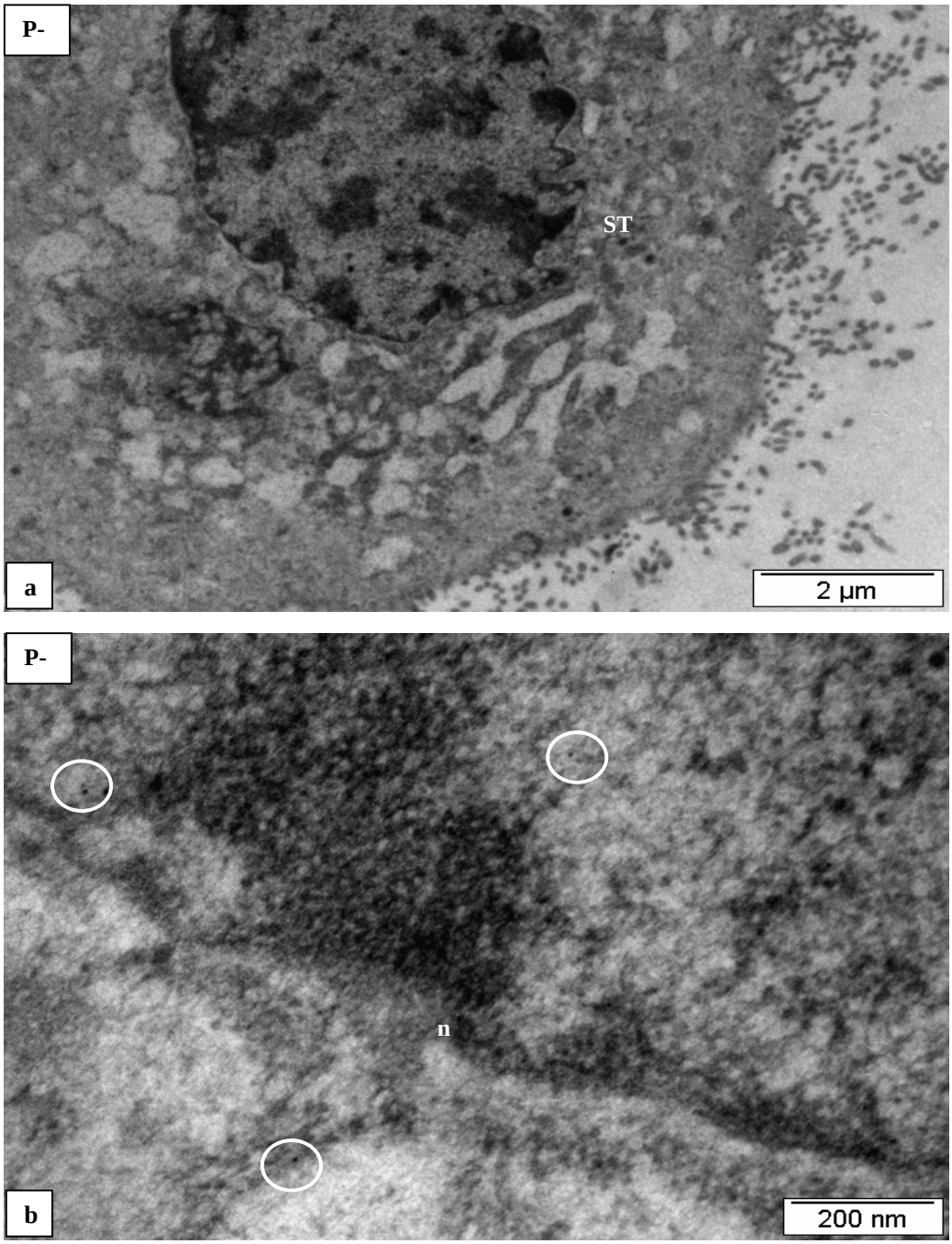


Figure 3.73: Electron micrographs depicting VEGF immunolocalisation (circle) in the P- group within (a-b) endoplasmic reticulum (ER) of syncytiotrophoblast (ST). Note nucleolemma (n).

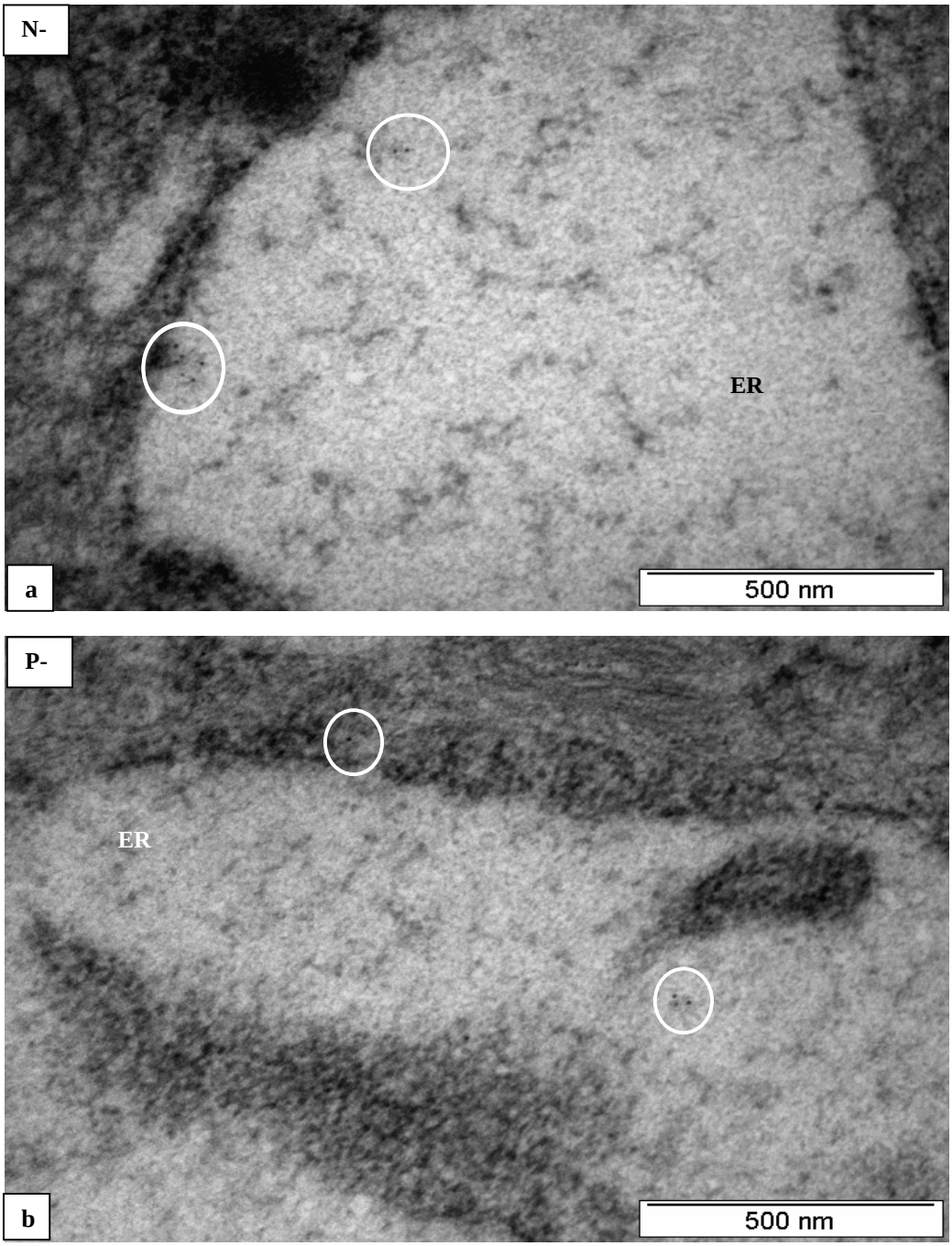


Figure 3.74: Electron micrographs depicting VEGF immunolocalisation (circle) across N- and P- groups within (a-b) endoplasmic reticulum (ER) of syncytiotrophoblast (ST).

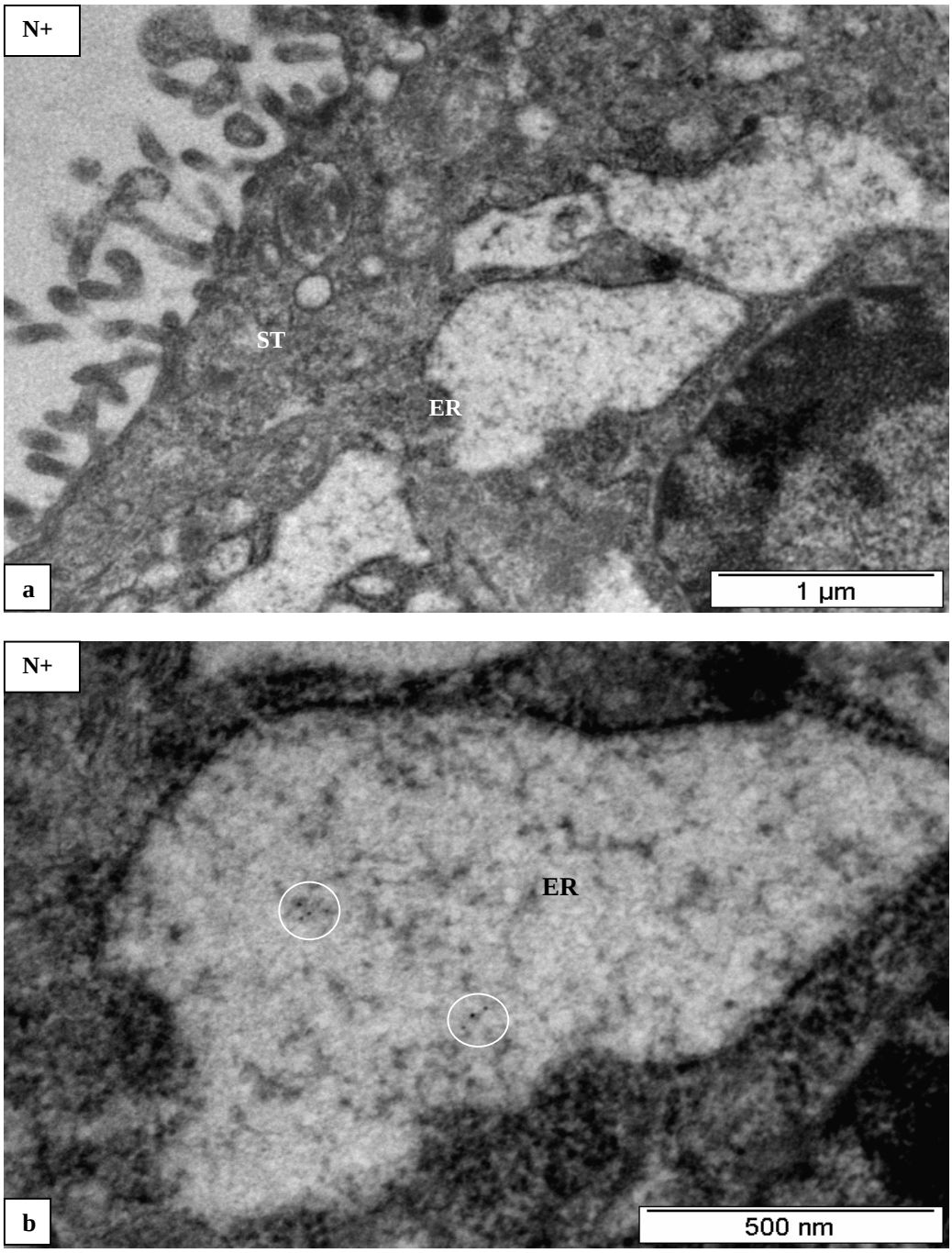


Figure 3.75: Electron micrographs depicting VEGF immunolocalisation (circle) in the N+ group within (a-b) endoplasmic reticulum (ER) of syncytiotrophoblast (ST).

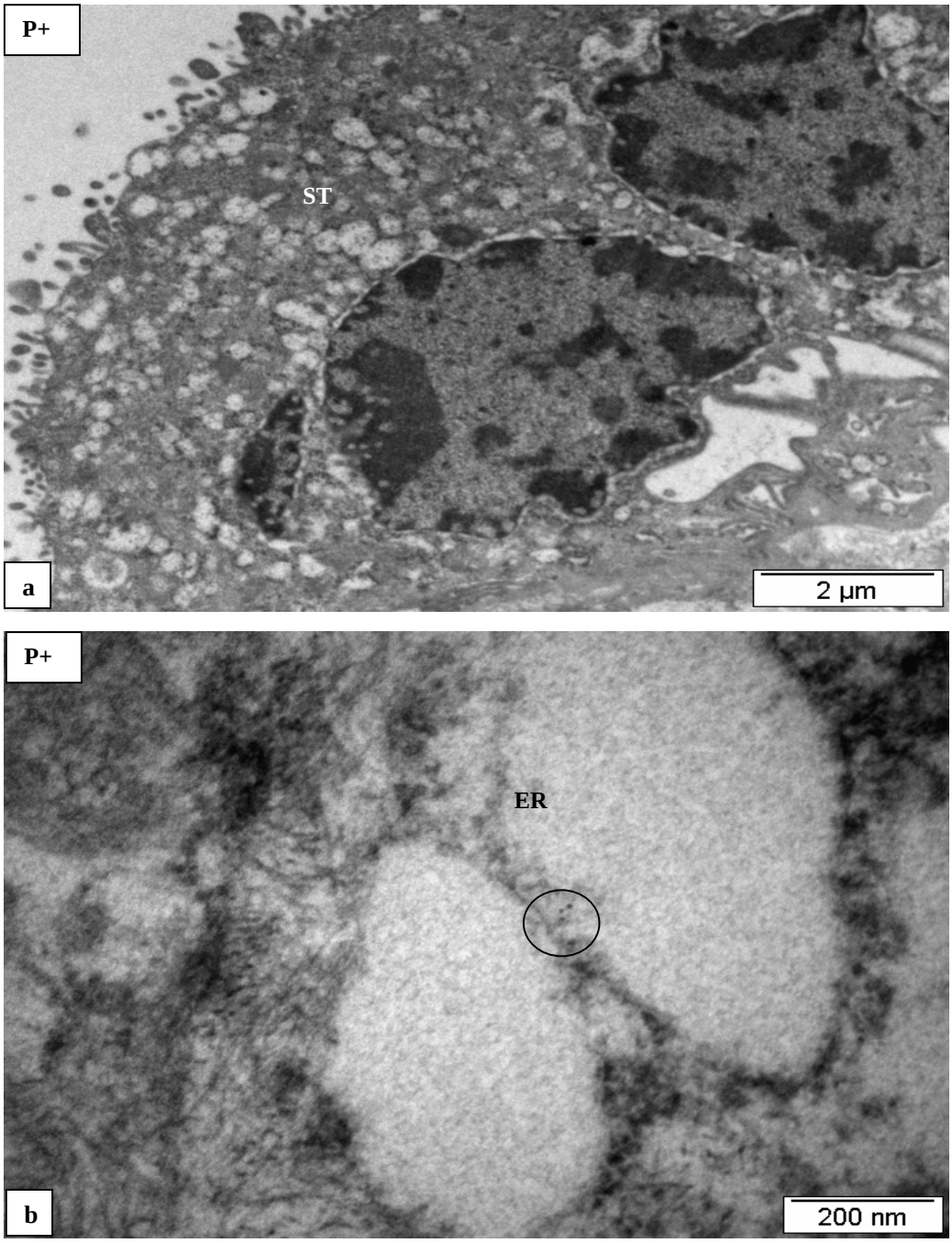


Figure 3.76: Electron micrographs depicting VEGF immunolocalisation (circle) in the P+ group within (a-b) endoplasmic reticulum (ER) of syncytiotrophoblast (ST).

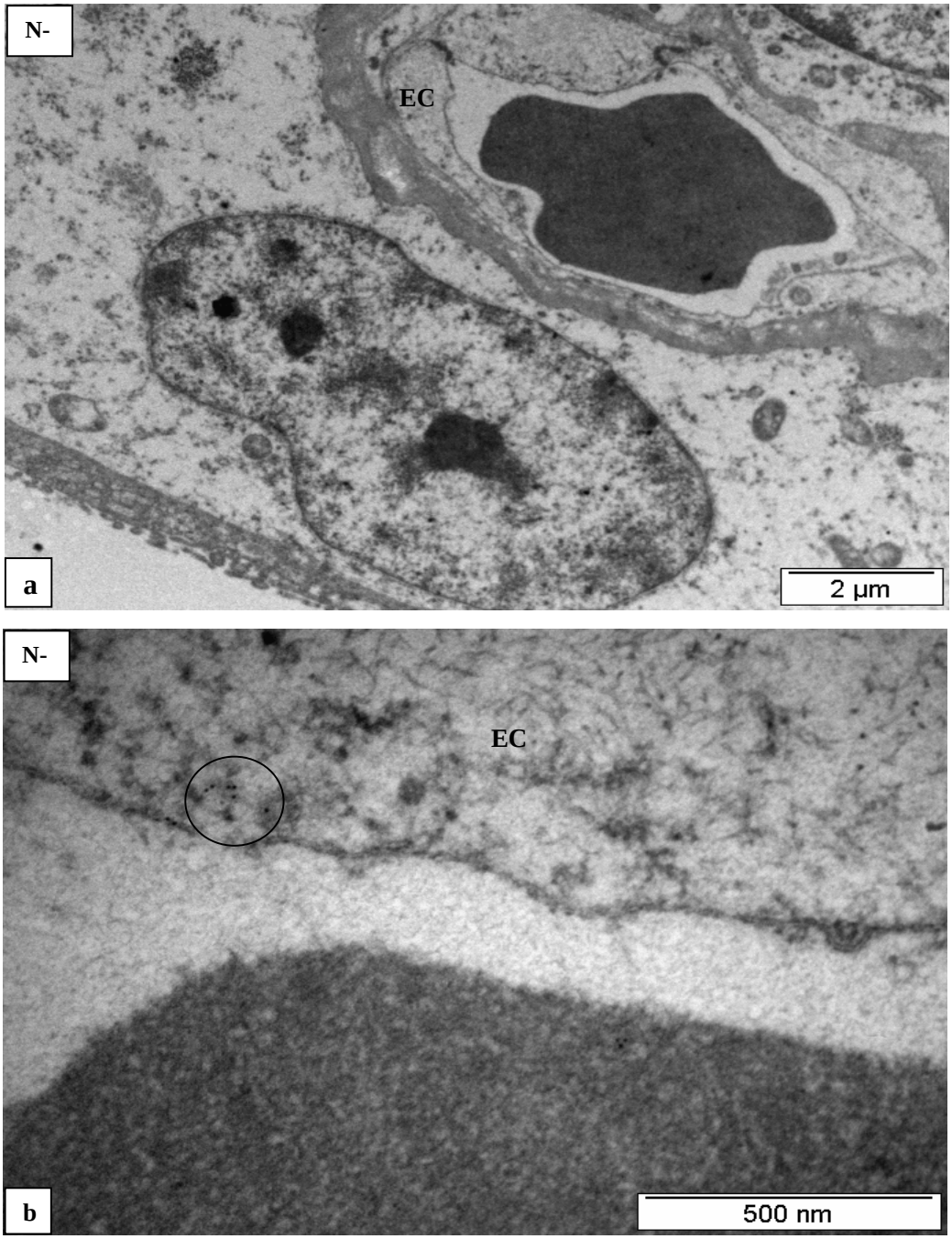


Figure 3.77: Electron micrographs depicting VEGF immunolocalisation (circle) in the N- group within (a-b) endothelial cells (EC).

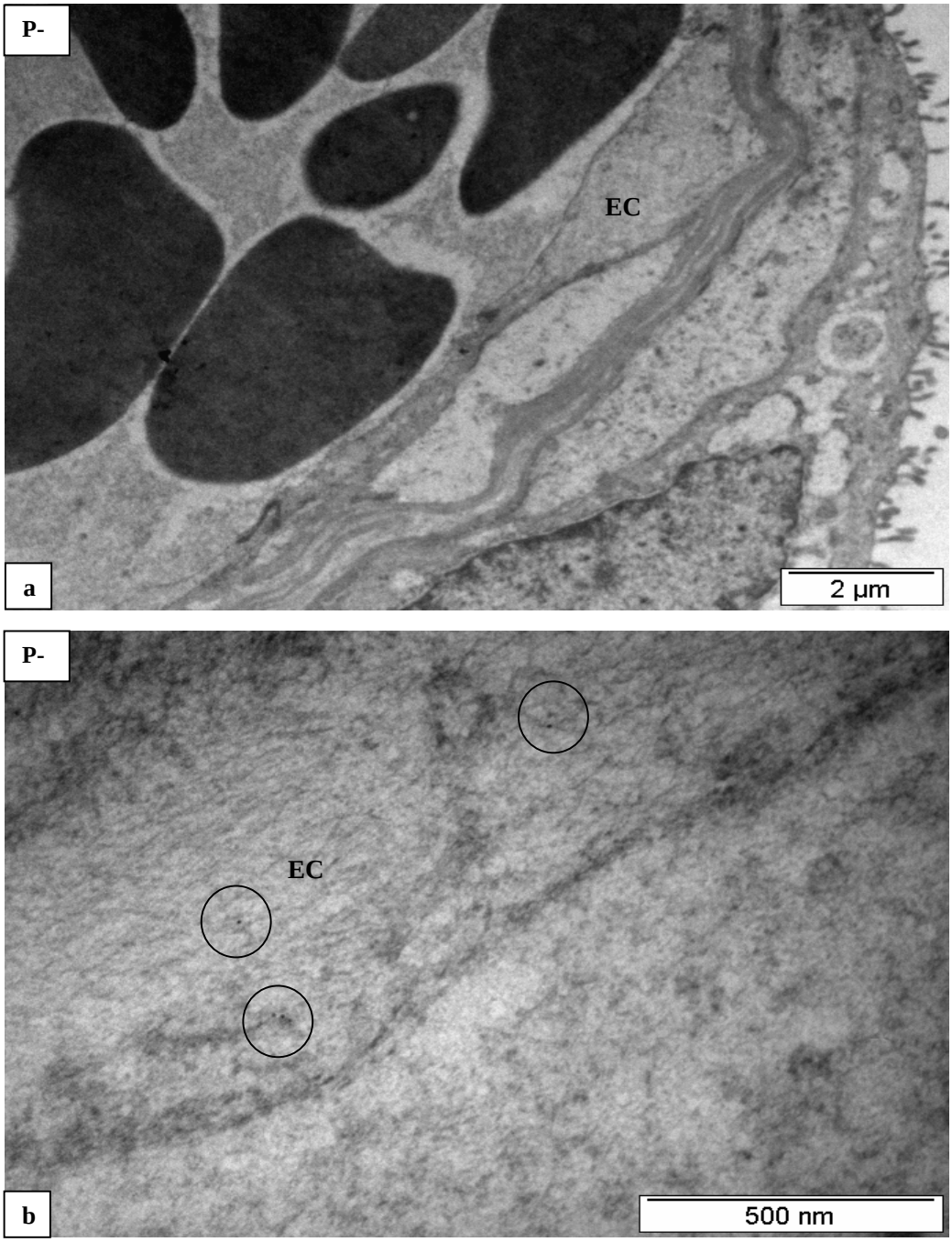


Figure 3.78: Electron micrographs depicting VEGF immunolocalisation (circle) in the P- group within (a-b) endothelial cells (EC).

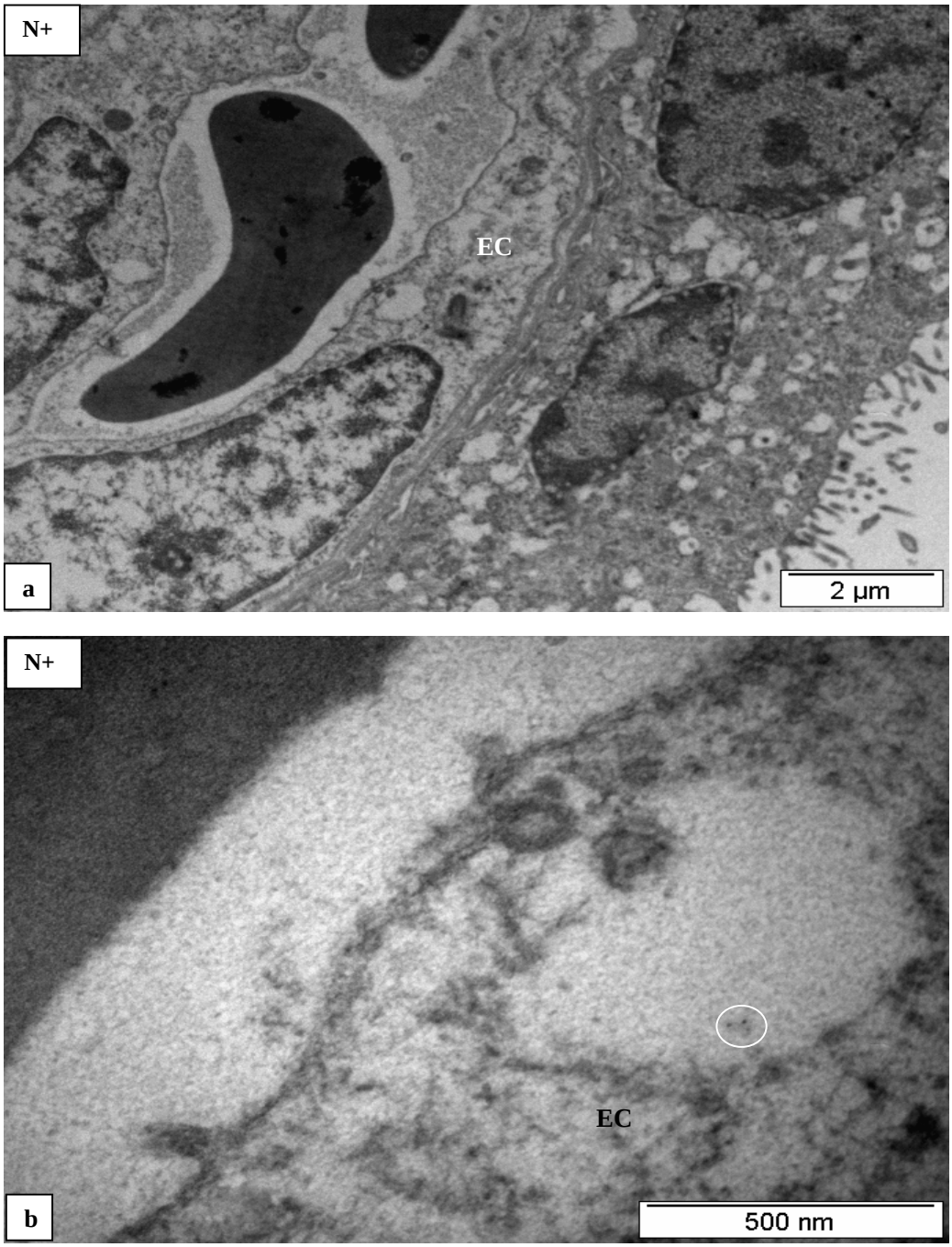


Figure 3.79: Electron micrographs VEGF immunolocalisation (circle) in the N+ group within (a-b) endothelial cells (EC).

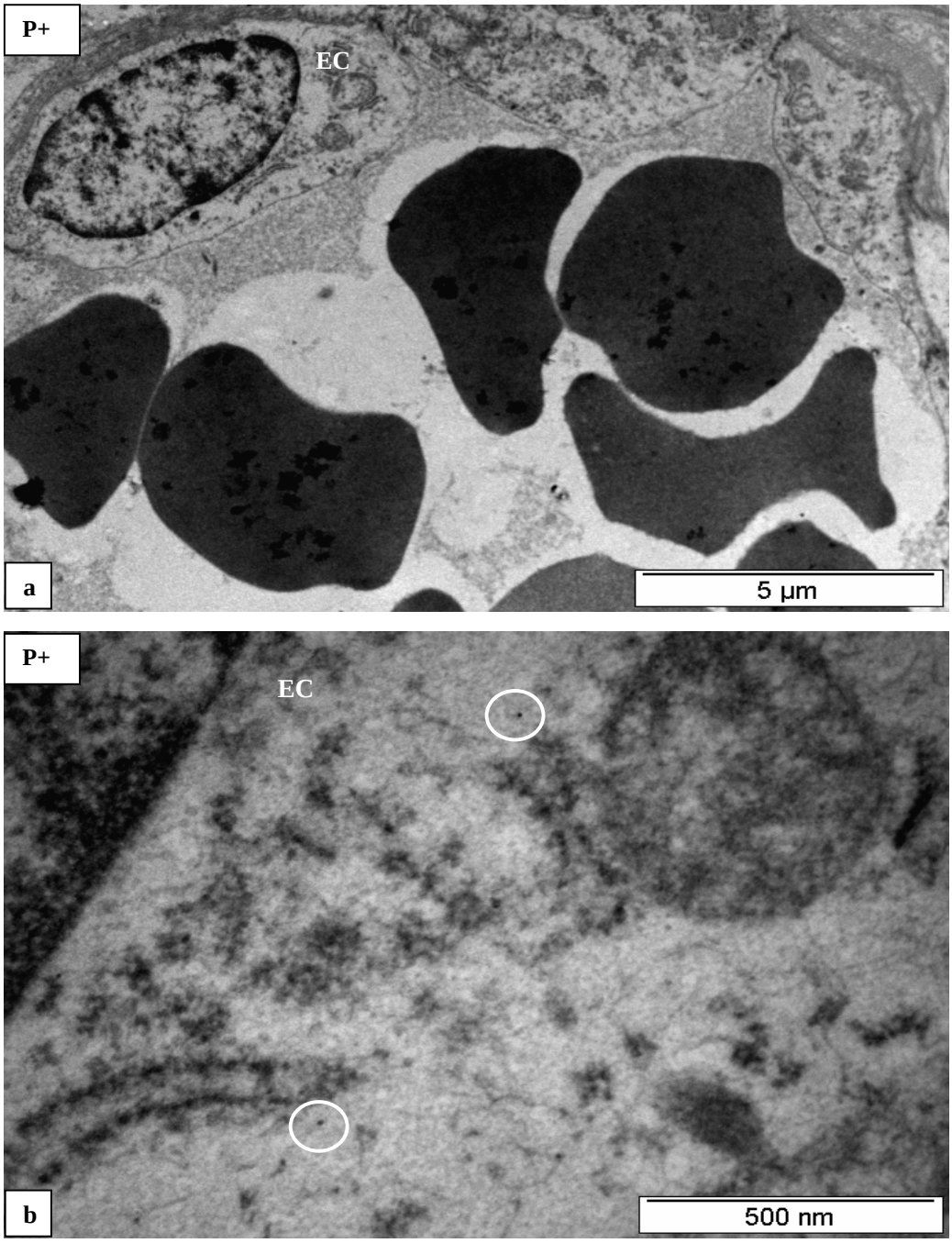


Figure 3.80: Electron micrographs VEGF immunolocalisation (circle) in the P+ group within (a-b) endothelial cells (EC).

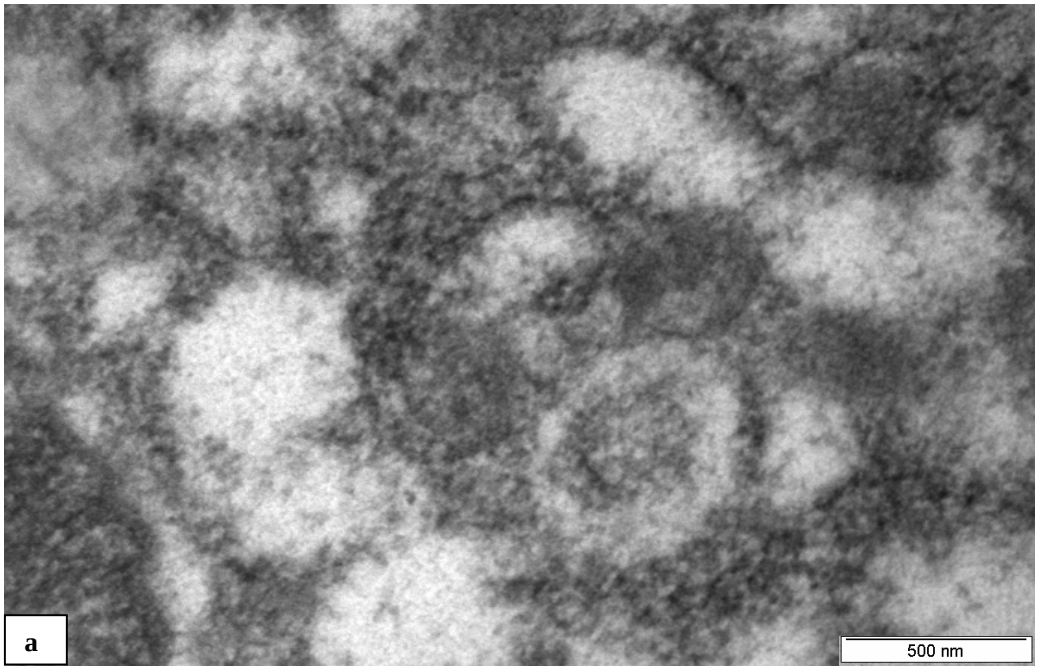


Figure 3.81: Electron micrographs depicting (a) the absence of gold particles in control tissue.

3.5 Genetic analysis of pro- and anti-angiogenic factors

The mRNA levels of pro and anti-angiogenic factors were evaluated using real time PCR and representative images of the real time PCR are shown in **Figs. 3.82-83**.

3.5.1 Two-way Analyses of Variance

There was no significant effect between the HIV negative (normotensive and pre-eclamptic pregnancies) *vs* the HIV positive (normotensive and pre-eclamptic pregnancies) groups for the expression of sFlt-1 [($F(1,72)=0.01$, $p=0.92$]; sEng [$F(1,73)=0.14$, $p=0.71$]; VEGF [$F(1,70)=0.01$, $p=0.93$] and PlGF [$F(1,73)=1.17$, $p=0.28$] respectively.

3.5.1.1 Placental sFlt-1

However, a significant effect of type of pregnancy (normotensive and pre-eclamptic) of sFlt-1 expression [$F(1,72)=11.09$, $p=0.001$] was noted. Simple main effect analyses indicates that the pre-eclamptic had higher LN (sFlt-1) measurements (mean=9.32, 95% CI: 9.70-8.93) compared to the normotensive pregnancies (mean=8.36, 95% CI: 8.78-7.94) **(Fig. 3.84a)**. Finally, no interaction between HIV status and type of pregnancy was noted [$F(1,72)=0.068$, $p=0.794$].

3.5.1.2 Placental sEng

Similarly, a significant effect of type of pregnancy (normotensive vs pre-eclampsia) was shown for sEng expression ($F(1,73)=11.09$, $p=0.001$); showing that the pre-eclamptic had higher LN measurements (mean=10.05, 95% CI:10.40-9.71) compared to the normotensive pregnancies (mean=10.85, 95% CI:11.18-10.52) **(Fig. 3.84b)**. Finally, no interaction between HIV status and type of pregnancy was noted [$F(1,73)=0.001$, $p=0.977$].

Mean placental concentration of both sFlt-1 and sEng were elevated in the P+ group compared to P- groups (**Fig. 3.84a-b**). In addition, analyses of the RNA*later* solution used for storage of placental sample revealed no significant effect on type of pregnancy (normotensive and pre-eclamptics) for the expression of sFlt-1 ($p=0.5130$) (**Fig. 3.86a**), whilst a significant difference ($p<0.05$) between the N- *vs* the N+ was shown for sEng (**Fig. 3.86b**).

3.5.1.3 Placental PlGF

There was no significant effect of the type of pregnancy (normotensive and pre-eclamptics) of PlGF expression [$F(1,73)=1.33$, $p=0.253$] **(Fig. 3.85b)** or between HIV status and type of pregnancy. [$F(1,73)=1.305$, $p=0.257$].

3.5.1.4 Placental VEGF

There was no significant effect of type of pregnancy (normotensive vs pre-eclampsia) of VEGF expression [$F(1,70)=0.372$, $p=0.544$] **(Fig. 3.85a)**or between HIV status and type of pregnancy [$F(1,70)=0.651$, $p=0.422$].

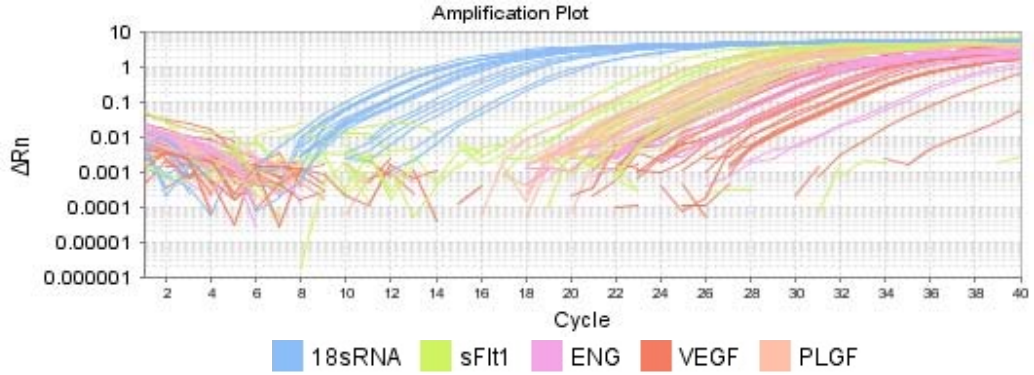


Figure 3.82 Representative image of real time PCR showing the relationship between 18sRNA *vs* the pro-angiogenic and anti-angiogenic factors.

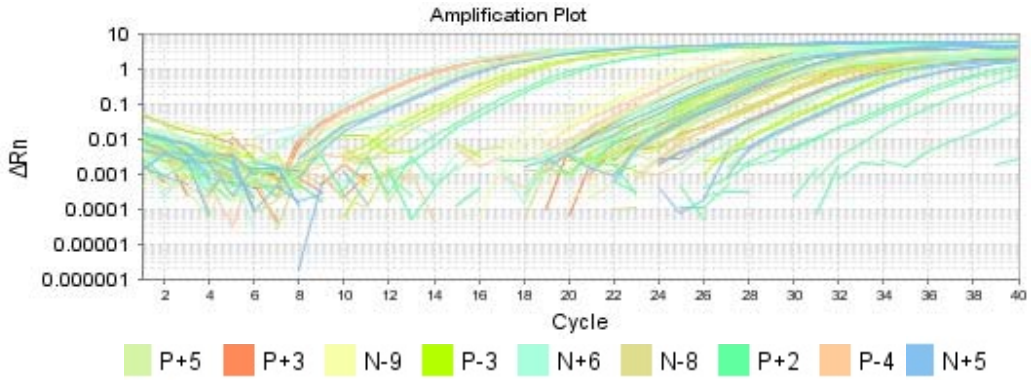


Figure 3.83 Representative image of real time PCR showing the relationship between 18sRNA *vs* the pro-angiogenic and anti-angiogenic factors between study groups.

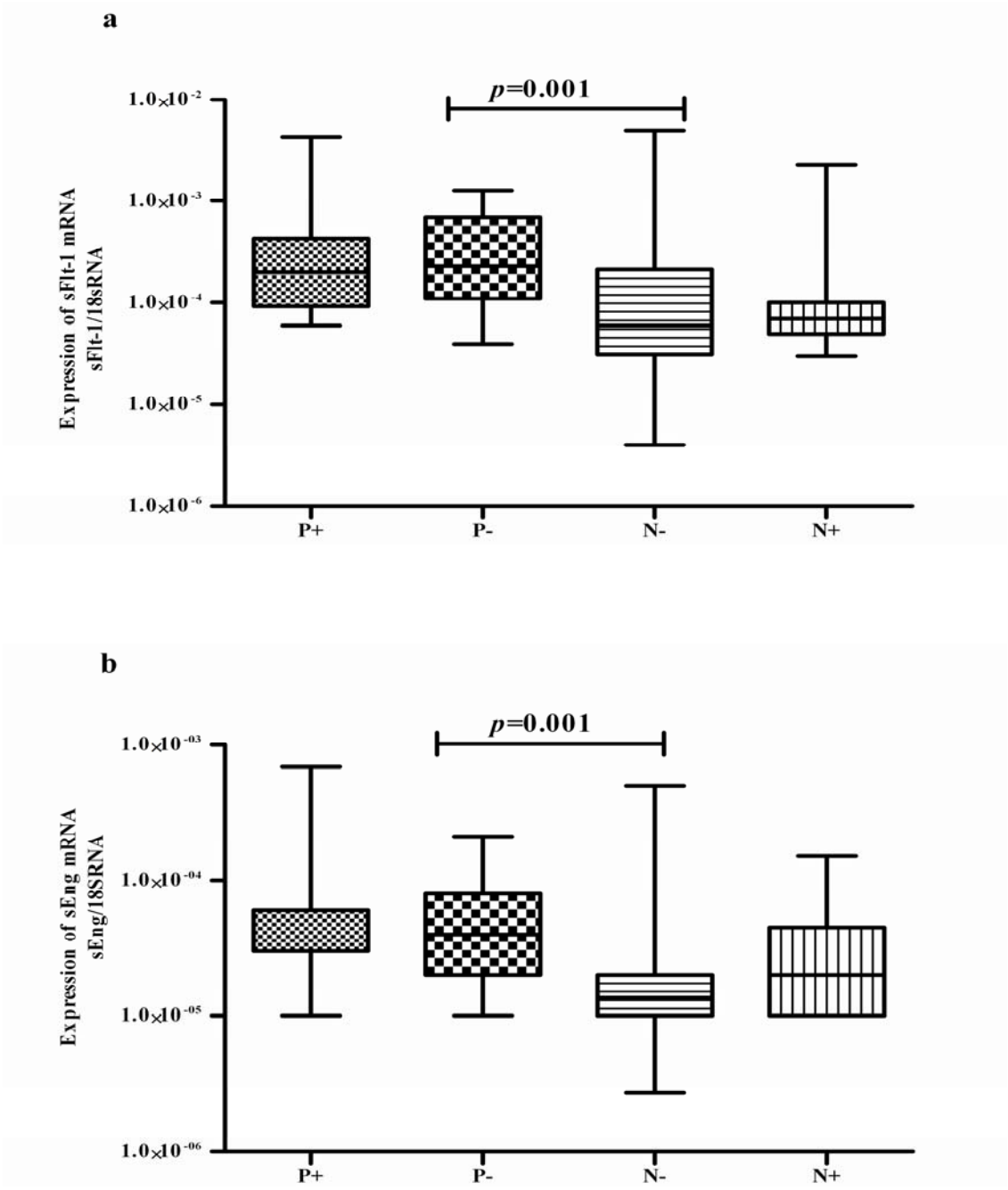


Figure 3.84 Anti-angiogenic placental concentrations using log transformed data (a-b, median and interquartile range): (a) sFlt-1/18S mRNA and (b) sEng /18S mRNA in HIV positive pre-eclamptic (P+); HIV negative pre-eclamptic (P-); HIV negative normotensive pregnant (N-) and HIV positive pregnant women (N+).

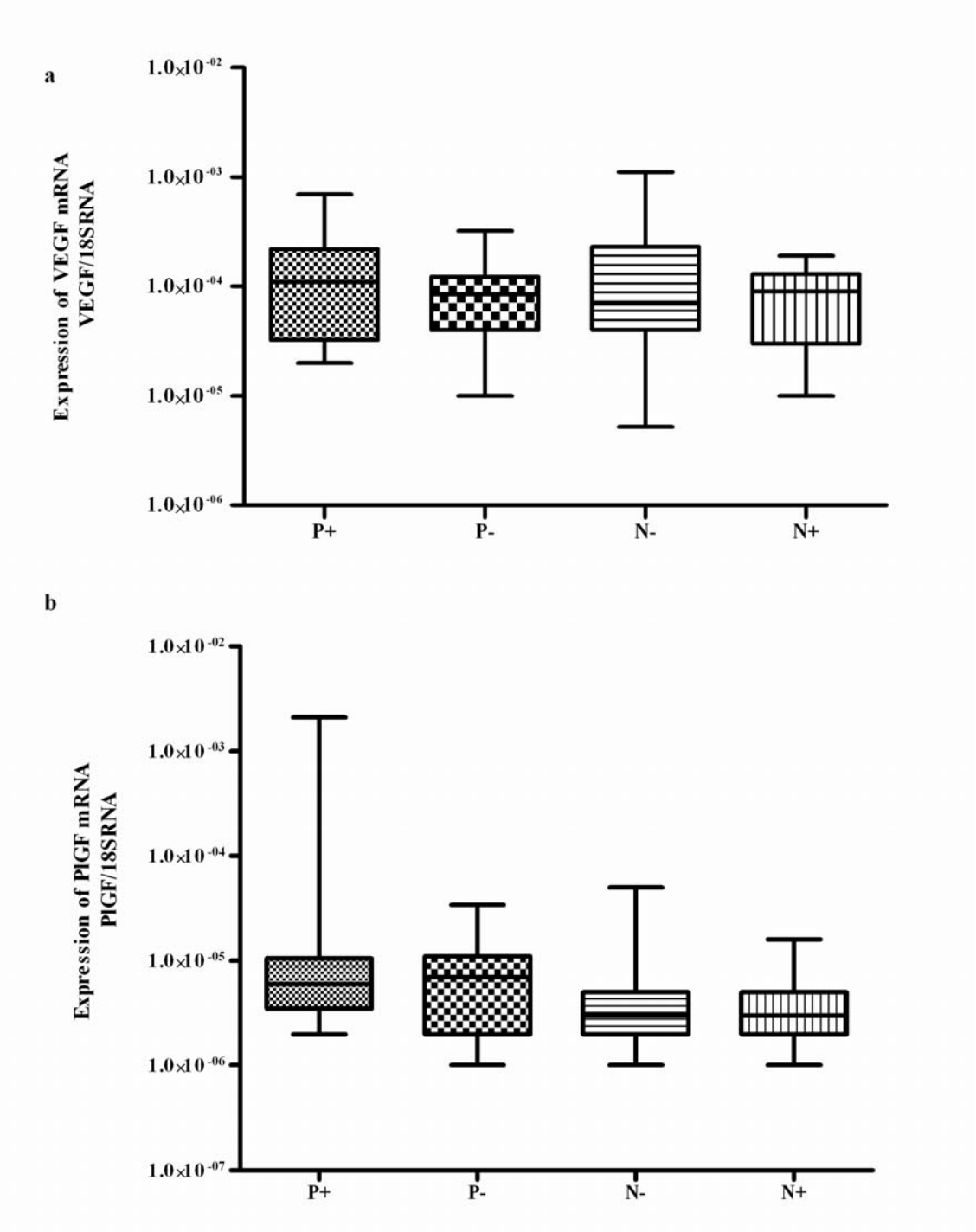


Figure 3.85 Pro-angiogenic placental concentrations using log transformed data (a-b, median an dinterquartile range): (a) VEGF /18S mRNA and (b) PlGF /18S mRNA (AU) in HIV positive pre-eclamptic (P+); HIV negative pre-eclamptic (P-); HIV negative normotensive pregnant (N-) and HIV positive pregnant women (N+).

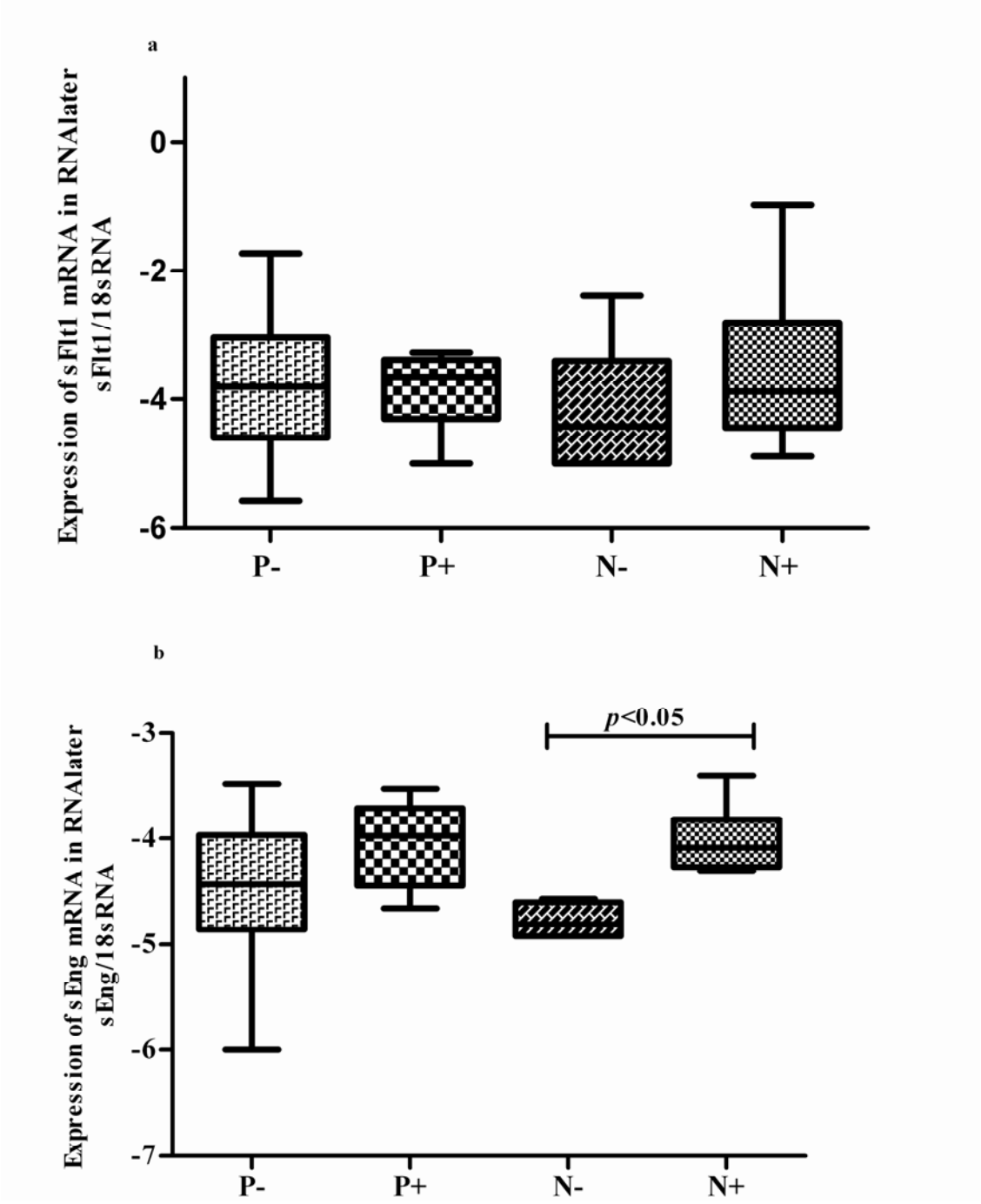


Figure 3.86 Placental RNA*later* concentration of anti-angiogenic factors using log transformed data (a-b, median and interquartile range): (a) sFlt-1/18sRNA and (b) sEng/18sRNA (AU) within HIV positive pre-eclamptic (P+); HIV negative pre-eclamptic (P-); HIV negative normotensive pregnant (N-) and HIV positive pregnant women (N+).

3.6 Quantisation of serum pro- and anti-angiogenic factors using enzyme-linked immunosorbent assay (ELISA) techniques

3.6.1 Two-way Analyses of Variance

The results indicated no interaction between HIV negative *vs* HIV positive groups for the expression of sFlt-1 [F(1,69)=3.86, p=0.05]; sEng [F(1,67)=0.44, p=0.51]; VEGF [F(1,55)=0.22, p=0.64] and PlGF [F(1,67)=0.08, p=0.77] respectively. All data are shown in **Table 3.10; Figs. 3.87a-b; 3.88a-b**.

3.6.1.1 Circulating sFlt-1 levels

However, a significant interaction between the effects of sFlt-1 on pregnancy (normotensive vs pre-eclampsia) [F(1,69)=5.79, p=0.019] was noted. Simple main effect analyses indicated higher sFlt-1 measurements in the pre-eclamptic (mean=14043.5, 95% CI: 12181.8-15905.1) compared to the normotensive pregnant women (mean=10891.6, 95% CI: 9056.0-12727.3). Bonferroni post hoc analyses showed a significant effect between the P- and N+ (p=0.001, **Fig. 3.87a**) only. There was no effect between HIV status and type of pregnancy [F(1,69)=0.039, p=0.193].

3.6.1.2 Circulating sEng levels

Similarly, a significant effect of type of pregnancy (normotensive vs pre-eclampsia) of sEng levels [F(1,67)=10.56, p=0.02] was noted, indicating higher sEng levels in pre-eclamptics (mean=26.81, 95%CI: 20.80-32.83) compared to normotensive (mean=13.08, 95%CI: 7.152-

19.01) groups (**Fig. 3.87b**). Bonferroni post hoc analyses showed a significant difference between N- vs P+ (p=0.02), P- vs N+ (p=0.007) and P+ vs N+ (p=0.001). However, no significant effect between HIV status and type of pregnancy was noted [F(1,67)=0.017, p=0.897].

3.6.1.3 Circulating PlGF levels

Data for PIGF was not normally distributed, thus analyses of PlGF is based on a natural logarithm (LN) transformation of the data. There was a significant effect of type of pregnancy (normotensive vs pre-eclampsia) of PlGF circulating levels [F(1,67)=6.09, p=0.02]. This effect on PlGF showed that pre-eclamptic (mean=4.88, 95% CI: 4.42-5.34) compared to the normotensive (mean=5.67, 95%CI: 5.22-6.11) groups had lower PIGF LN transformed levels (**Fig. 3.88a**). However, no effect was shown between HIV status and type of pregnancy [F(1,67)=1.674, p=0.201].

3.6.1.4 Circulating VEGF levels

Serum VEGF levels were undetected by both the standard and ultrafiltration processes.

3.6.1.5 Circulating TGF-β_1 levels

Data for TGF-β_1 was normally distributed. There was no significant effect of HIV status and type of pregnancy (normotensive and pre-eclamptic) [F(1,79)=0,14p=0.905]. Similarly, no significant effect of type of pregnancy (normotensive vs pre-eclamptics) of circulating TGF-β_1

levels [F(1,79)=1.348, *p*=0.249] were noted. Finally, there was no significant effect of HIV status on type of pregnancy [F(1,79)=0.904, *p*=0.344, **Table 3.10; Fig. 3.88b**]

3.6.1.6 Circulating sEng/TGF- β_1 ratio

There was no significant effect of HIV status and type of pregnancy (normotensive and pre-eclamptic) [F(1,79)=0,649*p*=0.423]. However, a significant effect of type of pregnancy (normotensive vs pre-eclamptics) of circulating TGF-β_1 levels [F(1,79)=9.237, *p*=0.003] was shown. Bonferroni post hoc analyses revealed a significant difference between P- vs N+ (*p*=0.03) and the P+ vs N+ (*p*=0.023). Finally, there was no significant effect of HIV status on type of pregnancy [F(1,79)=1.137, *p*=0.289 , **Table 3.10; Fig. 3.89b**].

3.6.1.7 Circulating sFlt-1/PlGF ratio

Raw data were not normally distributed across the smallest comparative groups, thus a natural logarithm transformation was performed. There was no significant effect between HIV status and type of pregnancy (normotensive and pre-eclamptics) of sFlt-1/PlGF levels (*p*=0.745). Likewise, no significant effect of type of pregnancy (normotensive and pre-eclamptics) of sFlt-1/PlGF levels was noted (*p*=0.301, **Fig. 3.89b**). In addition, there was no significant effect between HIV status and type of pregnancy (*p*=0.096).

3.6.1.8 Circulating sFlt-1 + sEng/ PlGF ratio

There was a significant effect between HIV status and type of pregnancy (normotensive and pre-eclamptics) of sFlt-1 + sEng/ PlGF levels (p=0.04). However, no significant effect of type of pregnancy (normotensive and pre-eclamptics) of sFlt-1+sEng/PlGF levels was noted (p=0.161, **Fig. 3.90**). Finally, no significant effect between HIV status and pregnancy was noted (p=0. 105).

However, the anti-angiogenic ratio levels of sFlt-1/PlGF were notably higher in the HIV negative pre-eclamptics compared to the HIV negative normotensive pregnancies (**Fig. 3.89b**), whilst no observable difference was evident for the HIV positive cohorts. Likewise, there was no noticeable difference in the levels of sFlt-1+sEng/PlGF between the pre-eclamptics (HIV negative and positive) compared to the normotensive pregnancies (HIV negative and positive), (**Fig. 3.90**).

3.6.2 Correlation analyses of circulating factors

3.6.2.1 Circulating levels of sFlt-1 *vs* sEng

A Pearson correlation coefficient was computed to assess the relationship between circulating levels of sFlt-1 and sEng across all study groups. There was a modest, positive correlation (**Fig. 3.91a**) between the circulating levels of sFlt-1 and sEng across the study population (r=0.530, p=0.000).

3.6.2.2 Circulating levels of sFlt-1 and PlGF

A Pearson correlation coefficient was computed to assess the relationship between circulating levels of sFlt-1 and PlGF across the study groups. There was a moderate, negative correlation between the circulating levels of sFlt-1 and PlGF across the study population (r=-0.475, p=0.000, **Fig. 3.91b**). Thus, increases in circulating sFlt-1 concentrations are correlated with decreases in circulating PlGF concentrations.

3.6.2.3 Circulating levels of sEng and TGF-β_1

A Pearson correlation coefficient was computed to assess the relationship between circulating levels of sEng and TGF-β1 across all study groups. However, there was no correlation between these factors (r=0.094, p=0.4).

3.6.3 Descriptive statistics of pro- and anti-angiogenic factors

Mean serum levels of sFlt-1 and sEng were higher in both the HIV negative pre-eclamptic and HIV positive pre-eclamptic women (p<0.05) compared to the normotensive groups. Similarly, mean serum levels of PlGF were higher in the normotensive compared to the pre-elamptic groups.

Table 3.10 Comparison of pro- and anti-angiogenic factors of maternal serum across study groups

	Normotensive Pregnant Women		Pre-eclamptic Pregnant Women		
	HIV negative (N-)	**HIV positive (N+)**	**HIV negative (P-)**	**HIV positive (P+)**	**p value**
n	27	31	27	25	
[*]**sFlt-1(pg/ml)**	10747.6±4476	10117.1±5453	15527.8±6489	12173.4±5498	*p*=0.019
[*]**sEng(ng/ml)**	15.2±3.5	11.6±3.1	26.1±3.1	29.6±3.5	*p*=0.02
[#]**PlGF(pg/ml)**	488.6(183.9848.3)	207.1(102.6-358.6)	202.2(47.9-490.4)	229.3(74.3-615.9)	*p*=0.02
[*]**TGF-β1 (pg/ml)**	29580.34±9466.5	31461.43±7121.17	33292.93±6739.78	31830.79±8318.59	ns
[*]**sEng/ TGF-β1 (pg/ml)**	0.61±0.60	0.35±0.20	0.88±0.79	0.92±0.74	*p*=0.003
sFlt-1/PlGF (pg/ml)	21.4(7.2-50.8)	41.6(14.5-140.7)	66.5(28.3-136.9)	37.3(14.8-106.2)	ns
sFlt1+sEng/ PlGF (pg/ml)	12392(8792-15700)	9422(7014-16914)	12988(6859-17276)	10772(8345-13527)	ns

[*]Significance: p< 0.05, n=110, Parametric data: mean ±SD; non parametric data: median and inter-quartile ranges,

[#] Refers to actual data and not log transformed data

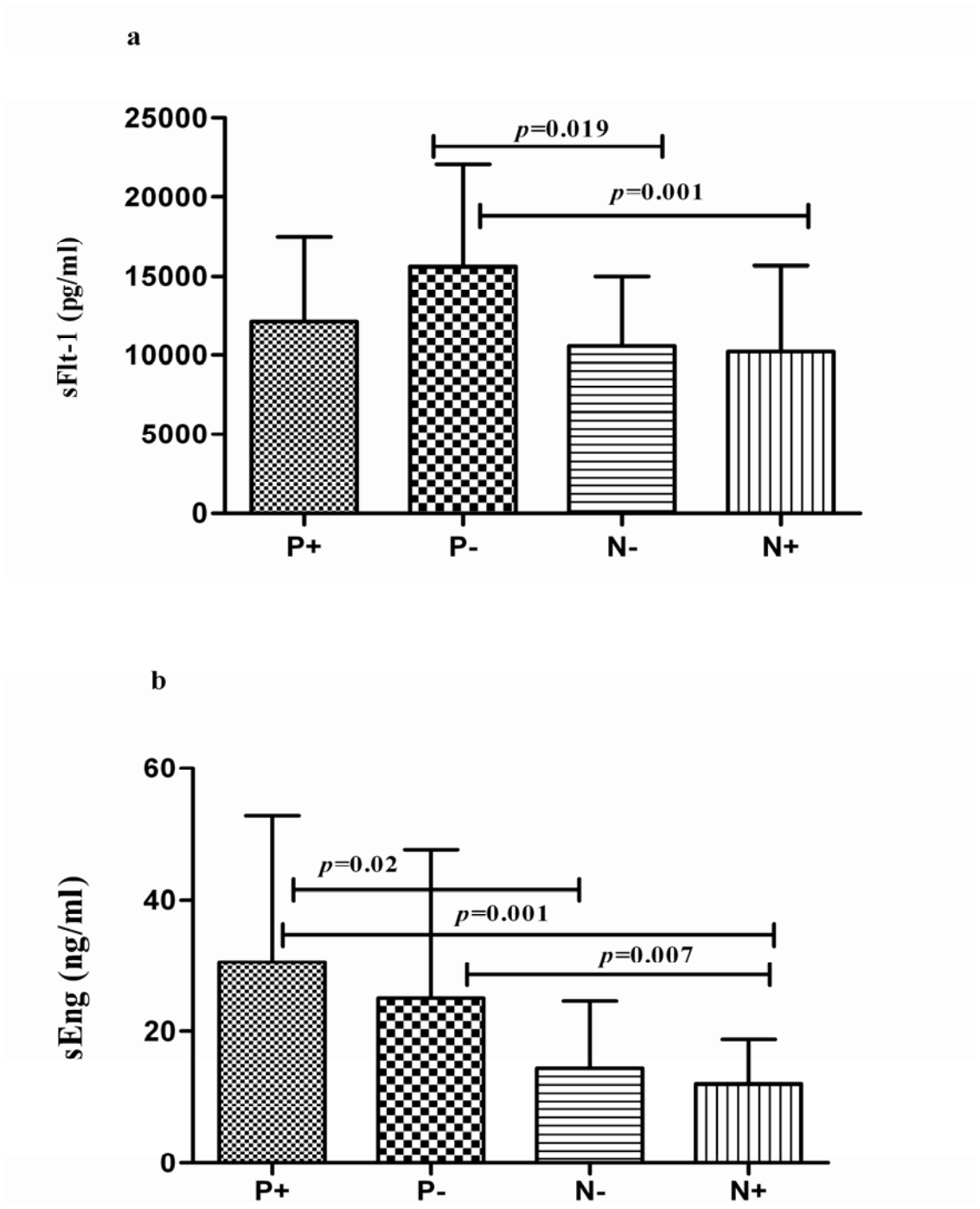


Figure 3.87: Anti-angiogenic serum concentrations (a-b, mean ± SD): (a) sFlt1 (pg/ml) and (b) sEng (ng/ml) in HIV positive pre-eclamptic (P+); HIV negative pre-eclamptic (P-); HIV negative normotensive pregnant (N-) and HIV positive pregnant women (N+).

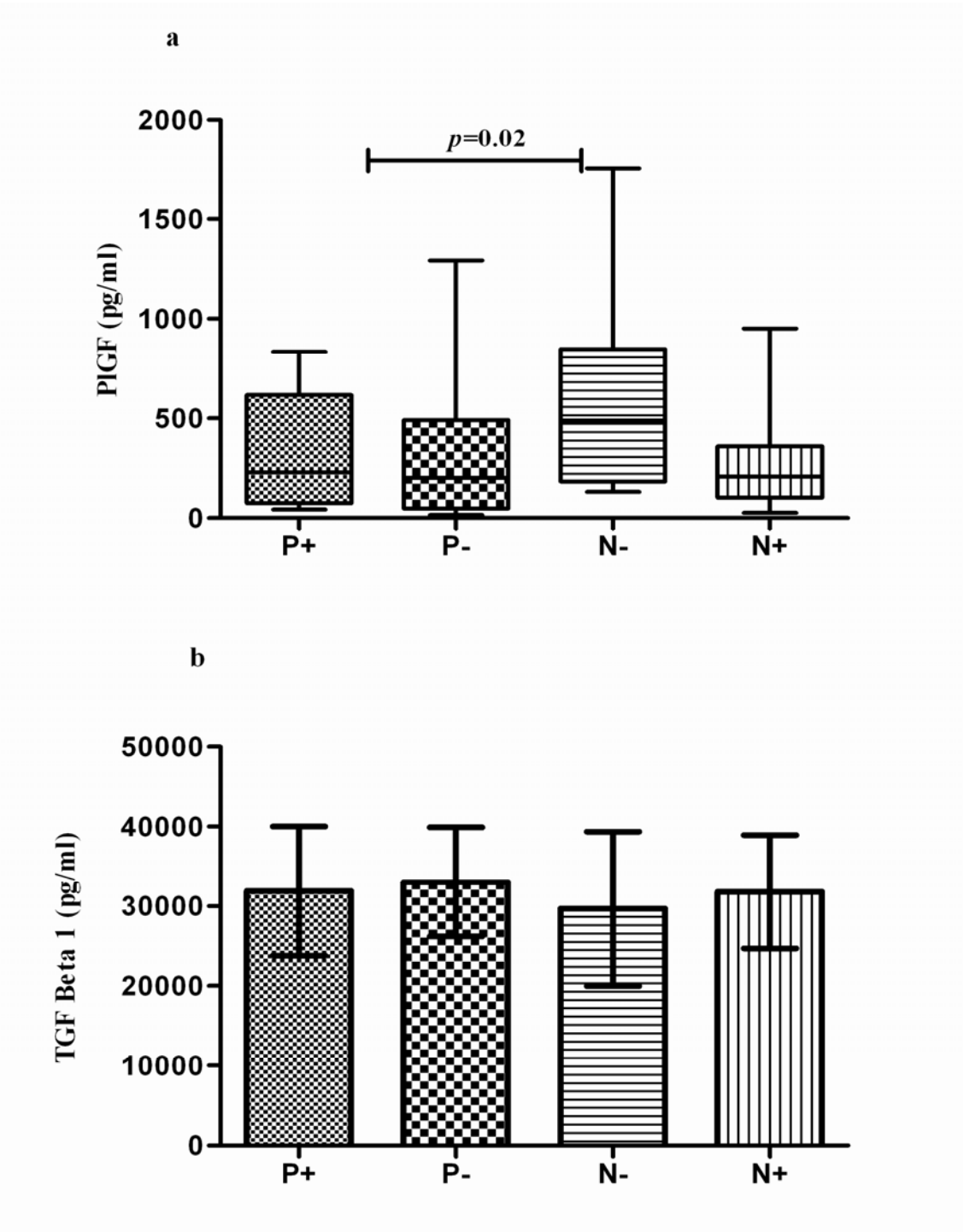


Figure 3.88: Pro-angiogenic serum concentrations: (a) PlGF (median interquartile range, pg/ml) and (b) TGF beta 1 (mean ± SD, pg/ml) within HIV positive pre-eclamptic (P+); HIV negative pre-eclamptic (P-); HIV negative normotensive pregnant (N-) and HIV positive pregnant women (N+).

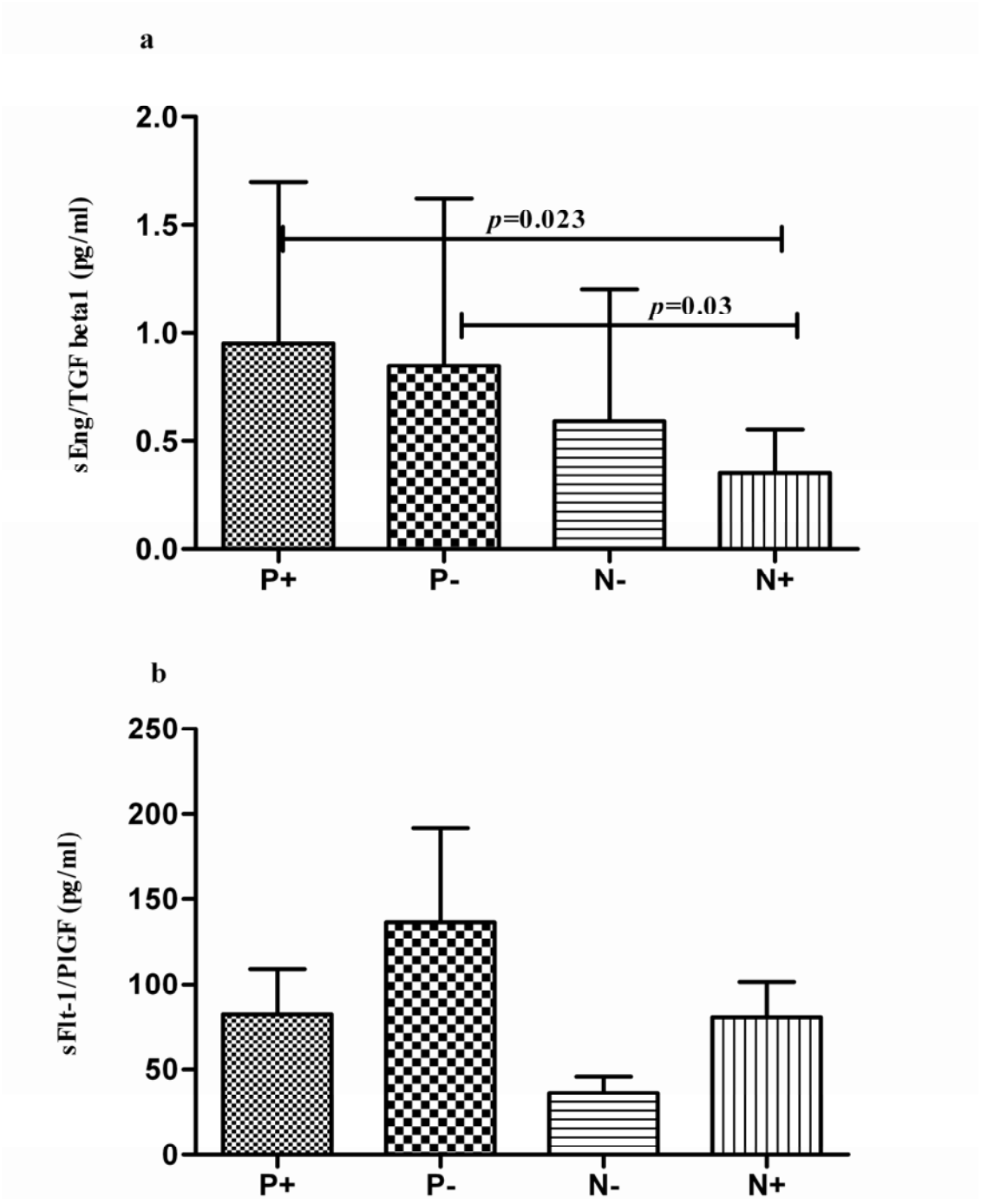


Figure 3.89: Anti-angiogenic ratio serum concentrations: (a) sEng/ TGF-β_1 mean ± SD and (b) sFlt-1/PlGF (median and inter-quartile range)in HIV positive pre-eclamptic (P+); HIV negative pre-eclamptic (P-); HIV negative normotensive pregnant (N-) and HIV positive pregnant women (N+).

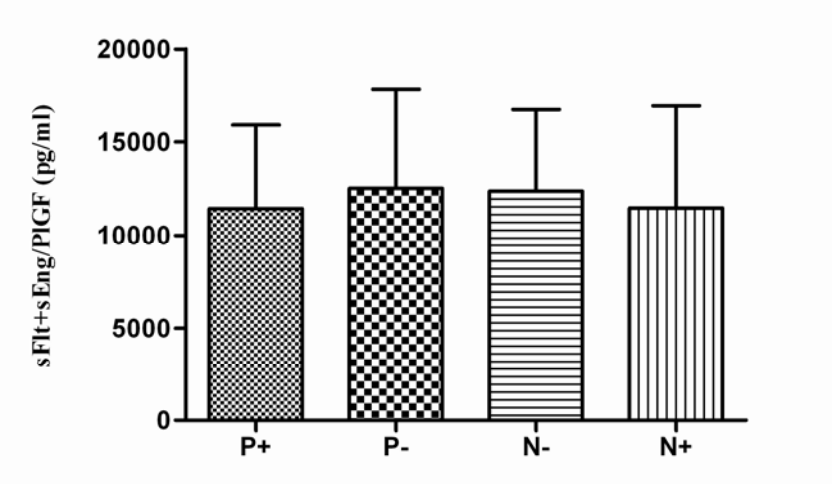


Figure 3.90: Anti-angiogenic ratio [(sFlt-1+sEng)/PlGF] serum concentration (mean ± SD) in HIV positive pre-eclamptic (P+); HIV negative pre-eclamptic (P-); HIV negative normotensive pregnant (N-) and HIV positive pregnant women (N+).

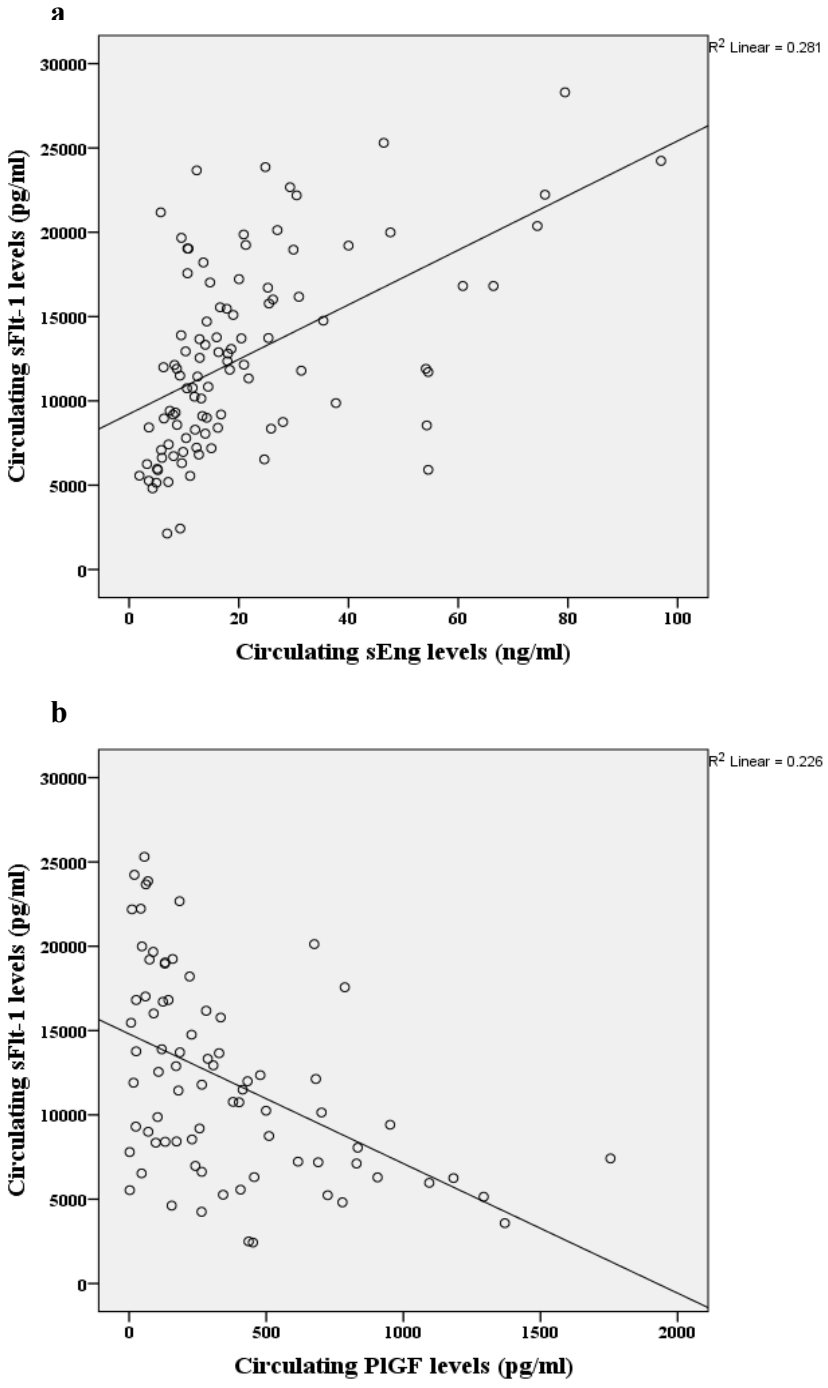


Figure 3.91 Pearson's correlation analyses (a) circulating sFlt-1 (pg/ml) *vs* sEng (ng/ml) and (b) sFlt-1 *vs* PlGF (pg/ml) across all study groups.

3.7 Comparison between serum and placental levels of pro- and anti-angiogenic factors

3.7.1 Correlation analyses of sFlt-1, sEng, PlGF and VEGF expression between serum and placenta

A Spearman's rho correlation coefficient was computed to assess the relationship between circulating and placental expression of sFlt-1, sEng, PlGF and VEGF across all study groups. There was a weak correlation between circulating and placental concentration for sFlt-1, sEng and PlGF (r=0.26, p=0.031; r=0.42, p<0.001 and r=-0.3, p=0.014) respectively. Additionally, sub-group correlation analyses for sEng within the P- group, showed a strong, positive correlation between circulating vs placental expression (r=0.52, p=0.035). However, sub-group analyses for PlGF within the P+ group revealed a strong, negative correlation between serum *vs* placental expression (r=-0.54, p=0.04). A scatter-plot summarizes the results for sFlt-1 and sEng (**Fig. 3.92a-b**).

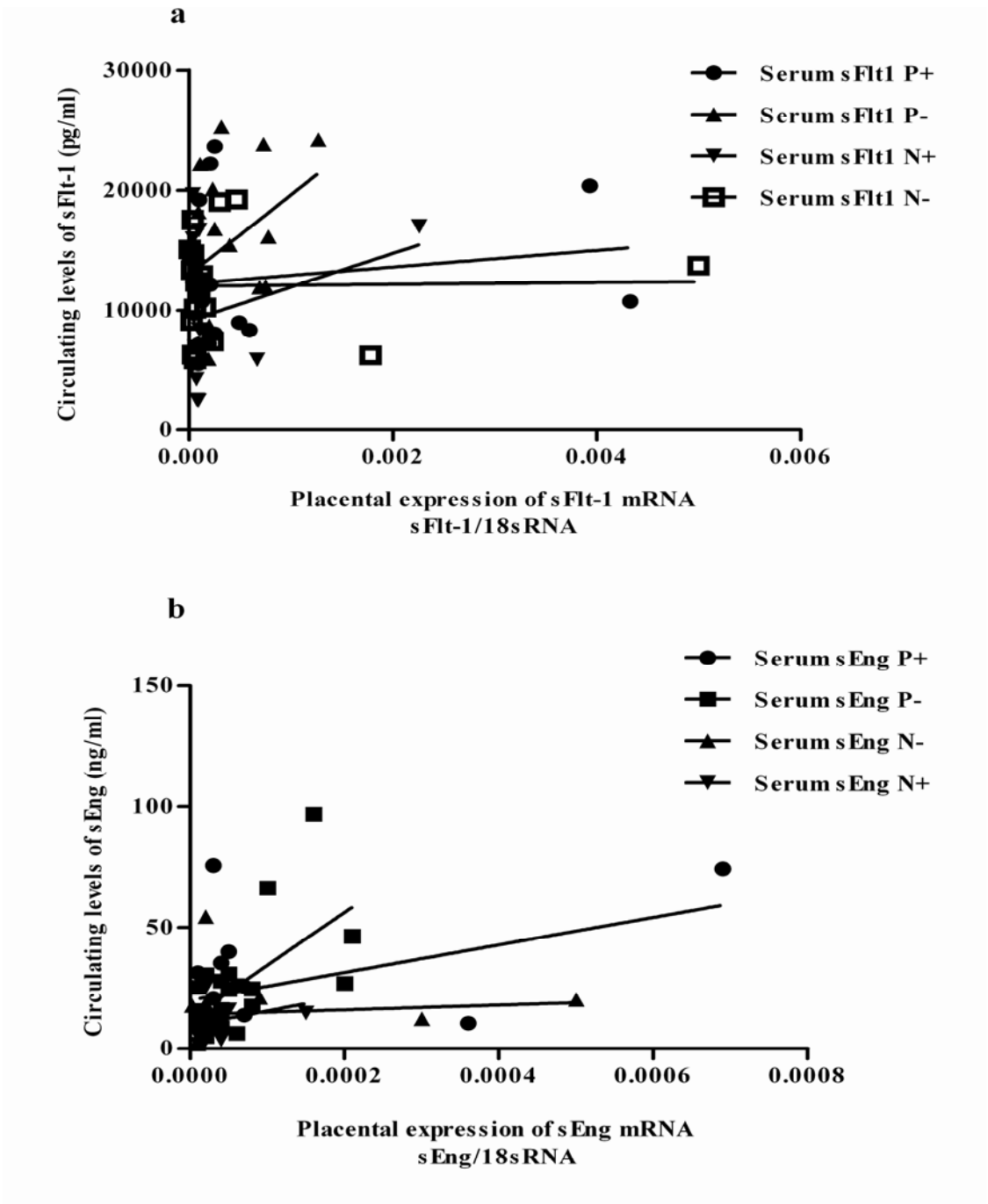
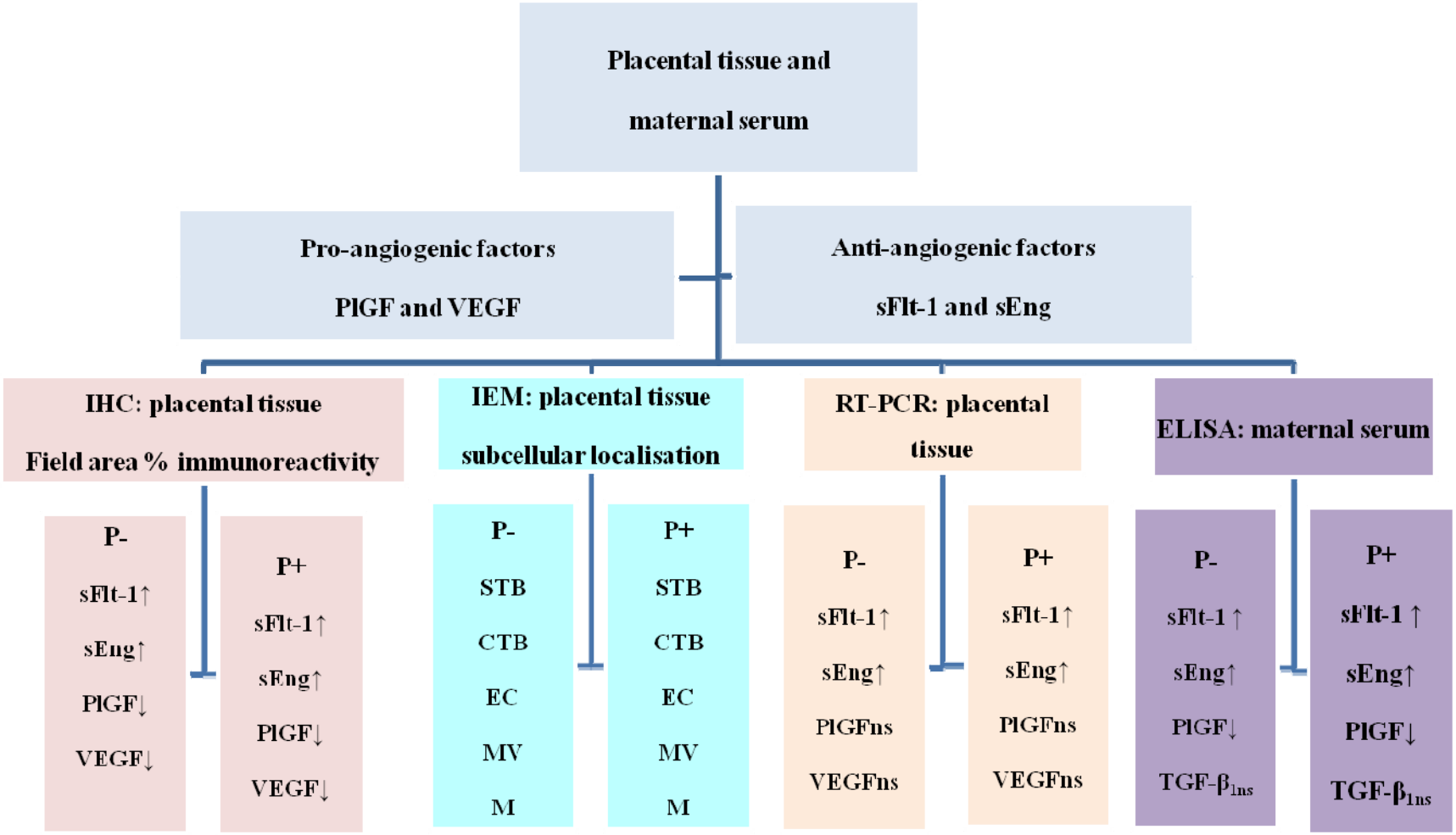


Figure 3.92 Spearman's rho correlation analyses between serum and placental expression: (a) sFlt1 and (b) sEng within HIV positive pre-eclamptic (P+); HIV negative pre-eclamptic (P-); HIV negative normotensive pregnant (N-) and HIV positive pregnant women (N+). Data has been log transformed to ensure normality between the groups

Summary of results for HIV-associated pre-eclampsia (P- and P+ groups)



CHAPTER 4

Discussion

Systemic inflammation combined with angiogenic variations, oxidative tension and vascular reactivity are key features that epitomise normotensive pregnancies (Myatt and Webster, 2009). In contrast, these features are intensified during pre-eclampsia with consequential collapse of compensatory systems. The resultant hypoxic microenvironment of pre-eclampsia in turn stimulates the discharge of anti-angiogenic factors (sFlt-1 and sEng), inflammatory cytokines, and apoptotic waste into the maternal circulation (Redman and Sargent, 2000, Cindrova-Davies, 2009, Maynard *et al.*, 2003, Rajakumar *et al.*, 2012, Sargent *et al.*, 2003). Excess levels of both sFlt-1 and sEng have been associated with pre-eclampsia development (Mutter and Karumanchi, 2008). Thus, it is presumed that these anti-angiogenic proteins antagonise the effects of VEGF, PlGF and TGF-β, respectively, leading to defective placentation, culminating in an elevated maternal inflammatory response and subsequent endothelial dysfunction, that characterises pre-eclampsia (Levine and Karamanchi, 2005, Venkatesha *et al.*, 2006, Mutter and Karumanchi, 2008, Maynard *et al.*, 2003).

This is the first study that examines the effect of HIV infection on the expression of pro-and anti-angiogenic factors in pre-eclampsia at term. In South Africa, HIV infection is the leading cause of maternal mortality and morbidity in pregnancy (Saving Mothers Report, 2012). The results of our study demonstrate that HIV infection has no significant effect on type of pregnancy (normotensive and pre-eclamptic), with regards to the expression of PlGF, VEGF, sFlt-1 and sEng. Variations between the maternal circulating levels of pro-angiogenic (VEGF, PlGF & TGF-β_1) and the anti-angiogenic factors (sFlt-1 & sEng) that occur in pre-eclamptic (P- and P+) and normotensive (N- and N+) pregnancies are however, demonstrated.

In developed countries, materno–fetal morbidity and mortality may be avoided by early identification of pre-eclampsia and the subsequent delivery of the pre-term placenta. On the contrary, in developing countries like South Africa, pre-eclampsia is usually diagnosed at term due to late patient presentation at the tertiary hospital under study. Within this low socio-economic setting, the early identification of pre-eclampsia development is often not possible, contributing to insufficient prenatal care. Consequently, data from developed countries on pro- and anti-angiogenic proteins mirror pre-term, in contrast to term pregnancies in our study. Nevertheless, our study is novel in that elevations in sFlt-1 and sEng, irrespective of HIV infection are sustained in serum of pre-eclamptics at term, emphasising their utility as discriminatory factors for disease detection and management.

4.1 Clinical Characteristics

Since placental delivery is the only cure for pre-eclampsia, its clinical management is dependent on gestational age and disease severity. In our study, pre-eclamptic groups (P+ and P-) delivered at a slightly earlier gestational period compared with the normotensive groups. Our data revealed that placental weight for the pre-eclamptics was greater than the normotensive groups. This is surprising, as one would have expected the placental weights to be lower in view of the fact that the pre-eclamptics delivered at a lesser gestational age and that pre-eclampsia is associated with IUGR. Although we did not correlate fetal growth with gestational age following delivery, there is circumstantial evidence that women with gestational hypertension and mild pre-eclampsia tend to have slightly bigger babies and larger placental masses than their normotensive counterparts at birth (personal communication, J Moodley). It is plausible to hypothesise that a mild increase in blood pressure can cause a concomitant

increase in placental perfusion pressure and increased oxygen supply, resulting in increased placental size.

Whilst classical studies implicate elevated sFlt-1 and sEng levels in the development of pre-eclampsia (Levine and Karumanchi, 2005, Levine *et al*., 2006, Maynard *et al*., 2003), more recent epidemiological analysis reports that placental weight may not be a risk indicator for the placental dysfunction evident in pre-eclampsia (Eskild and Vatten, 2010). The latter study, together with others, suggests that whilst the placenta is a rich angiogenic contributor, the maternal circulatory angiogenic imbalance evident in pre-eclampsia may be ascribed mainly to placental hypoxia.

As per inclusion criteria, our study demonstrates higher levels of systolic and diastolic blood pressures in pre-eclamptics compared with normotensive pregnancies, irrespective of the HIV status. Recent studies examining the relationship between blood pressure and maternal angiogenic factors in normotensive pregnancies, suggests that elevations in blood pressure at term may be ascribed to increases from the second to third trimesters, however this link may be less pronounced in pre-eclampsia (Troisi *et al.*, 2008). In addition, Troisi *et al*., (2008) illustrated a linear association between blood pressure and maternal sEng concentrations, with a reciprocal effect on PlGF concentrations in normotensive pregnancies. In our study, the elevated blood pressure observed within the pre-eclamptic cohorts may be ascribed to the increased levels of sFlt-1 and sEng.

More recently, sequential changes in maternal circulating levels of sFlt-1, sEng, and PlGF and their link to blood pressure and endothelial function was demonstrated (Noori *et al.*, 2010),

suggestive of a supportive role for pro-angiogenic biomarkers in managing pre-eclampsia related vascular resistance. A positive correlation between maternal circulating levels of sEng and blood pressure was demonstrated, whilst sFlt-1 levels were strongly associated with proteinuria (Masuyama *et al.*, 2007). This association demonstrates their possible role in the severity of pre-eclampsia, however highlighting that their roles may differ in the pathophysiology of pre-eclampsia development. Thus, it is plausible that sEng contributes to the development of pre-eclampsia by increasing blood pressure.

The molecular basis for the angiogenic imbalance evident in pre-eclampsia remains to be elucidated (Maynard *et al.*, 2008a). In addition, the association between clinical parameters with the maternal angiogenic profiles are limited. However, an evaluation by Troisi *et al*., (2008) suggests that an elevation in blood pressure from mid- to late gestation is possibly related to a raised anti-angiogenic profile at delivery. Moreover, an association was shown between increasing maternal age and an elevated anti-angiogenic profile [(sFlt1+ sEng)/PlGF] in normotensive compared to pre-eclamptic pregnancies (Staff *et al.*, 2009). Additionally, a higher anti-angiogenic profile correlation with lower gestational age and birth weight was demonstrated in pre-eclampsia compared to normotensive pregnancies. However, due to the paucity of data, a large epidemiological study will possibly clarify the current association between maternal clinical parameters with circulating anti-angiogenic profiles and may dictate the severity of this maternal disorder.

4.2 Histology

Normal pregnancy can only be sustained if the placenta is capable of ensuring adequate gas and nutrient exchange between the maternal-fetal interface, thereby enhancing the development of a healthy fetus (Gude *et al.*, 2004). Histological analyses of the full term placentae in our study revealed the typical architecture of placental villi across all study groups. Additionally, we noted other features such as syncytial knots and bridges, perivillous and intravillous fibrinoid deposition. Moreover, the syncytial knots and bridges were observed more frequently within the pre-eclamptic compared to the normotensive prgnancies. Our observations concur with previous studies (Majumdar *et al.*, 2005), who demonstrated an increase in syncytial knot formation, cytotrophoblast proliferation and stromal fibrosis of villi in pre-eclampsia compared to normotensive pregnancies.

Syncytial knots and bridges are both focal accumulation of nuclei arising from local syncytial hyperplasia, trophoblast degeneration (Tenney-Parker changes), mal-perfusion of placental villi or the structural appearance of hypoxia, ischaemia or hypertension (Bernischke *et al.*, 2006). More specifically, syncytial bridges occur between adjoining villous surfaces with consequent syncytial fusion (Cantle *et al.*, 1987). These features have also been ascribed to tangential sectioning across syncytiotrophoblastic surfaces (Cantle *et al.*, 1987, Burton and Jones, 2009). However, other studies have shown that these syncytial nuclei accumulation are apoptotic or aponecrotic, hence they should be referred to as apoptotic knots (Huppertz *et al.*, 2002, Huppertz *et al.*, 1998). These nuclei essentially characterise different apoptotic phases. In addition, syncytial nuclei accumulations have also been reported as extrusion sites for old or dying nuclei that are later pinched off together with some surrounding cytoplasm and released into maternal circulation (Cantle *et al.*, 1987).

In our study, the qualitative increase in the occurrence of syncytial knotting in pre-eclamptic cohorts may be regarded as nuclei accumulation due to Tenney-Parker changes since full term placentae were utilised (Bernischke *et al.*, 2006). In addition, the qualitative increase of syncytial knotting in pre-eclamptic cohorts may also be ascribed to hypoxia. This corroborates previous reports that knotting occurs more frequently under hypoxic conditions such as hypertensive disorders (Heazell *et al.*, 2007), maternal anemia (Kosanke *et al.*, 1998) and high altitude (Alia *et al.*, 1996). Studies have linked pre-eclampsia to decidual vasculopathy and villous hypermaturity, characterised by increased syncytial knotting (Kraus *et al.*, 2004, Taché *et al.*, 2011). In our study, the qualitative observation of a more frequent occurrence of syncytial knots within pre-eclamptics compared to normotensive pregnancies is possibly associated with villous hypermaturity.

Our study also shows the occurrence of Langhans, Rohr, perivillous and intravillous fibrin deposition across all study groups. Fibrinoid deposition is a classical feature of normotensive term placentae (Bernischke *et al.*, 2006). Aggregation of platelets has been linked to the early phases of perivillous fibrinoid deposition with the subsequent deterioration of syncytiotrophoblast (Kaufmann *et al.*, 1996, Fox, 1997). The cause of fibrinoid formation has been attributed to intervillous circulatory factors (collagens, fibronectin and laminin) and to intrasyncytial compromise. Intrasyncytial causes have been linked to hypoxia and acidosis, resulting in syncytial deterioration and the consequent syncytial replacement by fibrinoid. Intervillous circulatory factors on the other hand, are components of the trophoblast basal lamina that instigate coagulation. Thus, focal syncytiotrophoblast deterioration and the resulting trophoblast deficiency appear to be regulated by the development of fibrinoid. In our

study, the prominent perivillous fibrinoid deposition within the pre-eclamptic study groups may be ascribed to either intervillous circulatory or intrasyncytial causes, which typically epitomise the HELLP and hypoxic features of pre-eclampsia.

In our study, intravillous fibrinoid deposition with complete obliteration of villous stroma was pronounced in the pre-eclamptic compared to normotensive pregnancies. This deposition was observed mainly within exchange villi, suggestive of a deficient exchange of nutrient and gas occurring at this materno-fetal interface. Other studies have observed this anomaly in diabetes (Verma *et al.*, 2010) and fetal erythroblastosis (Hellman and Hertig, 1938). Intravillous fibrinoids are also perceived to arise from placental deterioration aggravated by aging and immunological mechanisms (Bernischke *et al.*, 2006). In our study, however, no qualitative difference was noted between the HIV positive and HIV negative cohorts.

Notably, immunologic disorders may possibly impinge on the syncytiotrophoblast barrier, thereby enhancing its permeability to antigen-antibody complexes and the consequent deposition of fibrinoids within villous stroma (Kaufmann *et al.*, 1996). This may be relevant in the high HIV endemic region of sub-Saharan Africa, as syncytial deterioration may be involved in vertical transmission. In this study, our qualitative observation of cytotrophoblast proliferation within the pre-eclamptic groups concurs with the observations by Fox (1997). The quantity of cytotrophoblast/Langhans' cells is enhanced by the severity and duration of pre-eclampsia (Fox, 1997). This feature was also observed in conditions with immature placentae, erythroblastosis (Hellman and Hertig, 1938), maternal anaemia (Kosanke *et al.*, 1998) and diabetes mellitus (Verma *et al.*, 2010).

In addition, in our study, the qualitative observation of cisternal pools of dilated endoplasmic reticulum within the HIV positive cohorts was noted. Recent studies have implicated that this endoplasmic stress may be due to HIV infection, possibly via the induction of endoplasmic reticulum stress pathways (Maingat *et al.*, 2011).

4.3 Placental and circulating levels of pro- and anti-angiogenic factors

Pro-angiogenic factors VEGF, PlGF and TGF-β together with their antagonist, sFlt-1, are highly expressed within the placental tissue, suggestive that angiogenic regulation is essential for normal placental growth and thus a healthy fetus (Clark *et al.*, 1998a, Mutter and Karumanchi, 2008). In our study, the immunolocalisation of pro-angiogenic VEGF and PlGF and anti-angiogenic sFlt-1 and sEng confirms the placenta as a site of production. Regardless of pathology, the site of immunolocalisation of these factors remained consistent across study groups, suggestive of their characteristic physiologic expression.

To date, studies have explored both serum and plasma concentrations of these factors during pre-eclampsia and compared it to normotensive pregnancies. Our investigation is the first study to explore the effect of HIV status on circulating levels of these factors in pre-eclampsia, compared to normotensive pregnancies. It is also novel in that it subcellularly localises these factors within the placenta. In our study, however due to the small sample size, we were unable to demonstrate a relationship between placental mRNA expression of PlGF, sFlt-1 and sEng with serum immunoexpression.

VEGF is a powerful endothelial-specific mitogen and its role in angiogenesis is well documented (Ferrara and Keyt, 1997, Ferrara, 2004, Ferrara and Davis-Smyth, 1997). It

functions via two high affinity receptor tyrosine kinases Flt-1 and VEGFR-2. In addition, PlGF a member of the VEGF family binds to Flt-1 thereby supplementing the pro-angiogenic effects of VEGF. More recently, a soluble isoform and splice variant of Flt-1, sFlt-1 was identified (Maynard *et al.*, 2003). These investigators demonstrated an excess production of sFlt-1 by the pre-eclamptic placenta with their subsequent discharge into the maternal circulation. This variant is characterised by the presence of a ligand binding domain and absence of a trans-membrane and cytoplasmic domain (Maynard *et al.*, 2003).

4.3.1 Placental sFlt-1

The release of VEGF, PlGF and TGF-β and their antagonists' sFlt-1 and sEng into maternal circulation is dependent upon both their autocrine and paracrine regulation. Our study is consistent with previous data reporting sFlt-1 immunolocalisation in the syncytio-, cytotrophoblasts, syncytial knots, endothelial cells (exchange and conducting villi) and some stromal cells of the placentae in both normotensive and pre-eclamptic pregnancies (Tsatsaris *et al.*, 2003, Helske *et al.*, 2001, Munaut *et al.*, 2012, Ahmad and Ahmed, 2004, Munaut *et al.*, 2008). Similar to Tsatsaris *et al*, (2003), our study also reports immunodetection of sFlt-1 in the extravillous trophoblast cell population. In addition, sFlt-1 was immunolocalised sub-cellularly within endoplasmic reticulum, mitochondria and cytoplasm of syncytio and cyto-trophoblasts, as well as endothelial cells across all study groups.

To date, there is no available literature on the subcellular localisation of sFlt-1 in the placenta. The HIV immune status of our study groups did not affect sFlt-1 immunoexpression in the exchange (p=0.53) and stem villi (p=0.052). However, the immunoexpression of sFlt-1 in exchange villi differed significantly between the normotensive and pre-eclamptic cohorts

(p=0.003). In contrast, the stem villi remained unaffected. Post hoc analyses revealed an up-regulation of sFlt-1 immunoreactivity in P- compared to the N- (p=0.04). The field area percentage of sFlt-1 immunoreactivity was stronger within the pre-eclamptic (P- and P+) compared to the normotensive (N- and N+) pregnancies (exchange and conducting villi). Additionally, our study reports an intensity upregulation of sFlt-1 immunoexpression in pre-eclampsia compared to normotensive pregnancies. These intensity of sFlt-1 immunoexpression in our study are consistent with previously published data (Tsatsaris *et al.*, 2003, Helske *et al.*, 2001, Munaut *et al.*, 2012, Ahmad and Ahmed, 2004, Tripathi *et al.*, 2008, Taché *et al.*, 2011).

More recently, studies utilising immunohistochemical procedures demonstrated a positive relationship between sFlt-1 immunoexpression and the severity of placental lesions in hypertensive disorders such as placental size, and hypermaturity of villi (Taché *et al.*, 2011). In addition, Taché *et al*., (2011) demonstrated a strong association between increased placental sFlt-1 immunoexpression and hypermature villi along with the severity of hypertensive syndromes. It is possible that villous hypermaturity may be associated with hypoxia and the subsequent increased placental production of sFlt-1. Notably in our study, this elevated sFlt-1 immunoreactivity observed within the pre-eclamptic cohorts is consistent with our pathological observations of immature intermediate villi and syncytial knot predominance, features indicative of a hypoxic microenvironment of pre-eclampsia.

The hypothesis that sFlt-1 has a causative role in pre-eclampsia has also been ascribed to its release from placental trophoblasts as a consequence of hypoxia (Ahmad and Ahmed, 2004, Nagamatsu *et al.*, 2004). Currently, the exact manner in which oxygen facilitates these events remains unclear. A possible factor that may be implicated in the development of these events is

the hypoxia-inducible factor-1 (HIF-1), a transcription factor that activates gene transcription in reaction to low oxygen (Wang and Semenza, 1993). Placental derived sFlt-1 is elevated during both physiological and pathological hypoxia, through the effect of HIF-1, thereby contributing to pre-eclampsia development (Nevo *et al.*, 2006). The link between sFlt-1 and hypoxia may also be ascribed to the inadequate remodelling of spiral arterioles by trophoblasts during early placentation in pre-eclampsia. This chronic hypoxic microenvironment contributes to the increased placental discharge of sFlt-1 with the subsequent maternal symptoms of pre-eclampsia (Karumanchi and Bdolah, 2004).

In our study, strong sFlt-1 immunoreactivity was observed in trophoblasts and villous endothelial cells in both normotensive and pre-eclamptic study groups, with their up-regulation being predominant within the pre-eclamptic cohort's. It is plausible to hypothesise that sFlt-1 has a dual placental function. On the one hand, it exerts a paracrine control over placental angiogenesis; whilst on the other hand, an autocrine mode of action is exerted over trophoblast invasion, indicative of the endothelial mitogenic effect of the VEGF ligand receptor structure as well as other roles when located in non-endothelial cells (Vuorela *et al.*, 1997). Our findings therefore corroborate previous reports (Yang *et al.*, 2008, Helske *et al.*, 2001, Vuorela *et al.*, 1997), however we also report no difference in the sFlt-1 immunoexpression between HIV negative and positive groups. Additionally, the elevated immunoexpression of sFlt-1 within syncytial knots from pre-eclamptic placenta in our study conforms to previous studies (Nevo *et al.*, 2006, Tripathi *et al.*, 2008). This is suggestive that syncytial shedding may serve as a conduit through which sFlt-1 enters the maternal circulation, contributing to the negative effect of sFlt-1 on maternal vessels.

Therefore, we hypothesise that the deficient trophoblast activity during pre-eclampsia, irrespective of the HIV status, may be due to this elevated sFlt-1 levels. Finally, the immunoexpression of sFlt-1 in villous endothelial cells negatively influences the normal angiogenic functions inclusive of capillary formation and fusion of angiogenic cell cords, resulting in the endothelial dysfunction that characterises pre-eclampsia.

4.3.2 Circulating sFlt-1

Our study confirmed previous reports of elevated serum levels of sFlt-1 in the pre-eclamptic compared with normotensive groups, suggestive of a corresponding reduction in the pro-angiogenic factors, thereby affecting normal placentation and vascularisation (Maynard *et al.*, 2003, Romero *et al.*, 2008, Staff *et al.*, 2005, Robinson *et al.*, 2006, Troisi *et al.*, 2008, Varughese *et al.*, 2010). The hypothesised role of sFlt-1 in pre-eclampsia development is robustly supported by animal studies, wherein the administration of exogenous sFlt-1 stimulates this maternal disorder (Maynard *et al.*, 2003).

Our data is therefore consistent with the postulated role for elevated sFlt-1 and sEng as circulating antagonists of VEGF, PlGF and TGF-β in pre-eclampsia development. It is plausible the elevated serum sFlt-1 and reduced PlGF levels add to the enhanced permeability of the vascular endothelium. In our study, sFlt-1 concentrations was higher in the pre-eclamptic (P- and P+) compared with normotensive pregnancies (N- and N+). However, when grouped according to HIV status, sFlt-1 was higher in the HIV negative groups (N- and P-) compared to the HIV positive groups (N+ and P+), suggesting an ameliorative role of HIV infection in pre-eclampsia development.

Our findings therefore concur with the pioneer report by Maynard and co-workers (Maynard *et al.*, 2003), who highlighted the role of sFlt-1, an antagonist of both VEGF and PlGF in the pathogenesis of pre-eclampsia. We therefore support the hypothesis that via its neutralisation effect, sFlt-1 adds to the insufficient vascularisation of the placenta. This higher level of sFlt-1 in pre-eclampsia may be implicated in the deficient physiological remodelling of spiral arterioles in the placental bed, with the consequential inadequate placental perfusion (Brosens *et al.*, 1972, Roberts and Redman, 1993).

In contrast, our increased circulating sFlt-1 levels are inconsistent with that reported by Srinivas *et al*., (2010). These investigators demonstrated no difference at term in the expression of sFlt-1 between pre-eclamptics compared to normotensive pregnancies (Srinivas *et al.*, 2010). The latter study negates the use of sFlt-1 as a predictor test for pre-eclampsia development, especially at term. However, they report sEng elevation with the concomitant reduction in PlGF in pre-eclampsia (Srinivas *et al.*, 2010), suggestive of their possible use as discriminatory factors between pre-eclamptic and normotensive pregnancies. One would expect a reliable (or sensitive or specific) predictive marker to be discriminatory at the time of diagnosis. However, if a serum biomarker is unable to discriminate pre-eclampsia at term, then the possibility of it being reliable, predictor of disease onset, is low.

Similar to our findings in serum, plasma sFlt-1 levels in women of Indian origin were also elevated (Kulkarni *et al.*, 2010). Similar studies conducted on maternal plasma from Zimbabwean normotensive and pre-eclamptic women revealed higher sFlt-1 and lower VEGF levels in the pre-eclamptic cohorts and unaffected PlGF levels (Muy-Rivera *et al.*, 2005). This

is in contrast to our findings, which show increased circulating sFlt-1 levels, decreased PlGF levels and undetectable VEGF levels.

Studies evaluating the expression of sFlt-1 levels during the second trimester have demonstrated an increase in their expression, suggestive that this elevation may herald the clinical appearance of pre-eclampsia (Wathen *et al.*, 2006). Similarly, others have reported that both early and late-onset pre-eclampsia are related to the increased sFlt-1 plasma levels and decreased PlGF levels, with these variations being prominent in early onset instead of in late-onset pre-eclampsia (Wikstrőm *et al.*, 2007, Govender *et al.*, 2012). Recent studies have also supported the role of plasma sFlt-1 and sEng in the aetiology of pre-eclampsia (Chedraui *et al.*, 2009).

Hypoxia inducible factor 1 (HIF-1) is upregulated in placental trophoblasts during pre-eclampsia (Rajakumar *et al.*, 2003). Of note, both Eng and Flt-1 genes consist of HIF-1 binding sites. Others have also suggested placental hypoxia may be the source of increased sFlt-1 levels observed in pre-eclamptics (Granger *et al.*, 2002, Hubel, 1999, Ahmad and Ahmed, 2004). This therefore implies that the elevation of both sFlt-1 and sEng in our study is probably related to the increased expression of HIF-1 that characterises pre-eclampsia.

Furthermore, pre-eclampsia has also been ascribed to an upregulation of TNF-α stimulated by hypoxia (Conrad *et al.*, 1998). Irregular placental perfusion due to the deficient invasion of trophoblasts may be responsible for elevations of maternal circulatory TNF-α. These findings concur with Ahmad and Ahmed (2004), suggesting that the discharge of sFlt-1 from placental explants may be due to TNF-α. Notably, these investigators report a synergistic mode of action

implicating additional cytokines in the discharge of sFlt-1. Thus it is reputed that in pre-eclamptic placentae, the elevated production of cytokines stimulates the placental production of sFlt-1, subsequently preventing angiogenic events and impairing the maternal vasculature. Similarly, other studies have supported the role for sFlt-1 with concomitant reduction in circulating free PlGF in pre-eclampsia development (Shibata *et al.*, 2005).

The presence of a human specific placental-derived variant (sFlt-14) was also shown to be elevated in maternal circulation of pre-eclamptics (Sela *et al.*, 2008). These investigators have hypothesised that a novel VEGF inhibitor is evident in human placentae, suggestive of a protective mechanism to non-endothelial cells from adverse VEGF signalling. The syncytial knots are the main source of circulating sFlt1-14, thus it is plausible that the predominant occurrence of syncytial knotting in pre-eclamptic placentae may provide an additional understanding of why circulating levels of sFlt-1 (now called sFlt1-14) are elevated during the second and third trimesters of normotensive pregnancies, and with a concomitant higher elevation in pregnancies complicated by pre-eclampsia. These findings emphasise the role of the syncytial knots in understanding the etiology of pre-eclampsia development.

Moreover, ethnicity has been implicated in the expression of sFlt-1 during first pregnancies compared to second pregnancies (Wolf *et al.*, 2005). Additionally, Wolf et al., (2005) report higher sFlt-1 levels in primigravid Hispanic compared to Caucasian women, suggesting that ethnicity may confer a greater risk of altering sFlt-1 levels in pre-eclampisa development. Our study demonstrates variations in the expressions of pro- and anti-angiogenic factors in African women. In order to validate the effect of ethnicity on the expressions of pro- and anti-

angiogenic factors in pre-eclampsia, a larger sample size or an epidemiological approach is required.

Additionally, it may be hypothesised that anomalies in endothelial function may be due to the vasoactive effect of trophoblasts on nearby villous endothelial cells. More recently, a relationship between elevated circulating levels of endothelin (ET-1) a powerful vasoconstrictive peptide, concurrent with elevations in sEng and sFlt-1 in pre-eclamptic pregnancies was demonstrated (Aggarwal *et al.*, 2012). These elevated levels correlated with severity of hypertension and proteinuria, suggestive of the possibility of an interaction between these pathways in the development of pre-eclampsia.

It is thus postulated that endothelin-1, prostacyclin, nitric oxide and sFlt-1 are produced, thereby activating endothelial mediated vasoconstriction, vascular permeability, endothelial-mediated aggregation of platelets, and the subsequent manifestations of pre-eclampsia (Aggarwal *et al.*, 2012). Elevated sFlt-1 concentrations may be due to the production by villous endothelial cells, concurring with previous reports (Zeng *et al.*, 2001, Brockelsby *et al.*, 1999). This suggests that the biologic function of VEGF via Flt-1 prevents endothelial production and migration mediated by VEGFR-2, thereby decreasing endothelial-dependent relaxation of uterine vasculature.

4.3.3 Placental sEng

Endoglin, a co-receptor for both TGF-β_1 and TGF-β_3 is highly expressed in syncytiotrophoblasts and endothelial cells (Lim *et al.*, 2009, Lopez-Novoa, 2007). It is identified as a pro-angiogenic factor that regulates vascular remodelling and homeostasis via

the endothelial nitric oxide synthase pathway (Lim *et al*., 2009, Maynard *et al*., 2008a, Lopez-Novoa, 2007, Maynard *et al*., 2005). Soluble endoglin however, is identified as an anti-angiogenic factor that weakens the affinity of TGF-β_1 to bind to its receptor on the cell surface, diminishing TGF-β_1 signalling and thereby activating eNOS with a resultant vasodilation. *In vitro* studies highlighted the inhibitory role of sEng on endothelial function, whilst *in vivo* studies on pregnant rats allude to an adenovirus-assisted elevation in both sFlt1 and sEng, which function in concert to enhance the effects of either molecule resulting in pre-eclampsia (Venkatesha *et al.*, 2006, Luft, 2006). The role of sEng in pre-eclampsia was recently highlighted (Venkatesha *et al*., 2006, Levine *et al*., 2006), indicating increased circulatory sEng levels in pre-eclampsia compared to the normotensive pregnancies. However, this elevation was dependent on the presence of sFlt-1 for its stimulatory effect in the aetiology of this syndrome.

sEng is produced abundantly in the placenta (Hu *et al.*, 1998, Sporn and Roberts, 1992). Our study is consistent with this sEng-rich placental profile. We additionally provide a composite profile of sEng immunolocalisation within the syncytio-and cytotrophoblasts and extravillous trophoblast cell populations as well as the endothelial cells. Our study further demonstrates a sub-cellular immunolocalisation of sEng within endoplasmic reticulum, mitochondria and cytoplasm of syncytio and cyto-trophoblasts, as well as within endothelial cells across all study groups. To date, this is the first study highlighting the ultrastructural localisation of sEng in the placenta. To our knowledge, it is also the first study that has morphometrically analysed the immuno-localisation and immunoreactivity pattern of sEng in the placenta of HIV-associated normotensive and pre-eclamptic pregnancies. In our study, HIV infection did not affect sEng immunoexpression in exchange villi (p=0.088) whilst an interaction was noted in stem villi

(p=0.001) across all groups. However, the percentage immunoexpression of sEng differed significantly between the normotensive *v*s the pre-eclamptic cohorts in exchange (p=0.02) and stem (p<0.001) villi.

This elevated immunoexpression pattern within placental trophoblasts in our study is consistent with observations by Yang *et al*. (2008), as observed in *in vitro* hypoxic conditions. Our results, together with the latter study, support the angiogenic imbalance theory that characterises pre-eclamptic placentae. The exact cause of elevation of placental endoglin expression in pre-eclampsia is unknown. However, studies have ascribed this elevated placental sEng expression during early stages of first trimester pregnancies, but reduced levels during the late first and second trimester pregnancies possibly resulting from hypoxia (Yinon *et al*., 2008). This elevation of sEng expression may be due to failure of down regulation of the endoglin gene expression in the second trimester, or it may be a compensatory mechanism or pro-angiogenic effect. It can thus be speculated that this elevation probably prevents the differentiation and subsequent invasion of trophoblasts, resulting in an elevated placental release of sEng into the maternal circulation.

Earlier studies have established endoglin as a transmembrane protein with a molecular weight of 68kD (Gougos and Letarte, 1990), however, sEng is reported as a disulphide-linked dimer (Raab *et al.*, 1999). Studies using western blot analyses have identified a 65kD soluble form and a 90kD full length Eng and a 70kD soluble and 110-150 kD full length Flt-1 protein during hypoxia (Jeyabalan *et al.*, 2008, Venkatesha *et al.*, 2006, Yang *et al.*, 2008). Thus, these high molecular weight proteins possibly exist as dimers, polymers and/or complexes that contribute to the upregulation of sEng levels during pre-eclampsia.

4.3.4 Circulating sEng

Our study demonstrates elevated circulatory sEng concentrations irrespective of the HIV status, in the pre-eclamptics compared to normotensive pregnancies. Our findings are consistent with elevated sEng levels in maternal serum in previous reports (Levine *et al.*, 2006, Reddy *et al.*, 2009, Yang *et al.*, 2008, Maynard *et al.*, 2003, Masuyama *et al.*, 2007, Jeyabalan *et al.*, 2008, Sandrim *et al.*, 2008) and in plasma (Chedraui *et al.*, 2009, Kim *et al.*, 2009, Stepan *et al.*, 2007). However, studies using plasma for detection of sEng levels (Kim *et al.*, 2009) did not concur with the latter. Kim *et al*., (2009) were unable to correlate sFlt-1 with proteinuria, but demonstrated that sEng was strongly linked with early-onset and severe pre-eclampsia together with fetal growth restriction.

In addition, studies have shown a strong correlation between maternal circulatory sEng and sFlt-1 concentrations (Stepan *et al.*, 2007, Noori *et al.*, 2010, Masuyama *et al.*, 2007), suggesting that both these anti-angiogenic factors work synergistically but with unique role to induce the symptoms of pre-eclampsia. In our study, albeit term pregnancies, we also demonstrate a positive correlation of circulating sFlt-1 with sEng levels and a negative correlation of circulating sFlt-1 with PlGF levels. More recently, studies conducted in high risk women (≤34 weeks gestational age), show that plasma sEng levels was robustly linked with sFlt-1 and PlGF levels, suggesting a common conduit for pre-eclampsia development (Rana *et al.*, 2012a). This prospective clinical study further demonstrated increased concentration of sEng in women who initially present to obstetric triage thereby implying a greater possibility of pre-term delivery. These findings further suggest that the measurement of plasma sEng levels in high risk women may positively influence the early detection of pre-eclampsia.

Earlier reports utilising sEng as an essential intermediary of endothelial dysfunction in pre-eclampsia has sparked great interest in the exploration of its utility as a predictor of pre-eclampsia development (Venkatesha *et al.*, 2006). To date, various investigators have shown elevated levels of sEng in maternal circulation prior to the onset of pre-eclampsia in an otherwise healthy, nulliparous population (Rana *et al.*, 2007, Levine *et al.*, 2006). Recent observations indicate that normotensive, multiple gestational pregnancies would result in increased placental tissue, thereby placing these pregnancies at risk of developing pre-eclampsia (Maynard *et al.*, 2010). These investigators also demonstrated elevated serum sFlt-1 levels in multiple compared to singleton gestations (Maynard *et al.*, 2008b).

In addition, in normotensive pregnancies the concentration of sEng increases in proportion to placental size (Romero *et al.*, 2008, Maynard *et al.*, 2010). This implies that during normal pregnancies both sEng and sFlt-1 increase with gestational age, implying that as placental size increases, so does the expression of these molecules. It is therefore plausible that since sEng is placental-derived, the larger placental weight characterising multiple gestations will contribute to increased sEng concentrations. These reports therefore suggest that multiple gestations may confer a greater risk in pre-eclampsia development.

Nonetheless, recent studies have reported a reduction of circulating sEng levels during the second trimester (Petzold *et al.*, 2011, Rana *et al.*, 2007, Stepan *et al.*, 2008, Zhao *et al.*, 2010), contradicting previous reports (Maynard *et al.*, 2010). Earlier reports have, however, demonstrated that pregnancies complicated with triploidy or molar anomalies have larger placentae, thus expressing high sEng and sFlt-1 levels (Stepan and Faber, 2007). Concurrent with this hypothesis, multiple pregnancies were associated with an elevated anti-angiogenic

profile in contrast to singleton pregnancies (Bdolah *et al.*, 2008). Thus, it is possible that the placental release of both sFlt-1 and sEng is possibly stimulated by low oxygen levels (Nagamatsu *et al.*, 2004).

The disproportion of angiogenic function is more prominent in pre-term than term pregnancies (Levine and Karamanchi, 2005, Vatten *et al.*, 2007). Thus, we hypothesise that since the placenta is a major angiogenic source, a large placenta will produce more pro-and anti-angiogenic factors and *vice versa*. In our study, placental size was higher in the pre-eclamptic (HIV negative and positive) compared to the normotensive groups (HIV negative and positive). The results of our study, albeit term pregnancies, concurs with findings of Maynard *et al*., (2010), in that we show increased placental weight within the pre-eclamptic cohort, with subsequent elevations in sEng and sFlt-1 levels. However, a recent epidemiological study confirmed that placental mass is unrelated to placental dysfunction in pre-eclampsia (Eskild and Vatten, 2010).

The relationship between maternal angiogenic and uterine Doppler factors were recently investigated (Petzold *et al.*, 2011). A physiological reduction of maternal sEng levels between first and second trimester corresponds to the reduction of utero-placental resistance in normotensive pregnancies. Petzold *et al*., (2011) also show an inverse association between sEng and uterine Doppler factors during the second trimester. These findings suggest an autonomous prognostic utility of sEng in placental pathology when compared to Doppler sonography, indicating its value as an independent biomarker.

Consequently, the anti-angiogenic effects of sEng evident in pre-eclampsia occurs via its interaction with TGF-β_1, resulting in the inhibition of the endothelial attachment and the subsequent loss of endothelial pro-angiogenic and vasodilatory effects of TGF-β_1 (Luft, 2006, Lopez-Novoa, 2007, Salahuddin *et al.*, 2007, Venkatesha *et al.*, 2006). Our study, however, demonstrated no significant differences in circulating TGF-β_1 although a significant difference was noted for the sEng/TGF-β_1 anti-angiogenic ratio between the HIV negative pre-eclamptics compared to HIV negative normotensives (p=0.003). Our results for TGF-β_1 expression albeit higher, were similar to previous reports (Ayatollahi *et al.*, 2005, Huber *et al.*, 2002). However, earlier reports have shown an elevation in the expression of plasma TGF-β_1 among pre-eclamptics compared to normotensive pregnancies, suggesting a possible role in pre-eclampsia development (Djurovic *et al.*, 1997).

Circulating TGF-β_1 levels has been postulated to be equivalent to that found at the maternal-fetal interface, suggesting an interchangeable biological role of both (Ayatollahi *et al.*, 2005). During normotensive pregnancies TGF-β_1 acts as a regulatory factor in fetal allograft survival, by limiting primary immune responses, whilst within the maternal circulation, it may regulate T cell responses (Ayatollahi *et al.*, 2005). However, in our study, the expression of TGF- β_1 was unaffected by HIV status and type of pregnancy. Notably, the sEng/TGF-β_1 ratio analyses in our study was unaffected by HIV status but significantly affected by type of pregnancy (normotensive and pre-eclamptic, p=0.003). This anti-angiogenic ratio (sEng/TGF-β_1) was higher among the pre-eclamptics (P- and P+) compared to normotensive (N- and N+) groups, suggesting a possible role for TGF-β_1 in the pathogenesis of pre-eclampsia. Our findings regarding sEng and the anti-angiogenic ratios are consistent with previous studies (Rana *et al.*, 2007, Levine *et al.*, 2006, Lim *et al.*, 2009). In addition, observation of elevated sEng levels

and the lack of significant difference of TGF-β_1 levels in our study concur with findings of Lim *et al*., (2009).

Effective placentation during normal pregnancy occurs through the effects of TGF-β_1 on trophoblast invasion. Reduced circulating TGF-β_1 levels observed in normotensive second trimester pregnancies (Lim *et al.*, 2008), concurs with reports by (Lim *et al.*, 2009). Hence, these reports suggest that screening of both sEng and TGF-β_1 levels during the second trimester may possibly serve as predictor tests for identifying those at risk of developing pre-eclampsia.

Endothelial dysfunction has been associated with the antagonistic effects of sFlt-1 on VEGF as well as the activation of the eNOS pathway (Kendall *et al.*, 1996). Alteration of this pathway in pre-eclampsia is negatively associated with sFlt1-1 levels (Sandrim *et al.*, 2008), suggesting a pathophysiological role of nitrites in pre-eclampsia development. It is also presumed that sEng reduces arterial vasodilatation stimulated by TGF-$\beta_{1/3,}$ which is mediated by NO. Thus, both sEng and sFlt-1 are likely involved in the pathogenesis of pre-eclampsia, with the subsequent inhibition of endogenous NO production. Our findings of elevated circulatory sEng and sFlt-1 levels albeit in term pregnancies, implicate their role as discriminatory diagnostic tests rather than a predictor test for pre-eclampsia development.

Placental oxidative stress contributes to the elevation of both sFlt-1 and sEng levels; however, this mechanism remains unclear. Recently, this elevation was demonstrated to be synchronised with an elevation in DNA damage-inducible 45 alpha (Gadd45α) and p38 mitogen-activated protein kinase (MAPK) in pre-eclamptics, compared to normotensive pregnancies (Luo *et al.*, 2011). Furthermore, knockdown studies provide evidence that both Gadd45α and MAPK are

protective during hypoxic and reoxygenation conditions, suggestive of an inhibitory role in oxidative stress and apoptosis but a stimulatory role *in vitro* angiogenesis. This has led to the presumption that regulatory signalling pathways compromised by hypoxia/reoxygenation possibly stimulates Gadd45α, subsequently activating MAPK with the resultant elevation in the expression of sFlt1 and sEng. This may likely form an additional route to understand the source of elevation in both sFlt-1 and sEng during pre-eclampsia.

4.3.5 Syncytial knots: sFlt-1 and sEng

The strong immunoreactivity of sFlt-1 and sEng located within syncytial knots in our study concurs with recent reports (Rajakumar *et al.*, 2012). Since the syncytial knots are rich sources of both sFlt-1 and sEng, as shown in our immunolocalisation results and others (Rajakumar *et al.*, 2012, Guller *et al.*, 2011), it is reasonable to hypothesise that their detachment, shedding and deportation as microparticles within the maternal circulation would contribute to their elevated levels in pre-eclampsia (Redman and Sargent, 2000, Redman and Sargent, 2009). Furthermore, these free, transcriptionally active syncytial debris are an autonomous source of both sFlt-1 and sEng delivery into the maternal circulation.

4.3.6 Placental PlGF

Our study has shown that PlGF immunoreactivity was observed within endothelial cells of fetal vessels (arteries, veins and capillaries), in accordance with previous studies (Vuorela *et al.*, 1997, Helske *et al.*, 2001). However, a weak pro-angiogenic immunoexpression was observed within syncytio- and cytotrophoblast cells with a slightly stronger immunoreactivity within extravillous trophoblast cells. Additionally, in our study PlGF was immunolocalised subcellularly within endoplasmic reticulum, mitochondria and cytoplasm of syncytio and cyto-

trophoblasts, microvilli plus endothelial cells across all study groups. This is the first study showing sub-cellular localisation of gold-conjugated PlGF in the placenta.

In our study, the HIV status had no significant effect on PlGF immunoexpression in exchange villi (p=0.08) although an interaction was noted in stem villi (p=0.003). The percentage PlGF immunoexpression was higher for both exchange (p<0.001) and stem villi (p<0.001) in normotensives (N+ and N-) compared to pre-eclamptics (P+ and P-).

Our immunohistochemical findings of reduced percentage PlGF immunoexpression in pre-eclamptics compared with normotensive pregnancies, are consistent with previous reports involving mRNA expression by *in situ* hybridisation (Kumazaki *et al.*, 2002, Clark *et al.*, 1998b) and immunohistochemistry techniques (Tsatsaris *et al.*, 2003, Yang *et al.*, 2008). The human placenta expresses VEGF and PlGF differently throughout gestation (Clark *et al.*, 1998b). However, in normotensive pregnancies, the placenta is a rich source of PlGF but not VEGF (Clark *et al.*, 1998b). These studies demonstrated that extravillous trophoblast cells adequately produce PlGF mRNA throughout gestation, whereas their production by villous trophoblasts decline during the second trimester and continues strongly until term. These reports by Clark et al., (1998b) concurs with recent studies (Romero *et al.*, 2008). On the other hand, VEGF was shown to be poorly produced by placental villi but highly expressed by decidual glands and macrophages. The angiogenic role of PlGF during the third trimester is likely the formation of terminal capillary loops (Cho *et al.*, 2003).

Clark *et al*. (1998b) further suggests that increased PlGF production by placental cells at term in normotensive pregnancies may be ascribed to the fully developed or large placentae and their

subsequent increased villous surface area of trophoblasts. However, in our study, placental size was greater amongst the pre-eclamptic compared to the normotensive term cohorts. The percentage immunoreactivity of both PlGF and VEGF was however greater in the normotensive (N- and N+) compared to pre-eclamptic (P- and P+) pregnancies.

Moreover, PlGF binds only to Flt-1 and not KDR whilst, VEGF binds to both receptors (Cao *et al.*, 1996). Earlier studies have shown that PlGF exerts its angiogenic effects by displacing VEGF from the decoy receptor, Flt-1 (Carmeliet *et al.*, 2001). Development and function of trophoblasts may be controlled by placental-derived PlGF through both autocrine and paracrine pathways only by Flt-1 receptors (Desai *et al.*, 1999). Thus, increased placental production of PlGF during normal pregnancy enhances their interaction with this decoy receptor, Flt-1 thereby supporting the angiogenic activity of VEGF by forming PlGF/VEGF heterodimers (Cho *et al.*, 2003, Clark *et al.*, 1998b).

In our study, the immunoexpression of PlGF observed within endothelial cells suggests a dual function. This may occur via a transverse diffusion from their site of production (trophoblasts) to the villous endothelial cells (site of action). In addition, a paracrine mode of action is also possible. Studies have implicated a cross reactivity of PlGF with the VEGF/PIGF heterodimers, thus indicating that the positive PlGF immunoreactivity observed may not be totally precise for PIGF homodimers (Vuorela *et al.*, 1997). Our study proposes that both P1GF and VEGF target villous endothelial cells, via separate systems. Thus, based on their immunolocalisation, it is possible that PIGF reacts to stimuli arising from maternal circulation whereas VEGF responds to stimuli from fetal tissues.

Studies conducted on placental trophoblasts cultured in 20% oxygen conditions report reduced production of PlGF, with the concomitant elevation of sFlt-1 and sEng by the pre-eclamptic placentae (Yang *et al.*, 2008). These reports suggest that this reduced PlGF concentration is most likely due to its increased susceptibility to the hypoxic microenvironment than sFlt-1 and sEng. It is possible that the pre-eclamptic placentae lack compensatory mechanisms due to a deficiency of endogenous protective agents, such as heme-oxygenase-1, and antioxidant agents such as glutathione peroxidase or superoxide dismutase (Yang *et al.*, 2008).

4.3.7 Circulating PlGF

In our study, we report a two-fold reduction of circulating levels of PlGF in the HIV negative pre-eclamptic compared with HIV negative normotensive pregnancies (p=0.02). These reductions in circulating PlGF levels were accompanied by the concurrent elevation of sFlt-1 and concur with previous reports (Tsatsaris *et al*., 2003, Maynard *et al*., 2003, Taylor *et al*., 2003, Muy-Rivera *et al*., 2005, Levine *et al*., 2004, Levine *et al*., 2006, Robinson *et al*., 2006, Koga *et al*., 2009, Varughese *et al*., 2010). This is probably due to the angiogenic theory that increased maternal circulatory sFlt-1 levels binds to both PlGF and VEGF possibly decreasing the circulating levels of PlGF and VEGF less than the required threshold necessary for vasculogenesis . In addition, other studies have postulated that pre-eclampsia may arise from the combined effect of reduced PlGF levels and sex hormone binding globulin (Thandani *et al*., 2004a). Unexpectedly, this trend was reversed in the HIV positive normotensive group. It is possible that the immune insufficiency stimulated by HIV infection contributes to this reduction. In order to adequately verify this reduction of circulating PlGF levels in HIV positive normotensive pregnancies, a larger sample size is required.

Our study was unable to show a significant difference between study groups for the anti-angiogenic ratio (sFlt-1/PlGF). However, we observed higher anti-angiogenic ratios in the HIV negative pre-eclamptics compared to the HIV negative normotensive pregnancies, whilst the difference observed for the HIV positive cohorts was negligible. This anti-angiogenic ratio profile (sFlt-1/PlGF)) characterises the stability between sFlt-1 and PlGF (Moore Simas *et al.*, 2007, De Vivo *et al.*, 2008). Additionally, it represents their anti-angiogenic role in pre-eclampsia, suggesting a possible diagnostic predictor test value for pre-eclampsia development. Our results, albeit term pregnancies, showed lower sFlt-1 and sFlt-1/PlGF ratios in the normotensive (N- and N+) compared with the pre-eclamptic (P- and P+) groups, indicative of an apparent trend towards a discriminatory diagnostic significance

4.3.8 Placental VEGF

In our study, the VEGF protein was immunohistochemically observed within endothelial cells, syncytio- and cyto- trophoblast cell populations. The distribution of the VEGF staining varied from being absent to strong, within the endothelial cells and trophoblast populations. Occasionally, VEGF immunoreactivity was observed within stromal cells. A moderate VEGF immunoprecipitate was located in extravillous trophoblast cells, corroborating earlier studies (Tsatsaris *et al.*, 2003, Chung *et al.*, 2004). In our study, VEGF was also immunolocalised subcellularly within endoplasmic reticulum, mitochondria and cytoplasm of syncytio and cyto-trophoblasts, as well as endothelial cells across all study groups. This is the first study documenting the subcellular localisation of VEGF in the placenta.

The immunolocalisation of VEGF has been reported to be variable. Some studies have shown immunolocalisation specifically within the endothelium (Vuorela *et al.*, 1997, Helske *et al.*,

2001), the syncytiotrophoblast (Ahmad and Ahmed, 2004) and extravillous trophoblasts (Shiraishi *et al.*, 1996). In contrast, investigators using *in situ* hybridization studies, showed a ubiquitous localization of VEGF mRNA within villous stroma, trophoblast and extravillous trophoblast cells (Clark *et al.*, 1998a, Kumazaki *et al.*, 2002). These findings have been corroborated by other studies with the intensity of immunoreactivity being greater in pre-eclampsia than normotensive pregnancies (Sgambati *et al.*, 2004).

The discrepancy in VEGF immunolocalisation observations may be ascribed to the variable specificity of the antibodies (monoclonal and polyclonal antibodies) utilised. The existence of VEGF/PlGF heterodimer cross reacting with the VEGF antibody is also possible (Vuorela *et al.*, 1997). In our study, a significant effect of HIV infection on VEGF immunoexpression in exchange (p=0.005) was noted in contrast to stem villi (p=0.374) was noted across all groups. In contrast, the percentage immunoreactivity of VEGF in our study differed significantly between the normotensive (N- and N+) compared with the pre-eclamptic (P- and P+) pregnancies in both exchange (p=0.001) and stem villi (p=0.003). The immunoexpression of VEGF was higher in normotensive (N- and N+) compared to pre-eclamptic (P- and P+) pregnancies. In addition, there was no correlation observed between exchange and conducting villi on the immunoexpression of sFlt-1, sEng, VEGF and PlGF, nonetheless, higher levels of immunoreactivity was expressed by the stem villi compared to the exchange villi. This lack of association may be obviated by use of larger sample size.

Hence, the immunoexpression patterns observed for both VEGF and PlGF within syncytio- and cytotrophoblast in our study is suggestive of their autocrine mode of action, and their subsequent diffusion to the endothelial cells as sites of action (Vuorela *et al.*, 1997). Others

have suggested that their production in trophoblast cells is possibly due to their involvement in hormonal and protein secretion (Talamentes and Ogren, 1988).

4.3.9 Circulating VEGF

In our study, maternal serum concentrations of free VEGF were below the detectable concentration of the commercially available ELISA tests that were utilised (31.2 pg/mL). Previous studies have also reported undetectable levels of serum VEGF in pregnant (normotensive and pre-eclamptic) women (Baker *et al.*, 1995 , Taylor *et al.*, 2003, Staff *et al.*, 2005). A plausible explanation for these undetectable levels is that free/circulating VEGF is bound to sFlt-1, which is highly expressed during pre-eclampsia. Thus, we were unable to conclude if variations of VEGF concentrations existed across study groups.

Earlier studies utilizing plasma have however, demonstrated that in pre-eclamptic compared to normotensive term pregnancies, the total VEGF concentrations circulating at 20ng/ml are elevated in contrast to reduced free VEGF levels (circulating at 2-10pg/ml) (Tsatsaris *et al.*, 2003). Notably, these investigators differentiated between free circulating VEGF and VEGF bound to circulating proteins. Confounding factors contributing to this variation are the use of maternal plasma or serum and the type of VEGF being evaluated (free, bound or total circulating VEGF). In terms of plasma and serum, discrepancies may be attributed to the coagulatory mechanism, whereby platelets or other blood cells may discharge VEGF (Jelkmann, 2001). Additionally, in pregnancies complicated by pre-eclampsia, it is likely that elevation of both Flt-1 and sFlt-1 concentrations occur as a result of the highly hypoxic microenvironment, thereby justifying the inconsistencies in VEGF concentrations between

investigations. A limitation of our study however, is the utility of term pregnancies when vascular remodelling is complete.

However, recent studies comparing term circulating VEGF, PlGF, sFlt-1 and sEng levels between normotensive and early and late-onset pre-eclampsia demonstrates a significant difference only for circulating sEng levels (Sezer *et al.*, 2012), contradicting previous reports (Rana *et al.*, 2007). In contrast, our study demonstrates significant differences for sFlt-1 sEng, and PlGF across all study groups. This included elevations in maternal circulating sFlt-1 and sEng levels in both the HIV positive pre-eclamptic and HIV negative pre-eclamptic pregnancies. However, maternal serum and plasma concentrations of sFlt-1 and sVEGFR-2 in normotensive pregnancies were recently shown to be variable (Oggè *et al.*, 2010). Therefore serum *vs* plasma assays need to be considered when commenting on the extrapolation of the data utilised for prognostic or risk assessment test.

4.4 Genetic analysis of placental sFlt-1, sEng, PlGF and VEGF

In our study, placental expression of both sFlt-1 and sEng were higher in pre-eclamptic compared to the normotensive pregnancies. Our findings concur with previous reports (Tsatsaris *et al.*, 2003, Venkatesha *et al.*, 2006, Shibata *et al.*, 2005, Toft *et al.*, 2008). However for the P- cohort, the expression of sFlt-1 was slightly lower compared to the normotensive group. A plausible explanation for this low mRNA expression level may be attributed to either the gestational age of our cohorts or our collection method of placental tissue, as syncytial microparticles may be lost in the RNAlater solution. Analyses of the RNAlater solution used for specimen collection confirmed the presence of sFlt-1 and sEng mRNA. The findings of our study concur with recent observations (Rajakumar *et al.*, 2012).

Additionally for sEng, the trend observed in our study corresponds to that of previous data (Jeyabalan *et al.*, 2008). It is unknown what causes this placental sEng elevation during pre-eclampsia. However, studies have reported variability in the expression of sEng throughout gestation (Yinon *et al.*, 2008). Its expression is relatively high during the first trimester, possibly due to the compensatory and pro-angiogenic effect, but tends to diminish during the second trimester, implicating hypoxia (Venkatesha *et al.*, 2006). It is also probable that sEng arises as an accidental N-terminal cleavage product by the membrane type 1 metalloproteinase (Ten Dijke *et al.*, 2008).

Consistent with previous reports, our findings demonstrate no significant difference between VEGF and PlGF expression (Toft *et al.*, 2008, Tsatsaris *et al.*, 2003). Microarray analyses conducted by Toft *et al*., (2008) concur with the results produced by RT-PCR techniques in our study, suggesting that microarray analysis may be a valuable tool for the exploration of gene expression. Additionally, the angiogenic inhibiting gene expression patterns observed by Toft *et al*., (2008) advocate that separate, or to some extent related biological events, underlie the development of pre-eclampsia.

In South Africa, the standard of care for HIV positive patients include HAART for the mother's own health, or dual antiretroviral therapy for the prevention of mother to child transmission. It is possible that treatment modalities may alter the angiogenic balance in favour of a restoration of angiogenesis in HIV compromised pre-eclampsia. Wimalasundera *et al*. (2002) proposed that HAART restores the immune response of HIV positive pre-eclamptic women. In our study, a concise anti-retroviral treatment history was unavailable.

4.5 Clinical value

Several studies have highlighted the significance of angiogenic factors in the diagnosis and prediction of pre-eclampsia development (Levine *et al.*, 2004, Levine *et al.*, 2006, Romero *et al.*, 2008, Rana *et al.*, 2007). Increased levels of sFlt-1 correspond to the decreased levels of VEGF and PlGF at the onset, as well as prior to the onset of pre-eclampisa (Levine *et al.*, 2004). Its utility as a predictor test is well documented (Romero *et al.*, 2008, Vatten *et al.*, 2007, Moore Simas *et al.*, 2007, Rana *et al.*, 2012b), emphasising their prognostic significance in the detection of patients with severe pre-eclampsia, thus reinforcing the clinical use of these biomarkers. More recently, the clinical values of the urinary sEng *vs* sFlt-1/PlGF ratios have been assessed as risk indicators for pre-eclampsia development (Buhimschi *et al.*, 2010). Elevated urinary sEng was evident in early pre-eclampsia, however its ability to establish the severity of pre-eclampsia is limited. The prognostic utility of sFlt-1/PlGF ratio and (sFlt1+ sEng)/PlGF, on the other hand, was shown to be more reliable than urinary sEng or either protein alone as a predictor test for pre-eclampsia development (Levine *et al.*, 2006).

Controversy exists however with the use of these factors in the prediction of pre-eclampsia development in high risk pregnancies (Powers *et al.*, 2010). Moreover, significantly higher circulating levels of sFlt-1, sEng and PlGF was demonstrated among women with multifetal gestations in contrast to other high-risk groups such as pre-existing diabetes, chronic hypertension and previous pre-eclampsia. Powers et al. (2010) suggest that whilst variations may exist between high-risk and low risk pregnancies, these are quite modest and may not be clinically valuable predictors.

Whilst a threshold value has been established for the predictor test of pro- and anti-angiogenic factors in pre-eclampsia development, one must be cautious as these reference values for maternal serum may not be directly extrapolated to plasma levels without standardisation. Additionally, it may be argued that pre-eclampsia has 'multiple' etiologies. Recent *in vivo* studies (Rana *et al.*, 2012a, Rana *et al.*, 2012b) and animal studies (Venkatesha *et al.*, 2006, Levine *et al.*, 2006) strongly recommend a biochemical re-definition of pre-eclampsia based on angiogenic factors, thereby rendering their use as a gold standard for the prediction studies. Whilst the search continues for the gold standard for prediction of pre-eclampsia development, a recent systematic study substantiates the prognostic utility of circulatory levels of VEGF, PlGF; sFlt-1 and sEng; proteomic biomarkers such as P1-P13, albumin, fibrinogen; and α-1-antichymotrypsin and SERPINA1 as potential biomarkers in the development of pre-eclampsia (Gastrich *et al.*, 2010).

4.6 Limitations of study

Limitations of our study include the "spot measurement" post delivery, gestational age, and storage of placental tissue in RNA*later*, the limited anti-retroviral treatment history as well as the small sample size. Pre-eclampsia is associated with defective trophoblast invasion occurring early in pregnancy hence a major limitation of this study is that it reflects a spot measurement obtained post delivery.

4.7 Summary

In our study, the expression of sFlt-1, sEng, PlGF and VEGF was confirmed using immunohistochemistry, RT-PCR and ELISA techniques. To our knowledge, this is the first report demonstrating that irrespective of the HIV status; sFlt-1 and sEng were elevated with the

concomitant reduction in PlGF in pre-eclamptic compared to normotensive pregnancies. The levels of VEGF were however, undetectable across all study groups. It is plausible that this lack of effect of HIV status on the factors under study may be attributed to the treatment regimen, as HAART is known to restore the immune response of HIV positive pre-eclamptic women. Additionally, this study is novel in that it ultrastructurally immunolocalises sFlt-1, sEng, PlGF and VEGF within the placenta. This immunoelectron localisation data corresponds to our immunohistochemical and placental mRNA data.

Our study further demonstrates strong immunoreactivity of both placental sFlt-1 and sEng in pre-eclampsia, with concurrent elevations in the maternal circulation. We also confirm a qualitative increase in the occurrence of syncytial knots in the pre-eclamptic compared to the normotensive pregnancies. These observations support the detachment of anti-angiogenic rich microparticles from syncytial knots and their subsequent deportation and elevation in the maternal circulation. Moreover, their consequent antagonistic effects on VEGF, PlGF and TGF-β, disrupts the vascular endothelial maintenance.

In our study the strong immunoreactivity of sFlt-1, sEng, PlGF and VEGF was observed in villous endothelial cells. In addition, a strong sFlt-1 and sEng but a weak PlGF and VEGF immunoreactivity was noted in syncytio- and cytotrophoblasts. This immunoexpression within trophoblasts is suggestive of their autocrine mode of action on normal trophoblast functions, including invasion, differentiation and production. It is plausible that the angiogenic imbalance observed in our study will impact on placental function by modifying trophoblast activity, thereby contributing to abnormal placentation.

Our study supports the hypothesis that pre-eclampsia is characterised by an imbalance between pro- and anti-angiogenic factors. Whether the pregnancy is complicated by immune insufficiencies or not, does not affect the role of the anti-angiogenic factors in pre-eclampsia development. Despite the lack of statistical significance, this study demonstrates a trend of the effect of HIV status on the circulating levels of sFlt-1 and sEng. An explanation for this discrepancy is the limited availability of the antiretroviral history. Nevertheless, the neutralising effect of HIV infection on the immune system may be insufficient in the development of pre-eclampsia.

Finally, in conclusion our study confirms the use of sFlt-1 and sEng as discriminatory prognostic indicators for pre-eclampsia at term. Albeit at term, our findings support these factors as biomarkers for the development of pre-eclampsia. Whilst the cause of pre-eclampsia may be ascribed to these anti-angiogenic factors, and their use as predictive tests may be warranted, the main cause of this maternal disorder remains an area of dynamic placental exploration. Thus the potential role that pro- and anti-angiogenic factors may have on this increased possibility may provide a route for further studies, and contribute to the current theories on the pathogenesis of pre-eclampsia.

4.8 Future directions

There is a paucity of data on the estimation of urinary sEng, PlGF and sFlt-1 in pre-eclampsia. A study involving the latter will contribute towards understanding whether a relationship exists between circulating and urinary factors. Additionally, it may be interesting to immunolocalise the HIV viral antigen at the materno-fetal interface, examining the horizontal transmission from mother to child.

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CHAPTER 5

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CHAPTER 6

Appendices

APPENDIX I: POSTGRADUATE PERMISSION (PG/07)

13 November 2007

Professor T Naicker
Optics and Imaging Centre
DDMRI
NRMSM

PROTOCOL. The Role of Soluble Fms-Like Tyrosine-Kinase-1, Vascular Endothelial Growth Factor and Placental Growth Factor in HIV Associated Ore-Eclamptic Pregnancies.
Nalini Govender Optics & Imaging, PHD, Student no. 9304501

Ref: PG009/07

Dear Professor Naicker

The Postgraduate Education Committee ratified the approval of the abovementioned study on the 13th November 2007.

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. (The Ethics application form is available on the LAN. **(V:) /User/Staff/General/Ethics)**

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

Yours sincerely

PROFESSOR P MOODLEY
Chair : Postgraduate Education Committee
c.c **N. Govender**

Nelson R Mandela School of Medicine, Faculty of Health Sciences,
Continuing Professional Development (CPD)

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nding Campuses:

APPENDIX II: INSTITUTIONAL ETHICS CLEARANCE (BF037/07)

UNIVERSITY OF
KWAZULU-NATAL

Research Office
BIOMEDICAL RESEARCH ETHICS ADMINISTRATION
Research Office
Room N40 - Govan Mbeki Building
University Road, WESTVILLE CAMPUS
KwaZulu-Natal, SOUTH AFRICA
Tel: 27 31 2604769 - Fax: 27 31 2604609
Email: [illegible] - Website: [illegible]

08 November 2007

Mrs N Govender
Durban University of Technology
Department of Human Biology
PO Box 1334
Durban 4000
Fax: 031-2604435

Dear Mrs Govender

PROTOCOL: The Role of Soluble Fms-Like Tyrosine-Kinase-1, Vascular Endothelial Growth Factor and Placental Growth Factor in HIV Associated Pre-Eclamptic Pregnancies: A South African Perspective. Mrs. Nalini Govender. Durban University of Technology. Ref: BF037/07.

The Biomedical Research Ethics Committee considered the abovementioned application and the protocol was approved by a **full sitting of the committee** at a meeting held on 15 May 2007 pending appropriate responses to queries raised. Your responses dated 16 October 2007 to queries raised on 19 September 2007 has 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 at **08 November** 2007.
We note the permission from the Hospital Manager and evidence of your HPCSA registration.

This approval is subject to the receipt of the following administrative issues:
Please submit the outcome of the HPCSA registration on receipt thereof.
Please produce a copy of the signed Indemnity Form for formalization of BREC records.
The faculty to confirm that the PhD in (Medical Scientist) qualification is on record/approved, or should it read Medical Science?

This approval is valid for one year from **08 November 2007.** To ensure continuous approval, an application for recertification should be submitted a couple of months before the expiry date. In addition, when consent is a requirement, the consent process will need to be repeated annually.

I take this opportunity to wish you everything of the best with your study. Please send the Biomedical Research Ethics Committee a copy of your report once completed.

Yours sincerely

PROFESSOR J MOODLEY
Chair: Biomedical Research Ethics Committee

APPENDIX III: PERMISSION FROM HOSPITAL MANAGER

HEALTH
KwaZulu-Natal

KING EDWARD VIII HOSPITAL
Private Bag X02, **CONGELLA** 4013
Corner of François & Sydney Road
Tel.:031-3603853, Fax: 031-2061457
Email.:Rejoice.khuzwayo@.kznti.gov.za
www.kznhealth.gov.za

Enq.:Miss. R.Khuzwayo
Ref.: KE 2/7/1/ (13/2007)
Research Programming

9 October 2007

Ms. Nalini Govender
Optics & Imaging Centre
Doris Duke Medical Research Institute
Nelson R. Mandela – School of Medicine

Dear Ms. Govender

Protocol: The Role of Soluble FMS- Like Tyrosine-Kinase-1, Vascular Endothelial Growth Facor and Placental Growth Factor In HIV Associated Pre-Eclamptic Pregnancies: A South African Perspective.

Your request to conduct the above-mentioned researches at King Edward VIII Hospital has been approved.

Please ensure the following:-

- That King Edward VIII Hospital receives full acknowledgment in the study on all publications and reports and also kindly present a copy of the publication or report on completion.
- Before commencement:
  - Discuss your research project with our relevant Directorate Managers
  - Sign an indemnity form at Room8, CEO's Complex, Admin. Block.

The Management of King Edward VIII Hospital reserves the right to terminate the permission for the study should circumstances so dictate.

Yours faithfully

SUPPORTED/~~NOT SUPPORTED~~

10/10/2007
DATE

DR. O.S.B. BALOYI
MEDICAL MANAGER

APPROVED /~~NOT APPROVED~~

11/10/07
DATE

MR. M. BHEKISWAYO
CHIEF EXECUTIVE OFFICER

cc: *All Directorate Managers: A&E/Critical Care/General Surgery/Internal Medicine/O&G/Orthopaedics/Paediatrics /Radiology/Specialty Services/Theatre*

PERMISSION TO CONDUCT A RESEARCH STUDY/TRIAL

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

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

1. Research proposal and protocol.
2. Letter giving provisional ethical approval.
3. Details of other research presently being performed by yourself if in the employ of KEH, (individually or as a collaborator).
4. Details of any financial or human resource implications to KEH, including all laboratory tests, EEGs, X-rays, use of nurses, etc. (See Addendum 1)
5. Declaration of all funding applications / grants, please supply substantiating documentation.
6. Complete the attached KEH Form - *"Research Details"*

Once the document has been signed it should be returned to **Mrs S Buccas:** Biomedical Research Ethics Administrator, Room N40, Govan Mbeki Building, Westville Campus, University of KwaZulu-Natal.

To: Chief Medical Superintendent / Hospital Manager

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

Site 1 address:
OBSTETRICS & GYNAECOLOGY UNIT
KING EDWARD HOSPITAL

Investigator/s:
Principal: NALINI GOVENDER
Co-investigator: PROF T. NAICKER
Co-investigator: DR S. MOODLEY

Signature of Chief Medical Superintendent/Hospital Manager:
Medical
Date: 19/07/2007

Site 2 address:

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

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

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

APPENDIX IV: LETTER OF INFORMATION AND CONSENT

INFORMATION GIVEN TO PARTICIPANTS

Title: "The Role of Soluble Fms-Like Tyrosine-Kinase-1, Vascular Endothelial Growth Factor and Placental Growth Factor in HIV Associated Pre-Eclamptic Pregnancies: A South African Perspective" (BF037/07)

Good day. Miss/ Mrs _____. My name is _____
Thank you for giving me the time to speak to you.

My colleagues and I are currently doing research on high blood pressure and pregnancy. Research is just the process to learn the answer to a question. In this study, we want to learn what causes this high blood pressure in pregnant women and what factors may be involved in causing this to happen.

When you are pregnant, the baby growing inside of you actually receives nutrition from a piece of tissue that is attached to your womb called the placenta. When the baby is born, this piece of tissue, which is usually called the afterbirth, is delivered. After the cord has been tied, the placenta is cut away and incinerated.

We are inviting you to participate and require your permission to be included in this research study**.** This is what your role will be in this study. We need you to donate a piece of the tissue called the placenta and perform some laboratory tests on it. This will be done after the placenta has been discarded and will not harm you or your baby in any way. In addition, we need to draw a small tube of blood from you so that we can look at how these different factors play a role in this disease. There are no risks involved in this study, however there may be slight discomfort experienced during the process of drawing blood from you. Please be aware that a qualified nurse will be available to draw out the blood.

There will be 4 different groups of patients taking part in this study, namely; HIV positive pre-eclamptic, HIV negative pre eclamptic, HIV positive normal and HIV negative normal. This study is limited to patients from King Edward VIII Hospital, Kwa-Zulu Natal.

We will be collecting placenta samples from 120 woman. All these samples are being collected here in this hospital from South African woman and will be studied at the Nelson Mandela Medical School. The results of this will benefit medical science and be used for a PHD degree. We will require nothing more from you except permission to use a piece of your placenta. The piece of placenta will be labeled, your identity will remain unknown, and the specimen will be processed for experimental procedures. Post-study the sample will be archived in wax. The remainder of the placenta not utilized for study purposes will be burnt as usual by KEH.

However, please note that you are not obligated in any way to agree to do this. You do have an option to say NO and will not be disadvantaged in any way. Confidentiality is guaranteed.

Thank you for your time and assistance in this study.

ULWAZI OLUNIKEZWA UMHLANGANYELI

INCWADI YOLWAZI

Title: "The Role of Soluble Fms-Like Tyrosine-Kinase-1, Vascular Endothelial Growth Factor and Placental Growth Factor in HIV Associated Pre-Eclamptic Pregnancies: A South African Perspective" (BF037/07)

Sawubona Nks/Nkk …….. Igama lami ngingu …….. Siyabonga lelithuba osinika lona lokuba sikhulume nawe.

Abalingani bami kanye nami siphezu kocwaningo lokunyuka kwezinga lamandla egazi emzimbeni-ihayihayi (high blood pressure) kanye nokuzithwala/ ukukhulelwa. Ucwaningo luyiyona ndlela nomgudu wokuthola izimpendulo zombuzo othile. Kulolu cwaningo sifuna ukufunda kabanzi ngembangela yokunyuka kwezinga lamandla egazi emzimbeni kulabo abakhulelwe kanye nezici nemithelela ebangela isimo sehayihayi.

Uma ukhulelwe, ingane ekhula ngaphakathi kuwe ithola ukondliwa nawowonke umsoco kwisikhwanyana ekhula kuso ingane, lesisikhwanyana sinamathela esibelethweni sibizwa ngokuthi umzanyana. Uma umntwana esezaliwe, umzanyana nawo kumele uphume ngaphandle kwesibeletho ungasali ngaphakathi. Emva kokuba inkaba isiboshiwe, umzanyana uyasikwa bese ulahlwa.

Siyakumema ke ukuba uhlanganyele nathi kulolucwaningo futhi sicela nemvume yakho yokuba ube yingxenye kuloluphando. Sidinga ukuba unikele ngesicubu esincane somzanyana wakho bese senza ucwaningo olujulile lokuhlola lomzanyana. Lokhu kuyokwenziwa umntwana esezaliwe nomzanyana usulahliwe, ngaleyondlela angeke kubeke umntwana wakho engcupheni noma ebungozini nanoma ngayiphi indlela. Nakhu okunye, kuyodingeka ukuba sidonse igazi elincane kuwe ukuze sibheke ukuba konke lokhu kunomthelela muphi kulesisimo sehayihayi.

Abukho ubungozi kulolucwaningo, kepha kungabakhona ubuhlungu obuncane obuzwayo ngalesisikhathi kudonswa igazi kuwe. Siyacela ukukwazisa ukuthi lokhu kudonswa kwegazi kuyokwenziwa amanesi aqeqeshiwe nasemthethweni, enza wona lomsebenzi.

Kuyoba nezingxenye zamaqoqo amane (4) eziguli ezehlukene ezizohlanganyela kulolucwaningo. Nantu uhla lwabo; abesifazane abanegciwane lesandulela ngculaza (HIV positive) abanehayihayi, abesifazane abangenalo igciwane lesandulela ngculaza (HIV negative) abanehayihayi, abesifazane abanegciwane lesandulela ngculaza (HIV positive) kodwa abangenayo ihayihayi bese kuba abesifazane abangenalo igciwane lesandulela ngculaza (HIV negative) abangenayo futhi ihayihayi. Lolucwaningo luyokwenziwa kuphela kulabo abayiziguli zase King Edward VIII Hospital, Kwa-Zulu Natal.

Sizoqoqa imizanyana yomame abangu-120 akade benehayihayi enganyukanga, bese kuba ngeyomame abangu-120 akade bene-BP enyukile. Lemizanyana iziqoqwa ezibhedlela komame baseMzansi Afrika, izobe isiyocwaningwa esikoleni sobudokotela i-Nelson Mandela. Ososayensi bezempilo bayozuza ngemiphumela yalolucwaningo. Akukho okunye esikudingayo kuwe ngaphandle kwemvume yokusebenzisa isicubu somzanyana wakho. Isicubu somzanyana siyobhalwa imininingwane yakho engeke yaziwa ngomunye umuntu bese sisetshenziselwa indlela okuyohlolwa ngayo. Emva kocwaningo amasampula omzanyana ayogcinwa ukuze asetshenziselwe ikusasa. Umzanyana ongasebenzanga ngesikhathi socwaningo, uyoshiswa njengokujwayelekile

Qaphelisisa ke, awuphoqiwe ukuba uvume ukuzibandakanya kulolucwaningo/ ukuba uhlanganyele kanye nathi. Unalo ilungelo/ igunya lokuba unqabe noma lokuba uthi QHA/ CHA, futhi ukunqaba kwakho angeke kukwenze ukuba ungabe usanakekelwa ngendlela efanelekile uma ulashwa. Akukho okuyodalulwa, konke kuyoba imfihlo phakathi kwethu nawe.

Siyabonga kakhulu ngesikhathi sakho, nosizo lwakho kulolucwaningo.

CONSENT DOCUMENT

Consent to Participate in Research

You have been asked to participate in a research study.

You have been informed about the study by ……. . You may contact ……. at …… 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 at **031-260 4769** if you have questions about your rights as a research subject. 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 **Date**
(Where applicable)

_____ _____

Signature of Translator **Date**
(Where applicable)

INCWADI YESIVUMELWANO

Isivumelwano Sokuzibandakanya/ Sokuhlanganyela kulolucwaningo

Usuke wacelwa ukuba uhlanganyele kulolucwaningo.

Usuke waziswa kabanzi ngalolucwaningo ngu …….. . Ungakwazi ukuxhumana no……..e……ngazikhathi zonke uma unemibuzo mayelana nalolucwaningo noma ngabe uyewathola ukulimala ngenxa yalolucwaningo.

Ungaxhumana/ ungathintana namahhovisi akwa **Biomedical Research** ase Nelson R Mandela School of Medicine kulenombolo **031-260 4769** uma unemibuzo mayelana namalungelo akho njengomuntu ozibandakanyayo kulolucwaningo. Ukuzibandakanya kwakho kulolucwaningo akusiyo impoqo, uyazinikela ngokuthanda kwakho, futhi angeke ujeziswe noma ulahlekelwe ilungelo lakho lokwelashwa uma ngabe ukhetha ukunqaba noma uyeka endleleni.
Uma uvuma ukuzibandakanya, uyonikezwa incwadi yesivumelwano kanye nencwadi yolwazi-ekuchazela kafushane ngalolucwaningo.

Lolucwaningo seluchaziwe kabanzi kimina ngomlomo, kanye nalolulwazi olungenhla, ngiyaqonda ngokuphelele ukuthi ukuzibandakanya kwami kulolucwaningo kusho ukuthini, futhi ngiyavuma ukuzibandakanya.

_____	_____
Isayini yoMhlanganyeli	**Usuku**
_____	_____
Isayini yoMhlanganyeli (Uma kudingeka)	**Usuku**
_____	_____
Isayini yoMtoliki (Uma kudingeka)	**Usuku**

APPENDIX V: DATA COLLECTION TOOL

ANGIOGENIC GROWTH FACTORS (VEGF/PLGF & SFLT-1): HIV/PRE-ECLAMPSIA STUDY

Study No: _____

Category: Normotensive: 1 Pre-eclamptic: 2
HIV Normotensive: 3 HIV Pre-eclamptic: 4

☺ **No exclusion criteria present** (check against list) □

Please place hospital sticker here

GENERAL HOSPITAL INFORMATION

Admission date		KEH number			
Diagnosis (tick) if PET	MILD: BP 140-149 90-109 Protein +		SEVERE: BP ≥ 160 110 Protein ++		

PATIENT DEMOGRAPHICS

Date of Birth		Age	
Lifestyle (tick)	Rural	Urban	

HIV Status	+ve	-ve
CD4 counts		%
Anti Retroviral Therapy	Yes	No
HAART		
PMCTC		

MATERNAL TREATMENT

Type of Treatment	**Yes**	**No**
Magnesium Sulphate		
Aldomet		
Monohydralozine		
Nifedipine		
Dihydralozine (nepresol)		
Labetalol		

CLINICAL DATA

Parity	P: G:	Weeks gestation on admission			
Highest BP	Systolic: Diastolic:				
Maternal weight at last AnteNatal Visit		Maternal height			
Oedema (tick)	ankle	Up to knee	Up to groin	Generalised (facial)	
Lab Results On Admission	**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	Urea			
		Creatinine			
		Uric acid			
	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					
Indication for delivery (tick one)	Maternal interest	Fetal Distress (CTG abnormal)	Combination of Maternal and fetal interest.		
	Explain above if relevant	Explain above if relevant	Explain above if relevant		
Method of Delivery (tick one)	Normal vaginal		Caesarean		
	Spontaneous		Elective		
	Induced		Emergency		
Complications in labour.	Eclampsia –related (tick)	Severe preeclampsia	Imminent eclampsia		
	Other (explain)				

BABY DETAILS AT BIRTH

APGAR	1 min		5 min	
Baby (tick)	Live		Stillborn	
	Perinatal death (1st 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)

Baby weight: _____ Maternal BP: _____

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

_____
_____
_____
_____

Date:_____ Inpatient / Outpatient visit: _____

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

Baby weight: _____ Maternal BP: _____

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

_____
_____
_____
_____

FOLLOW UP DATA FOLLOWING 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:

_____
_____
_____

CardioVascular Journal of Africa

Maternal imbalance between proangiogenic and antiangiogenic factors in HIV infected preeclampsia

--Manuscript Draft--

Manuscript Number:	CVJSA-D-11-00122R1
Full Title:	Maternal imbalance between proangiogenic and antiangiogenic factors in HIV infected preeclampsia
Article Type:	Original Article
Section/Category:	Basic Science Section
Keywords:	sFlt1; Preeclampsia; antiangiogenic factors; HIV.
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Manuscript Region of Origin:	SOUTH AFRICA
Abstract:	Angiogenic imbalance contributes to the development of preeclampsia. We evaluated the protein expression of the proangiogenic placental growth factor (PlGF) and transforming growth factor beta 1 (TGF-β1) compared with the antiangiogenic soluble fms-like tyrosine kinase receptor (sFlt1) and soluble endoglin (sEng) in HIV infected normotensive and preeclamptic pregnancies. Blood was obtained from 110 pregnant women, enrolled in 4 groups, viz., HIV negative normotensives (27); HIV positive normotensives (31); HIV negative preeclamptics (27) and HIV positive preeclamptics (25), and used to measure PlGF,TGF-β1, sFlt1 and sEng levels. Increased sFlt1 and sEng levels were associated with the preeclamptics (HIV negative and HIV positive) compared with their counterparts. Decreased PlGF levels was observed between the HIV negative preeclamptics versus HIV negative normotensives, but levels differed significantly (p= 0.02) amongst the normotensives (HIV negative and HIV positive). TGF-β1 remained unchanged across all groups. Higher sEng/TGF-β1 ratios were associated amongst the preeclamptics (HIV negative and HIV positive) compared with their counterparts. This study demonstrated increased sFlt1 and sEng levels in preeclamptic compared with normotensive pregnancies, irrespective of the HIV status.
Suggested Reviewers:	I Mackraj Associate Professor in Physiology, University of KwaZulu Natal Mackraji@ukzn.ac.za Prof I Mackraj has conducted recent work on the etiology of angiogenic factors in preeclampsia

	DP Naidoo Professor, University of KwaZulu Natal naidood@ukzn.ac.za Prof DP Naidoo is a cardiologist who is an expert in hypertension in pregnancy and medical complications.
Opposed Reviewers:	
Response to Reviewers:	Dear Professor YK Seedat RE: CVJSA-D-11-00122: "MATERNAL IMBALANCE BETWEEN PROANGIOGENIC AND ANTIANGIOGENIC FACTORS IN HIV INFECTED PREECLAMPTICS" I have the revised the manuscript. I have addressed and outline the rebuttal against each comment raised by the reviewer below. In view of statistical queries, all means ±SD have been amended under "Results (text and Figures)" to read as Median (interquartile range) in consultation with our institutional biostatistician. Additionally, for clarity Table II comparing proangiogenic and antiangiogenic factors of maternal serum across all study groups, has been inserted. These amendments do not alter the results of the manuscript previously submitted. RESPONSE TO REVIEWER #3 COMMENTS: Query 1- References: This needs to be uniform. For some references the full name of the journal is written, while for others the abbreviated form is used. Response 1: The authors apologise for this inconsistency. This has been addressed and changes have been made accordingly under List of References on Page 13-15. Query 2-Aim: The aim of the study as mentioned in the introduction is to confirm the role of these pro and antiangiogenic biomarkers in the setting of HIV, prior to advocating these as biomarkers for the early detection of pre-eclampsia. The average gestational age of recruitment in this study is 37 weeks. How does this late recruitment impact on meeting the study aim and the findings of the study? Response 2: The authors acknowledge the limitation of the recruitment of participants at term. Taking cognizance of this fact, the aims have been amended and highlighted accordingly on Page 4. Query 3-Methods: More information needs to be given regarding recruitment of patients. Were these patients recruited consecutively? What were the inclusion /exclusion criteria? Response 3: The authors have reviewed the methods and amended accordingly. Time of recruitment (highlighted on Page 4), inclusion and exclusion criteria have been included and are highlighted on Page 5. Query 4-Results: Query 4a: Line 8 page 6 should read CD4 Response 4a: This has been addressed and is highlighted on Page 6. Query 4b: There is note made of a significantly higher difference between HIV positive preeclamptic and HIV negative normotensive pregnant women. What is the reason for the comparison of these 2 groups and what is the significance of this? Query 4c: A comparison is made between the systolic and diastolic blood pressures of the pre-eclamptic and normotensive women in the groups. As expected by definition of pre-ecalmpsia in the methods these are significantly different. A more important comparison would be the comparison of the Bp in the HIV positive and HIV negative pre-eclamptic groups as this may serve as a parameter of the any differences of

severity of hypertension in the 2 groups that may have impact on the angiogenic factors.
Query 4d: A comparison is made between the concentrations of angiogenic factors and noted to be significantly different between HIV neg pre-eclamptic women and HIV positive normotensive women. What is the reason for comparing these 2 groups and what is the significance of this result.

Response 4b-d:

Inter-group analysis was conducted using the non parametric Kruskal-Wallis test. Where differences were found in the Kruskal-Wallis test, the Dunn's post-hoc Test was used to identify where these differences existed. This is addressed accordingly in the manuscript under "Methods" and "Results". These changes are highlighted on Page 6 (statistical analyses), in text of results on Pages 6-8 and Tables I and II respectively.

Response 4c only:

We have performed the non parametric Mann-Whitney test to address the query regarding the blood pressure as suggested. However, no significant difference was noted, thus this data was not included in the manusrcript. To note, the Kruskal-Wallis test was done to compare inter-group variations for both the angiogenic and antiangiogenic factors as previously outlined in the manuscript. However, this test revealed no significant differences between the HIV negative and HIV positive preeclamptic groups (Figure 1a-d).
Query 4e: Use of significantly lower difference and significantly higher difference is confusing to the reader and should be rephrased.

Response 4e:
This has been addressed throughout the manuscript.

Query 5-Discussion:

Query 5a: The average gestational age of recruitment/delivery of the pre-eclamptics was 37/38 weeks suggesting that most likely pre-eclampsia was mild and this may contribute to the findings of the placental weights.

Response 5a:
The authors acknowledge that preeclampsia may be early or late in presentation and may contribute to the variations in placental weight. This has been amended and highlighted on Page 9.

Query 5b: The conclusion in the 1st paragraph on page 12 that the results suggest the ratios may have a clinical significance as a predictor test for pre-eclampsia development is not clear. Can the authors' explain?

Response 5b
The authors acknowledge the limitation of this study, being term gestation. This has been amended and highlighted on Page 12.

Query 6-Table 1:
How the p-values are derived - which groups, or are all groups compared?

Response 6:
This has been addressed and highlighted on Page 5 under statistical analyses of the manuscript. P value ($p<0.05$) was determined via inter-group analyses using the Kruskal-Wallis test. The table has been amended and data is presented as median (interquartile range).

Thank you for considering my manuscript. I hope that all comments have been adequately addressed.

Yours Sincerely

	Nalini Govender

Maternal imbalance between proangiogenic and antiangiogenic factors in HIV infected preeclampsia

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Abstract

Angiogenic imbalance contributes to the development of preeclampsia. We evaluated the protein expression of the proangiogenic placental growth factor (PlGF) and transforming growth factor beta 1 (TGF-β_1) compared with the antiangiogenic soluble fms-like tyrosine kinase receptor (sFlt1) and soluble endoglin (sEng) in HIV infected normotensive and preeclamptic pregnancies.

Blood was obtained from 110 pregnant women, enrolled in 4 groups, viz., HIV negative normotensives (27); HIV positive normotensives (31); HIV negative preeclamptics (27) and HIV positive preeclamptics (25), and used to measure PlGF, TGF-β_1, sFlt1 and sEng levels.

Increased sFlt1 and sEng levels were associated with the preeclamptics (HIV negative and HIV positive) compared with their counterparts. Decreased PlGF levels was observed between the HIV negative preeclamptics versus HIV negative normotensives, but levels differed significantly ($p= 0.02$) amongst the normotensives (HIV negative and HIV positive). TGF-β_1 remained unchanged across all groups. Higher sEng/TGF-β_1 ratios were associated amongst the preeclamptics (HIV negative and HIV positive) compared with their counterparts.

This study demonstrated increased sFlt1 and sEng levels in preeclamptic compared with normotensive pregnancies, irrespective of the HIV status.

Keywords: sFlt1, preeclampsia, antiangiogenic factors, HIV

Preeclampsia, a clinical syndrome unique to human pregnancy, is characterized by new onset hypertension and proteinuria, which present after the 20th week of gestation [1-4]. Although several studies in the last few decades have investigated the pathogenesis of this disorder, limited progress has been made in establishing the exact cause [5, 6]. Currently, preeclampsia is reported to be a 2 stage disorder, viz., a preclinical/asymptomatic and a clinical stage [2, 7]. The first stage is characterized by abnormal placentation leading to a hypoxic placenta, oxidative stress and immune dysregulation whilst the second stage is characterized by the placental discharge of soluble factors, such as sFlt1 and sEng into the maternal circulation resulting in widespread endothelial dysfunction and the clinical syndrome of hypertension, proteinuria, intrauterine growth restriction (IUGR) and thrombocytopenia [2, 7-10].

Both preeclampsia and HIV infections are common conditions in sub Saharan Africa and major causes of maternal deaths [11]. Recent studies have reported that the persistent infection of HIV infected individuals contributes to the development of chronic arterial injury and the subsequent endothelial damage, atherosclerosis and thrombosis [12]. Furthermore, untreated HIV infected patients may be prone to endothelial dysfunction [12]. HIV infection also seems to affect the mechanisms implicated in the aetiology of preeclampsia and IUGR. Normal pregnancy is characterised by an altered immune sensitivity thereby allowing maternal resistance against any infection and fetal tolerance whilst preeclampsia is a hyper-active immune response [13, 14]. It is plausible that the immune insufficiency stimulated by HIV together with the normal immune changes of pregnancy may possibly reduce a predisposition to the immune hyper-reactivity that is associated with preeclampsia [17 – 19]. Thus it is not surprising that some reports have shown that a reduced rate of preeclampsia prevails amongst untreated HIV infected patients in comparison with those on highly active antiretroviral therapy (HAART) [14, 15].

The administration of HAART is reported to enhance maternal immune reconstitution by re-establishing the mother's immune response to fetal antigens, and consequently making the woman susceptible to the development of preeclampsia [14]. Conflicting reports however, have created uncertainty as to whether HIV infected pregnant women on HAART have lower rates of preeclampsia [16-18]. This uncertainty may impact on both maternal and perinatal morbidity and mortality in a geographical region with a high prevalence of HIV. Angiogenic biomarkers have been suggested for the early detection of preeclampsia in high income countries, despite the lack of robust evidence for their use [4, 19]. Thus, this study set out to examine the role of pro and antiangiogenic factors in the aetiology of preeclampsia in a setting of high rates of HIV.

Methods

Institutional ethical and regulatory approvals were obtained. Clinical characteristics such as maternal and gestational age, parity, maternal, baby and placental weight, and blood pressure were collected during antenatal recruitment. Venous blood samples were collected from 110 pregnant black African women at term attending a tertiary maternity unit in Durban, KwaZulu-Natal, South Africa. All blood samples were centrifuged within 2 h at 3500 rpm for 10 min at 4°C. Serum aliquots were then carefully transferred into new tubes and stored at -70°C until analysis. Enzyme-linked immunoassays for PlGF (1:2), TGF-β_1 (1:40), sFlt1 (1:5) and sEng (1:5) were performed in triplicate according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Fifty two of the blood samples were obtained from preeclamptics and 58 from normotensive pregnant women. These groups were further subdivided into HIV negative and HIV positive sub groups.

Inclusion criteria for preeclampsia were persistent systolic blood pressure ≥ 140 mmHg and diastolic blood pressure ≥ 90 mmHg at least 6h apart, after 20 weeks' gestation in a previously normotensive patient. Proteinuria was defined as urine protein concentration of ≥ 300 mg/dl or 1+ on a urine dipstick in at least 2 random specimens collected at least 4 h apart. Exclusion criteria for all groups was chorioamnionitis, chronic hypertension, eclampsia and abruptio placentae; intrauterine death, pre-gestational diabetes, gestational diabetes and chronic renal disease; systemic lupus erythematous, sickle cell disease and anti-phospholipid antibody syndrome; thyroid disease, cardiac disease and active asthma requiring medication during pregnancy and pre-existing seizure disorders.

Statistical Analysis

Inter-group analysis was conducted using the non parametric Kruskal-Wallis test. Descriptive statistics were utilized and outcome variables are presented as Median (interquartile range). Where differences were found in the Kruskal-Wallis test, the Dunn's post-hoc Test was used for multiple comparisons. A probability level of $p<0.05$ was considered statistically significant. All statistical analyses were conducted using GraphPad Prism® version 5.01.

Results

Clinical Characteristics

Clinical characteristics for the preeclamptic and normotensive participants (n=110) were divided into HIV positive (n=56) and HIV negative groups (n=54) respectively viz., a) HIV negative normotensive (N-): BP ≤ 120/80 mmHg (n=27); b) HIV positive normotensive (N+): BP ≤ 120/80 mmHg; CD4 < 200 (n=31); c) HIV negative preeclamptic (P-): BP ≥ 140/90 mmHg (n=27) and d) HIV positive Preeclamptic (P+): BP ≥ 140/90 mmHg; CD4 < 200 (n=25) (Table I). A significant difference was detected for maternal and gestational age, parity, maternal and placental weight, systolic and diastolic blood pressure (p<0.05) between all 4 groups (Kruskal-Wallis test, Table 1). Mean maternal age ranged between 23-30 years whilst the mean gestational age ranged between 37-39 weeks (Table I).

For maternal weight, the kruskal-wallis test showed an overall significance (p<0.05). The Dunn's multiple comparison tests identified the significant difference between the HIV positive preeclamptic and the HIV negative normotensive pregnant women only (p=0.0321; Table I). However, for placental weight (Table I), a significant difference was evident between the HIV positive preeclamptic and HIV negative normotensive pregnant women (p<0.0001), the HIV negative preeclamptic and HIV negative normotensive pregnant women (p<0.0001) and the HIV positive normotensive and HIV negative normotensive pregnant women (, p<0.0001; Table 1).

For systolic blood pressure (Table 1), a significant difference was evident between the HIV positive preeclamptic and HIV negative normotensive pregnant women (p<0.0001), the HIV positive preeclamptic and the HIV positive normotensive pregnant women (p<0.0001), the HIV

negative preeclamptic and the HIV negative normotensive pregnant women (p<0.0001) and the HIV negative preeclamptic and HIV positive normotensive pregnant women (p<0.0001). A similar pattern was observed for diastolic blood pressure as indicated in Table I.

Proangiogenic and Antiangiogenic Factors

Serum concentrations for all evaluated proangiogenic (PlGF and TGF-β_1) and antiangiogenic (sFlt1 & sEng) factors varied {Table II; Figs. 1(a-d) and 2(a-c)}. A significant difference was observed for sFlt1, sEng and PlGF (p<0.05) between groups (Figs. 1a-d). For sFlt1, the kruskal-wallis test showed an overall significance (p<0.05). The Dunn's multiple comparison test revealed a significant difference between HIV negative preeclamptic and HIV negative normotensive pregnant women (p=0.0061), and HIV negative preeclamptic and HIV positive normotensive pregnant women (p=0.0061).

A significant difference for sEng (Table II; Fig. 1) was evident between HIV positive preeclamptic and HIV positive normotensive pregnant women (p=0.0017), and HIV negative preeclamptic and HIV positive normotensive pregnant women (p=0.0017). Likewise for PlGF, a significant difference was found between HIV negative preeclamptic and HIV negative normotensive pregnant women (p=0.021), and the HIV negative normotensive and HIV positive normotensive pregnant women (p=0.021). However, TGF-β_1 did not differ significantly between groups (p=0.359; Fig. 1).

Antiangiogenic ratio analyses revealed a significant difference only for sEng/ TGF-β_1 ratios (p<0.05, Table II; Fig. 2a). Accordingly, the Dunn's multiple comparison test revealed a significant difference for both HIV positive preeclamptic and HIV positive normotensive pregnant

women (p=0.002), and HIV negative preeclamptic and HIV positive normotensive pregnant women (p=0.002).

Discussion

Since placental delivery is the only cure for preeclampsia, its clinical management is dependent on gestational age and disease severity. In our study gestational age and placental weight varied amongst the study groups. The preeclamptic groups (HIV positive and HIV negative) delivered at a slightly earlier gestational period compared with the normotensive groups. Our data revealed that placental weights for the preeclamptic groups were greater than the normotensive groups. This is surprising as one would have expected the placental weights in preeclamptics to be lower in view of the fact that the preeclamptics delivered at a lesser gestational age and the fact that preeclampsia is associated with IUGR. Alternatively, this higher placental weight may be attributed to the late onset of preeclampsia.

Although we did not correlate fetal growth with gestational age following delivery, there is circumstantial evidence that women with gestational hypertension and mild preeclampsia tend to have slightly bigger babies and larger placental masses than their normotensive counterparts at birth. It is plausible that mild increases in blood pressure cause a concomitant increase in placental perfusion pressure and increased oxygen supply resulting in increased placental size.

However a recent epidemiological analysis conducted by Eskild and Vatten (2010) reported conflicting data with regards to placental weight [20]. Preeclampsia is hypothesised to be due to placental dysfunction, however these investigators have suggested that placental weight may not

be risk indicator for the placental dysfunction evident in preeclampsia. In addition, the placenta is identified as the major angiogenic contributor, and that the imbalance evident in preeclampsia may be associated to placental hypoxia [20]. Thus, preeclamptic placenta is involved with the cause of the disease and is implicated in the production of elevated levels of sFlt1 and sEng [2, 21-23]. This elevation is believed to disrupt the balance of the proangiogenic factors, thereby decreasing their bioavailability with the subsequent vascular maladaptation of preeclampsia.

Our study further demonstrated variations between the proangiogenic (PlGF & TGF-β_1) and the antiangiogenic factors (sFlt1 & sEng) that occurred in preeclamptic (HIV negative and HIV positive) and normotensive (HIV negative and HIV positive) pregnancies, lending credence to the antiangiogenic theory of preeclampsia. To our knowledge, there are no available data that explores the relationship of HIV with circulating proangiogenic and antiangiogenic factors in preeclampsia.

VEGF is recognised as a powerful endothelial-specific mitogen and its significant role in angiogenesis is well documented [24-26]. It is functional through the 2 high affinity receptor tyrosine kinases VEGFR1 (Flt1) and VEGFR2 (Flk1). PlGF is also a member of the VEGF family which binds to Flt1 thereby supplementing the proangiogenic effects of VEGF [24-26]. However, a soluble isoform and a splice variant of Flt1 have been identified as sFlt which contains a ligand binding domain but lacks a trans-membrane and cytoplasmic domain [27]. Karumanchi and co-workers further demonstrated that an excess production of sFlt1 by the preeclamptic placental trophoblasts and the subsequent discharge into the maternal circulation, implicating its key role in the aetiology of this maternal syndrome [27].

Transforming growth factor-beta (TGF-β_1) comprising 3 isoforms, is important for the development of the embryo, inflammation-repair, and angiogenesis [28]. TGF-β_1 an isoforms expressed copiously in trophoblasts and endothelial cells, functions as an apoptotic and proliferative mediator of vascular endothelial cells, immunosuppression and production of cellular matrix [29]. Furthermore, TGF-β_1 contributes to the normal placentation through the control of trophoblast invasion [30]. However, in PE it affects trophoblast cell migration and influences spiral artery conversion by activating gene transcription and increasing the synthesis of matrix proteins [34]. It also decreases pericellular proteolysis by decreased synthesis of proteolytic enzymes such as the serine and matrix metalloproteinases (MMPs) and increases the synthesis of tissue inhibitors (TIMPs), thereby modifying the repertoire of cell adhesion receptors such as the integrins [34]. In the current study we were unable to demonstrate any significant difference for TGF-β_1 between the groups.

Endoglin (Eng) a coreceptor for both TGF-β_1 and TGF-β_3, is highly expressed by in syncytiotrophoblasts and endothelial cells [29, 31]. It is identified as a proangiogenic factor that regulates vascular remodelling and homeostasis via the endothelial nitric oxide synthase pathway [29, 31-33]. In contrast, sEng prevents the signalling pathway of TGF-β_1 and the endothelial stimulation of TGF-β_1 mediated nitric oxide synthase pathway, thereby obstructing the endothelial and capillary development [34, 35]. Consequently, the antiangiogenic effects of sEng evident in preeclampsia, occurs via its interaction with TGF-β_1 resulting in the inhibition of the endothelial attachment of TGF-β_1 and the subsequent loss of the endothelial proangiogenic and vasodilatory effects of TGF-β_1 [22, 31, 34, 35]. Thus, the clinical significance of elevated levels of sFlt1 and sEng and its role as powerful antiangiogenic factors through their interaction with the circulating levels of VEGF, PlGF and TGF-β_1 respectively is well established.

Our study confirmed previous reports of elevated serum levels of sFlt1 and sEng in the preeclamptic compared with normotensive groups [10, 27, 36]. In addition, we showed a significant difference for sFlt1, sEng and PlGF between all groups. Furthermore, sFlt1 and sEng was higher in the preeclamptic (HIV negative and HIV positive) compared with normotensive pregnancies (HIV negative and HIV positive). In our study, the HIV negative normotensive pregnant women had higher levels of PlGF compared with both the preeclamptic groups, confirming previous reports [21, 27, 37]. PlGF was reduced in the HIV positive normotensive versus both the HIV negative and HIV positive preeclamptic groups. Preeclampsia is associated with decreased levels of PlGF with the concurrent increase of sFlt1. This trend was observed in our study amongst the HIV negative normotensive versus the preeclamptic group. Unexpectedly this trend was reversed in the HIV positive normotensive group. It is therefore likely to assume that the immune insufficiency stimulated by HIV infection possibly reduces a predisposition to immune hyper-reactivity, forestalling the development of preeclampsia. Moreover, there was a significantly higher difference between the HIV negative vs the HIV positive normotensive group.

The sFlt1/PlGF ratio has diagnostic predictor test value for preeclampsia [38, 39]. Thus it is evident that the clinical significance of sFlt1/PlGF ratio, which represents the antiangiogenic role in preeclampsia, characterizes the stability between sFlt1 and PlGF. Our results showed lower sFlt1 and sFlt1/PlGF ratio in the HIV positive preeclamptic compared with the HIV negative preeclamptic groups, indicative of an apparent trend towards a diagnostic value. The imbalance that occurs in preeclampsia may be attributed to the immunological nature of the disease, however this requires further investigation.

Unlike sFlt1 in the HIV positive preeclamptics, sEng varied compared with the HIV negative preeclamptic groups. Furthermore, when combined as an antiangiogenic ratio (sEng/ TGF-β_1), a significantly lower difference was evident between the control and the preeclamptic groups. The HIV negative and HIV positive preeclamptic pregnant females showed higher sEng/ TGF-β_1 ratios compared with both the normotensive groups. The HIV positive preeclamptics showed a higher ratio compared with the HIV negative preeclamptic groups whilst the average ratios of the HIV positive normotensives were lower than the HIV negative normotensive pregnant women. These results albeit in term pregnancies suggest that these ratios may have a clinical significance during early pregnancy as a predictor test for preeclampsia development.

An elevation of plasma and platelet depleted plasma levels of TGF-β_1 in preeclampsia compared with normotensive pregnancy has been previously reported [40]. Our study, however, demonstrated no significant differences in serum TGF-β_1 with a concomitant significance of sEng/ TGF-β_1 ratio for the HIV positive preeclamptics *vs* HIV positive normotensives and the HIV negative preeclamptics *vs* HIV positive normotensives. Our results were similar albeit higher to that observed by Huber *et al.,* (2002) showing no significant difference in TGF-β_1 expression between the preeclamptic and normotensive groups [41]. Noteworthy, the sEng/ TGF-β_1 ratio analyses in our study implicated a role for TGF-β_1 in the pathogenesis of preeclampsia. However, a limitation to our study is the relatively small sample size, and this requires further investigation.

In conclusion, our study demonstrated elevations of both sFlt1 and sEng in preeclamptic compared with normotensive pregnancies, irrespective of the HIV status. Quantification of serum proangiogenic/antiangiogenic factors in HIV associated preeclampsia is novel.

Acknowledgement

This study was supported by the Durban University of Technology and the National Research Foundation (Grant Number 69086), South Africa. The authors wish to thank Mrs T Esterhuizen, Mr S Van der Linde and Dr S Naidoo for advice with statistical and ELISA analyses respectively.

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Figure

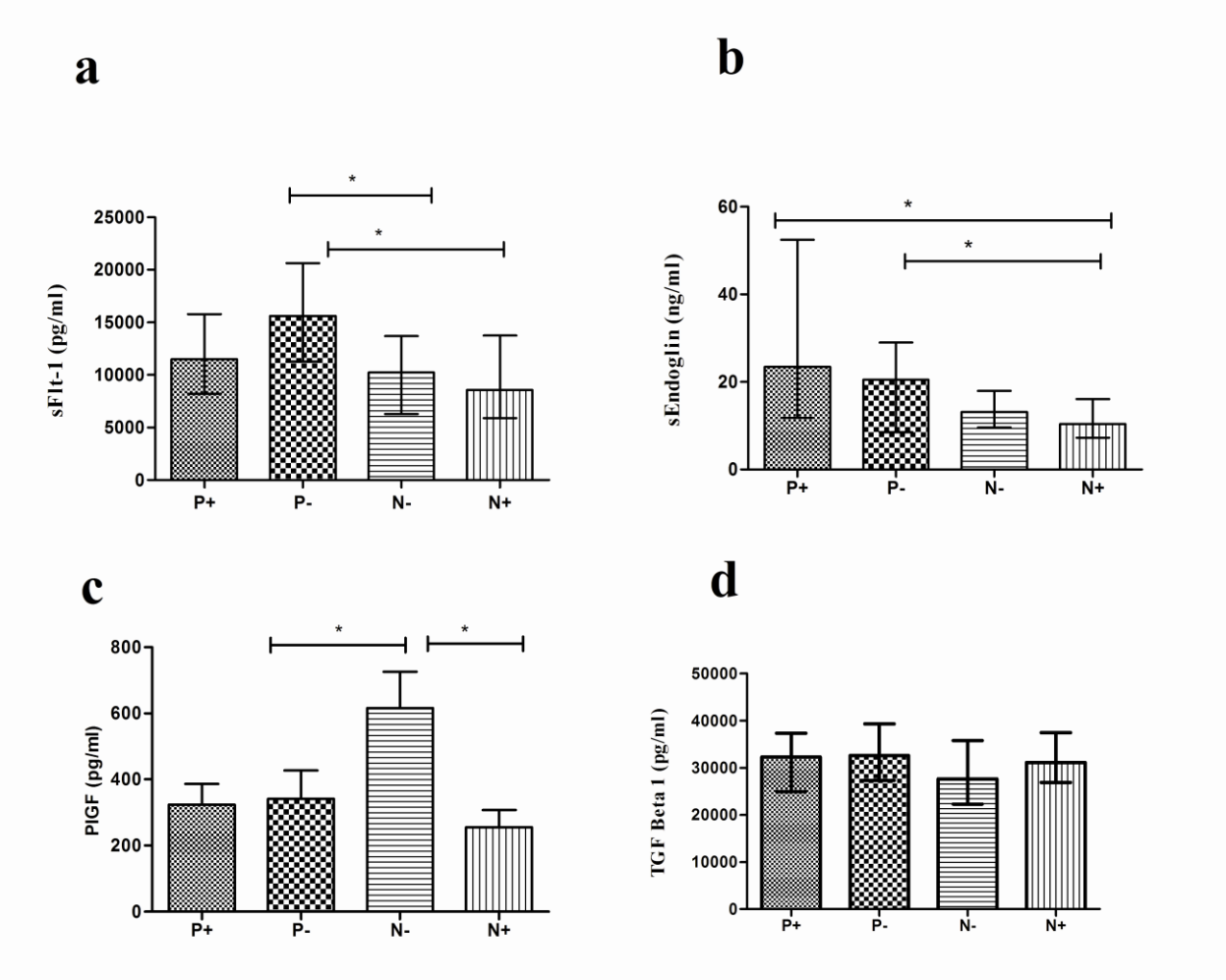

Fig.1. Proangiogenic and antiangiogenic serum concentrations (Medians with interquartile range): a. sFlt1 (pg/ml), b. sEng (ng/ml), c. PlGF (pg/ml) and d. TGF beta 1 (pg/ml); HIV positive Preeclamptic (P+); HIV negative preeclamptic (P-); HIV negative normotensive (N-) and HIV positive normotensive (N+)

Figure

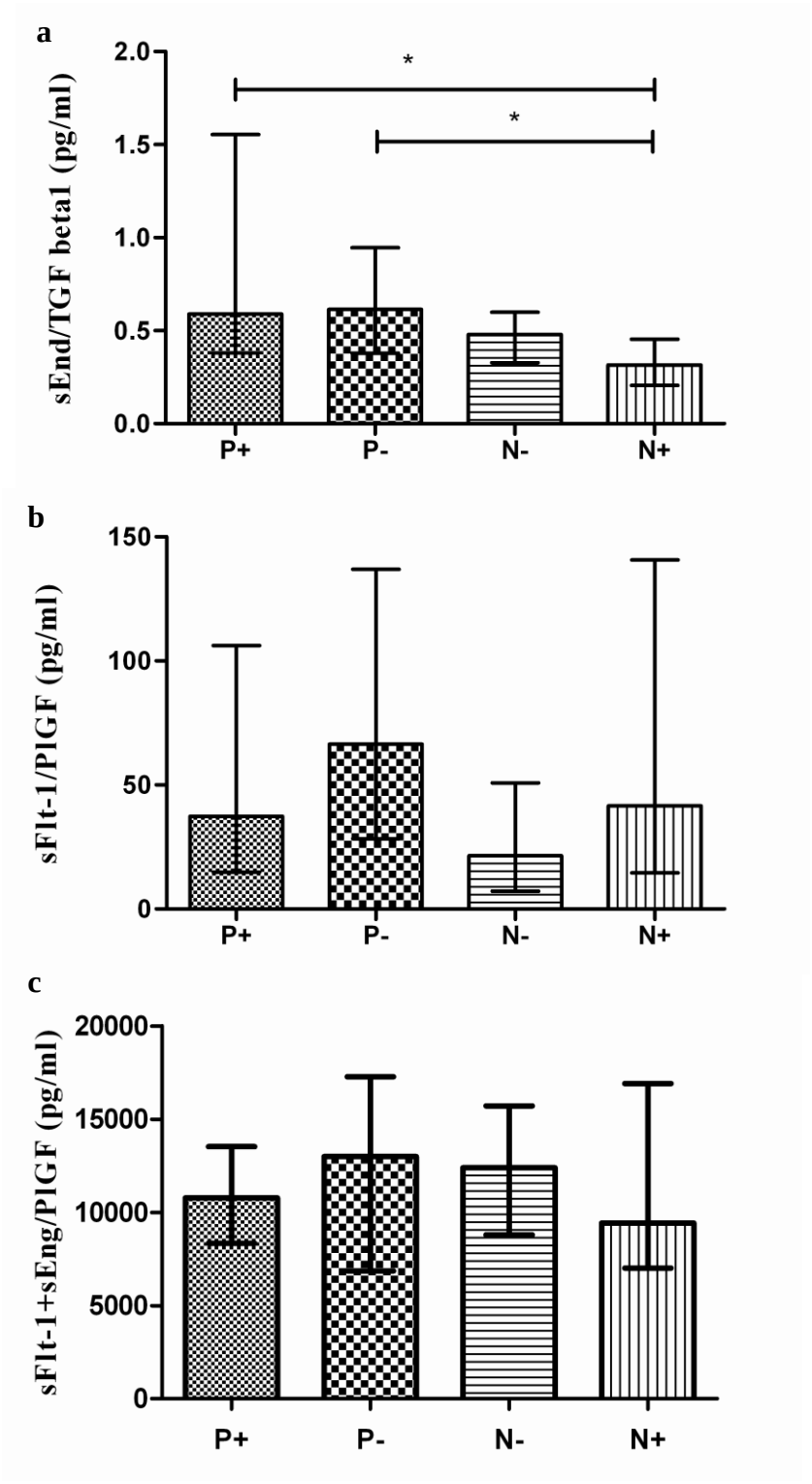

Fig. 2. Antiangiogenic ratio serum concentrations (Medians with interquartile range): a. sEng/ TGF-β_1, b. sFlt1/PlGF and c. (sFlt1 + sEng)/PlGF; HIV positive Preeclamptic (P+); HIV negative preeclamptic (P-); HIV negative normotensive (N-) and HIV positive normotensive (N+)

Table

Table I

Demographic and Clinical Profile of Patients recruited for Immunoassays

	Normotensive Pregnant Women (N-)	HIV Normotensive Pregnant Women (N+)	Preeclamptic Pregnant Women (P-)	HIV Preeclamptic Pregnant Women (P+)	P value
N	27	31	27	25	
Age (yrs)	24(21-26)	27(24-30)	25(20-32)	32(25.5-34)	p=0.0009*
Gestational age (wks)	38(38-39)	39(38-40)	38(37-40(	38(36-38)	p=0.0026*
#Parity	0(0-1)*	1(1-2)	1(0-1)	1(0.5-3)	p=0.0174*
Maternal weight (kg)	66(59-74)	74(65-82)	75(65-96)	82(64-106)	p=0.0321*
Birth weight (kg)	3.2(3-3.4)	3.4(3-3.7)	3.2(2.6-3.8)	2.9(2.7-3.4)	ns
Placental weight (g)	360(300-400)	470(380-500)	480(430-510)	480(380-515)	p<0.0001*
Systolic BP (mmHg)	110(108-115)	112(107-120)	154(150-162)	150(145.5-159)	p<0.0001*
Diastolic BP (mmHg)	70(67-73)	70(67-74)	94(90-104)	96(87-99.5)	p<0.0001*

Medians (range) are presented; Kruskal-Wallis test and the posthoc Dunns multiple comparison test was used for statistical analysis, n=110

*p<0.05

weeks (wks); kilograms (kg); grams (g)

Table

Table II

Comparison of proangiogenic and antiangiogenic factors of maternal serum across study groups

	Normotensive Pregnant Women (N-)	HIV Normotensive Pregnant Women (N+)	Preeclamptic Pregnant Women (P-)	HIV Preeclamptic Pregnant Women (P+)	P value
N	27	31	27	25	
sFlt-1(pg/ml)	10249(6308-13708)	8578(5898-13769)	15617(11257-20641)	11494(8203-15784)	p=0.006*
sEng(ng/ml)	13.14(9.6-17.92)	10.4(7.3-16)	20.5(8.5-29)	23(11.8-52.5)	p=0.002*
PlGF(pg/ml)	488.6(183.9848.3)	207.1(102.6-358.6)	202.2(47.9-490.4)	229.3(74.3-615.9)	p=0.021*
TGF-β1 (pg/ml)	27640(22308-35771)	31164(26916-37474)	32652(27295-39328)	32301(24983-37355)	ns
sEng/ TGF-β1 (pg/ml)	0.48(0.33-0.6)	0.32(0.2-.45)	0.62(0.38-0.95)	0.59(0.38-1.5)	p=0.002*
sFlt-1/PlGF (pg/ml)	21.4(7.2-50.8)	41.6(14.5-140.7)	66.5(28.3-136.9)	37.3(14.8-106.2)	ns
sFlt1+sEng/ PlGF (pg/ml)	12392(8792-15700)	9422(7014-16914)	12988(6859-17276)	10772(8345-13527)	ns

Medians (range) are presented; Kruskal-Wallis test and the posthoc Dunns multiple comparison test was used for statistical analysis, n=110

*$p<0.05$

Nanograms (ng); pictograms (pg)

Non significant (ns)

Dear Sir

Please find manuscript and figures of a research paper entitled “Maternal imbalance between proangiogenic and antiangiogenic factors in HIV infected preeclamptics."

I hereby confirm that there are no conflicts of interest, and that the paper has not been submitted to any other journal for publication. All the authors (Mrs N.Govender; Prof T.Naicker; and Prof J. Moodley) have contributed to the study, have seen the final version submitted here, and agree to its submission to the Cardiovascular Journal of Africa.

The article contributes to the increased understanding of pre-eclampsia by examining the proangiogenic and antiangiogenic factors and its association with HIV infection.

We look forward to your response.

Sincerely

Nalini Govender
Lecturer: Department of Basic Medical Sciences
Durban University of Technology

Elsevier Editorial System(tm) for European Journal of Obstetrics & Gynecology and Reproductive Biology
Manuscript Draft

Manuscript Number: EJOGRB-12-9082R1

Title: SOLUBLE FMS-LIKE TYROSINE KINASE-1 AND SOLUBLE ENDOGLIN IN HIV ASSOCIATED PRE-ECLAMPSIA

Article Type: Research Article

Section/Category: Maternal-Fetal Medicine

Keywords: sFlt-1; sEng; sEng; pre-eclampsia; HIV; HIV

Corresponding Author: Prof. Jagidesa Moodley, FCOG, FRCOG (UK)

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First Author: Nalini Govender

Order of Authors: Nalini Govender; Thajasvarie Naicker; Augustine Rajakumar; Jagidesa Moodley, FCOG, FRCOG (UK)

Abstract: OBJECTIVE: Preeclampsia is characterised by endothelial dysfunction combined with increased concentrations of sFlt1 and sEng, which antagonizes the biological effects of VEGF, PlGF and TGFβ1 respectively. This angiogenic imbalance may have a role in its aetiology. This study evaluated the expression of VEGF, PlGF, sFlt1 and sEng amongst the N-, N+, P- and P+ third trimester pregnancies.
METHOD: Serum and placental tissue from 76 pregnancies were quantitatively evaluated using ELISAs and RT-PCR respectively.

RESULTS: Placental sFlt1 expression differed significantly between the N- vs the P- groups (p=0.001). Similarly, sEng expression differed between the N- vs P- groups (p=0.001). No significant effect was shown between HIV status and pregnancy. Serum sFlt1 (p=0.02) and sEng (p=0.001) was up regulated in the P- compared to N- groups. Similarly, no significant effect was shown between HIV status and pregnancy.
Both VEGF and PlGF did not differ significantly between groups. Notably, sEng expression was elevated in both placenta and serum, whilst placental sFlt1 differed from serum. A weak but significant correlation between serum and placental concentration for sFlt1, sEng and PlGF (r=0.26, p=0.031; r=0.42, p<0.001 and r=-0.3, p=0.014) was observed.

CONCLUSIONS: This study is novel as it demonstrates an up-regulation of serum sFlt1 and sEng in preeclamptic compared to normotensive groups irrespective of the HIV status of the pregnancy. This implicates a contributory role of sFlt1 and sEng in preeclampsia development. The serum reduction of sFlt1 and sEng within the HIV positive compared to the HIV negative cohorts may advocate a neutralization of the immune hyperreactivity of preeclampsia.

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28 February 2013

Jake Holdridge
Journal Manager
European Journal of Obstetrics & Gynecology and Reproductive Biology

Dear Sir

RE: Minor Revision to Ms. No. EJOGRB-12-9082, "SOLUBLE FMS-LIKE TYROSINE KINASE-1 AND SOLUBLE ENDOGLIN IN HIV ASSOCIATED PRE-ECLAMPSIA"

I wish to convey my thanks for the favourably reception of the above manuscript by the reviewers. The authors have addressed all minor revisions as requested.

The authors would like to thank the reviewers for their valuable comments. These comments have been addressed as follows:

Reviewers' comments:

Specialty Editor's comment: The reviewers have been very favourable about this manuscript. Only minor revisions are suggested.
Query 1: Please correct the journals' names in the list of references: some are abbreviated, some not and some are partly abbreviated. The guide for authors calls for abbreviated names.
Response: This has been addressed under “References” in text on Pages 16-17

Reviewer 1 comments:
Query 1: Authors should clarify that nearly the entire study is with subjects presenting with term pre-eclampsia.

Response: The authors have addressed and clarified that all subject presented with term pre-eclampsia. This has been addressed in text, under "Results-Clinical characteristics" on Page 9, Line 8 of the manuscript.

Query 2: Comment on placental weights is warranted. In general, severe preeclampsia with SGA has smaller placentas. In this study, it does appear that placentas from preeclampsia are bigger - this is a bit unusual, consistent with the fact that most of disease is mild and term disease.
Response: All patients recruited in the preeclamptic groups were mild to moderate preeclamptics, thus the study population represent late-onset preeclampsia. This has been addressed in text under "Results-Clinical characteristics" on Page 9, Line 8 of the manuscript.
Additionally, gestational age was ≥ 36 weeks across all study groups. We take cognisance of the fact that it is unusual for placental weight to be greater among the PE group. However, our results corroborate Eskild and Vatten (2010). This has been addressed in text, under Comment, Page 15, and Lines 4-8 in the revised manuscript.

Query 3: It may be worthwhile for the authors to evaluate pre-eclampsia that presents early <34 weeks where greater abnormalities in angiogenic factors have been reported.
Response: The authors acknowledges this comment and will address this in future studies.

Query 4: How was the diagnosis of preeclampsia ascertained? Was the BP/proteinuria data obtained from medical records or was it based on ICD9 codes.
Response: This was a prospective study and patients were recruited on blood pressure and protein recordings on admission. Thus, diagnosis was based on South African ICD 10 coding standards. This has been addressed in text, under Methods, on Page 7 and Line 7.

Reviewer 2 Comments
Reviewer #2: The subject is original and interesting in our days. Preeclampsia remains actually unclear. The title is appropriate and the abstract is clear. The methodology is adequate and the results well discussed. Maybe the pro and antiangiogenic factors are not the unique in the association between preeclampsia and HIV.
Response: The authors thank the reviewer for the comments. No change to manuscript.

I hereby confirm that there is no conflict of interest, and that the paper has not been submitted to any other journal for publication. All the authors (Dr N.Govender; Prof T.Naicker; Dr A Rajakumar and Prof J. Moodley) have contributed to these revisions, have seen the final version submitted here, and agree to its re-submission to the European Journal of Obstetrics, Gynaecology and Reproductive Biology.

We look forward to the final acceptance of this manuscript.

Yours Sincerely

PROF T NAICKER

Soluble fms-like tyrosine kinase-1 and soluble endoglin in HIV associated preeclampsia

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Word Count: 2525

Number of Tables: 2

Number of figures: 5

Condensation

Increased serum sFlt1 and sEng concentrations in HIV negative pregnancies advocate their role in preeclampsia whilst in HIV positive pregnancies it may implicate an effect of HIV infection.

ABSTRACT

OBJECTIVE: Preeclampsia is characterised by endothelial dysfunction combined with increased concentrations of sFlt1 and sEng, which antagonizes the biological effects of VEGF, PlGF and $TGF\beta_1$ respectively. This angiogenic imbalance may have a role in its aetiology. This study evaluated the expression of VEGF, PlGF, sFlt1 and sEng amongst the N-, N+, P- and P+ third trimester pregnancies.

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RESULTS: Placental sFlt1 expression differed significantly between the N- *vs* P- groups (p=0.001). Similarly, sEng expression differed between the N- *vs* P- groups (p=0.001). No significant effect was shown between HIV status and pregnancy. Serum sFlt1 (p=0.02) and sEng (p=0.001) was up regulated in the P- compared to N- groups. Similarly, no significant effect was shown between HIV status and pregnancy.

Both VEGF and PlGF did not differ significantly between groups. Notably, sEng expression was elevated in both placenta and serum, whilst placental sFlt1 differed from serum. A weak but significant correlation between serum and placental concentration for sFlt1, sEng and PlGF (r=0.26, p=0.031; r=0.42, p<0.001 and r=-0.3, p=0.014) was observed.

CONCLUSIONS: This novel study demonstrates an up-regulation of serum sFlt1 and sEng in preeclamptic compared to normotensive groups irrespective of the HIV status of the pregnancy. This implicates a contributory role of sFlt1 and sEng in preeclampsia development. The serum reduction of sFlt1 and sEng within the HIV positive compared to HIV negative cohorts may advocate a neutralization of the immune hyperreactivity of preeclampsia.

Keywords: sFlt1, sEng, preeclampsia, HIV

Abbreviations

VEGF: vascular endothelial growth factor

PlGF: placental growth factor

sFlt1: soluble fms-like tyrosine kinase receptor

sEng: soluble endoglin

HIV: Human Immunodeficiency Virus

HIV negative normotensive: N-

HIV positive normotensive: N+

HIV negative preeclamptic: P-

HIV positive preeclamptic: P+

Introduction

Normal pregnancy requires immune mediators which are essential for successful implantation and development [1, 2]. Preeclampsia, a human pregnancy specific multi-organ disorder is characterized by incomplete placentation, endothelial dysfunction and an exaggerated immune system response [3]. The cause of preeclampsia remains unclear, therefore treatment is empiric and cure affected by fetal and placental delivery [4-6]. The angiogenic balance between proangiogenic VEGF and PlGF versus the antiangiogenic sFlt1 and sEng are important for effective vasculogenesis, angiogenesis and placental development during pregnancy [1]. Recent studies indicate that an imbalance between VEGF and PlGF versus sFlt1 and sEng leads to widespread endothelial dysfunction and the subsequent new onset hypertension, proteinuria, intrauterine growth restriction (IUGR) and thrombocytopenia [3, 7, 8].

In pregnancies complicated by HIV infection, an exacerbation of events such as recurrent miscarriages, preeclampsia, diabetes and preterm labor may occur [9]. Recent studies report that HIV infection contributes to the development of chronic arterial injury including endothelial damage, atherosclerosis and thrombosis [10]. However, the association between HIV infection and preeclampsia is contradictory [9, 11-13]. Wimalasundera *et al*., (2002), report a reduced rate of preeclampsia development amongst women who did not receive antiretroviral treatment than those on treatment [9]. Additionally, Highly Active Anti Retroviral Therapy (HAART) contributed to the immune restoration and led to preeclampsia development. HAART may also augment the risk of preeclampsia development by its direct toxicity on the liver [9, 14]. In contrast, a Brazilian study observed that predisposition to preeclampsia is reduced by HIV infection [12], whilst a South African study reports no association between HIV infection and preeclampsia development [15].

Despite this controversy, we hypothesize that it is biologically plausible that a combination of immune activation in preeclampsia and immune suppression during HIV infection may impact the equilibrium between proangiogenic and antiangiogenic factors. This imbalance may reduce the predisposition to immune hyperreactivity and consequentially the development and/or progression of preeclampsia. This novel study attempts to elucidate whether a paradigm shift in the balance of proangiogenic (VEGF and PlGF) and antiangiogenic factors (sFlt1 and sEng) exist in normotensive and preeclamptic pregnancies that are compromised by HIV infection.

Materials and Methods

Study population and sample collection

Following institutional ethical and regulatory permission, placental tissue and venous blood samples were obtained from 76 women at delivery from the Obstetric Unit of a regional hospital in South Africa. Preeclampsia was defined as a sustained systolic blood pressure ≥140mmHg and a diastolic blood pressure ≥90mmHg at least 4hrs apart, and proteinuria after 20 weeks' gestation in a previously normotensive patient (SAICD10 coding). New onset proteinuria was defined as urine protein concentration of ≥30mg/dl or 1+ on a urine dipstick in at least 2 random specimens collected at least 4hrs apart.

The clinical profiles are outlined (Table I). Of these patients, 35 were diagnosed with preeclampsia and 41 were healthy normotensive pregnant women. The preeclamptic and normotensive participants (n=76) were divided into HIV positive (n=37) and HIV negative groups (n=39) respectively viz., a) HIV negative normotensive (**N-**): BP≤120/80mmHg (n=20); b) HIV positive normotensive (**N+**): BP≤120/80mmHg; CD4<200 (n=21); c) HIV negative preeclamptic (**P-**): BP≥140/90mmHg (n=19) and d) HIV positive preeclamptic (**P+**): BP≥140/90 mmHg; CD4<200 (n=16).

Placental villi were collected post vaginal or caesarean section delivery. Placental tissue strips were stored in RNA*later* stabilizing solution (Ambion, Austin, TX) at -70°C until further analysis. Blood samples were centrifuged within 2hrs (3500rpm, 10min at 4°C). Serum aliquots were stored at -70°C until analysis.

RNA Extraction and Quantitative Real Time Polymerase Chain Reaction (RT-PCR)

RNA was extracted from placental tissue [16], and cDNA synthesis was performed using the Applied Biosystems cDNA synthesis kit according to the manufacturer's protocol (Applied Biosystem, Foster City, CA). Thereafter, RT-PCR was performed to determine the levels of mRNA expressions of sFlt1, VEGF, PlGF and VEGF using standard methods.

Enzyme-linked immunoassays (*ELISA)*

Serum concentrations for VEGF (1:2), PlGF (1:2), sFlt1 (1:5) and sEng (1:5) were estimated in triplicate by ELISA according to manufacturer's protocol (R&D Systems, Minneapolis, MN).

Statistical Analysis

A two-way ANOVA was done to examine the effect of HIV status and pregnancy (normotensive and preeclamptic) simultaneously, on the expression of sFlt1, sEng, PlGF and VEGF. All data were assessed for their distribution by the Shapiro-Wilks test. The homogeneity of variance between groups was assessed by the Levene's test for equality of error variances. Kruskal-Wallis and Dunns' post hoc test was used to compare the means of clinical parameters. Descriptive statistics were utilized and outcome variables are presented as median (interquartile range). Spearman's rho correlation coefficient was computed to assess the relationship between serum and placental expression of sFlt1, sEng, PlGF and VEGF across all study groups. A probability level of $p<0.05$ was considered statistically significant. All statistical analyses were conducted using IBM SPSS Statistics version 20 and GraphPad Prism® version 5.01.

Results

Clinical Characteristics of the study population (Table1)

A significant difference was detected for maternal and gestational age, placental weight, systolic and diastolic blood pressure across all groups ($p<0.05$; Kruskal-Wallis test, **Table 1**). All preeclamptic patients recruited were mild to moderate late onset preeclamptic groups. Gestational age was ≥36 weeks across all study groups. For placental weight, a significant difference was noted between P- and N- ($p=0.0181$, **Table 1**) groups. Systolic blood pressure showed a significant difference between the N- versus P- ($p< 0.0001$), and N+ versus P+ ($p< 0.0001$) groups. Similarly, diastolic blood pressure differed significantly between the N- versus P- groups ($p<0.0001$), and N+ versus P+ ($p<0.0001$) groups.

Serum levels of pro and antiangiogenic factors in pregnancies with or without HIV

Serum concentrations of sFlt1, sEng and PlGF are presented (**Table II**; **Figure 1A-C)**. There was no significant effect between HIV status and pregnancy (normotensive and preeclamptic) on sFlt1 [$F(1,64)=3.66$, $p=0.06$); sEng [$F(1,63)=0.62$, $p=0.43$] and PlGF [$F(1,63)=0.02$, $p=0.86$)] levels respectively.

A significant effect was noted between the type of pregnancy (normotensive *vs* preeclampsia) on sFlt1 levels [$F(1,64)=5.71$, $p=0.02$]. Higher sFlt1 levels was noted in preeclamptics (mean=14069.6, 95%CI: 12164.2-15975.1) compared to normotensives

(mean=10891.6, 95%CI: 9056.0-12727.3; **Figure 1A**). No significant effect was noted between HIV status and pregnancy [F(1,64)=0.031, *p*=0.861].

There was a significant effect of type of pregnancy (normotensive and preeclamptic) on the levels of sEng [F(1,63)=13.07, *p*=0.001]. Higher sEng levels was noted in preeclamptics (mean=3.01, 95%CI: 2.75-3.27) compared to normotensive (mean=2.35, 95%CI: 2.09-2.60) groups (**Figure 1B**). No significant effect was noted between HIV status and pregnancy [F(1,63)=0.33, *p*=0.568].

In contrast, there was no significant effect of type of pregnancy (normotensive and preeclamptic) on PlGF levels [F(1,63)=2.912, *p*=0.093]. Additionally, no significant effect was noted between HIV status and pregnancy [F(1,63)=0.303, *p*=0.584]. PlGF was greater among normotensives compared to preeclamptics (HIV positive and negative) (**Table II; Figure 1C**). Serum VEGF was undetected by both the standard and ultrafiltration processes.

Real-time PCR estimation of mRNA levels of pro and anti-angiogenic factors

Representative images of RT-PCR are shown (**Figures 2 and 3**). The relative placental mRNA expression levels presented as arbitrary units (AU), of sFlt1, VEGF, VEGF and PlGF were compared across the four groups and were normalised to the housekeeping gene, 18sRNA (**Figure 4A-B)**. Data are expressed as fold changes. There was no significant effect between the HIV negative (normotensives and preeclamptics) *vs* HIV positive (normotensives and preeclamptics) groups for the levels of sFlt1 [(*F*(1,72)=0.01, *p*=0.92]; sEng [*F*(1,73)=0.14, *p*=0.71]; VEGF [*F*(1,70)=0.01, *p*=0.93] and PlGF [*F*(1,73)=1.17, *p*=0.28] respectively.

However, a significant effect of type of pregnancy (normotensive and preeclamptic) on sFlt1 levels [$F(1,72)=11.09$, $p=0.001$] was noted. Simple main effects analyses indicated higher LN (sFlt1) levels among preeclamptics (mean=9.32, 95% CI: 9.70-8.93) compared to normotensives (mean=8.36, 95% CI: 8.78-7.94; **Figure 4A)**. There was no significant effect between HIV status and pregnancy [$F(1,72)=0.068$, $p=0.794$].

Similarly, a significant effect of pregnancy (normotensive and preeclampsia) was shown for sEng [$F(1,73)=11.09$, $p=0.001$]; showing that preeclamptics had higher LN levels (mean=10.05, 95% CI:10.40-9.71) compared to normotensive pregnancies (mean=10.85, 95% CI:11.18-10.52; **Figure 4B)**. Finally, no significant effect between HIV status and pregnancy was noted [$F(1,73)=0.001$, $p=0.977$].

Placental sFlt1concentration was elevated in the P+ compared to P- groups, whilst circulating sFlt1 remained unchanged (**Figure 1A, 4A**). Placental and serum concentrations of sEng were both elevated in preeclamptics (**Figure 1B, 4B**). Additionally, analyses of RNA*later* solution showed no significant effect ($p=0.5130$) for sFlt1 levels, whilst a significant effect was shown for sEng ($p<0.05$) between the N- *vs* N+ groups.

In contrast, there was no significant effect of type of pregnancy (normotensive and preeclamptic) on PlGF [$F(1,73)=1.33$, $p=0.253$] and VEGF [$F(1,70)=0.372$, $p=0.544$] levels respectively. Additionally, there was no significant difference between HIV status and pregnancy for both PlGF [$F(1,73)=1.30$, $p=0.26$] or VEGF [$F(1,70)=0.65$, $p=0.42$].

Correlation analyses

There was no correlation between serum and placental concentrations for sFlt1, sEng, VEGF and PlGF (r=0.26, p=0.031; r=0.42, p<0.00; r=-0.14, p=0.328and r=-0.3, p=0.014) respectively. A scatter-plot summarizes the results for sFlt1 and sEng (**Figure 5A-B**).

Comment

Our study supports the hypothesis that an imbalance between proangiogenic (PlGF and VEGF) and antiangiogenic (sFlt1 and sEng) factors are associated with preeclampsia development. We report no interaction between HIV and pregnancy for both the proangiogenic and antiangiogenic factors.

Our findings demonstrate that serum levels of sFlt1 and sEng were higher between the preeclamptic (HIV negative and HIV positive) compared to normotensive pregnancies (HIV negative and HIV positive; **Figure 1A-B**). For both sFlt1 and sEng, a significant difference was evident between the P- and N- groups (**Figure1A-B**). Additionally, slightly higher circulating levels of sFlt1 and sEng in HIV negative pregnancies (preeclamptic and normotensive) compared to HIV positive pregnancies, suggests a neutralization of the immune hyperreactivity of preeclampsia (**Figure 1A-B**). Our data supports previous reports that an up-regulation of sFlt1 and sEng play a significant role in preeclampsia development [7, 8, 17-19]. Moreover, the elevated sFlt1 levels may be responsible for the decreased vascular supply due to reduced angiogenesis.

Our data are in agreement with Karumanchi and co-workers [8] and highlights the role of sFlt1, an antagonist of both VEGF and PlGF in the pathogenesis of preeclampsia. Additionally, we report higher levels of sFlt1 among preeclamptics, suggestive of subsequent inhibition of

endothelial and trophoblastic invasion of spiral arterioles in placental bed [20] and decreased placental perfusion [21]. Several others have reported increased sEng levels in preeclampsia [22, 23], an elevation thought to be dependent on the presence of sFlt1 for its stimulatory effect. Soluble Flt1 is a spliced variant of the vascular endothelial growth factor receptor 1 (Flt1) and is deficient of the transmembrane and cytoplasmic domains of Flt1 [24]. It antagonizes the effects of VEGF and PlGF by depleting their availability to membrane bound receptors. The preeclamptic placenta expresses increased quantities of sFlt1 with the subsequent reduced levels of VEGF and PlGF [6, 8]. Recent studies investigating plasma concentrations of these proangiogenic/antiangiogenic factors have ascertained prognostic significance for the detection of patients with severe preeclampsia that necessitate preterm delivery, thus reinforcing their clinical use [25].

Alternatively endoglin, a co-receptor for the transforming growth factor beta (TGFβ) family has a functional role in the maintenance of the vascular system and the nitric oxide synthase pathway [26]. Soluble endoglin however, is an antiangiogenic factor that weakens the affinity of $TGF\beta_1$ to bind to its receptor on the cell surface, diminishing $TGF\beta_1$ signalling thereby activating eNOS with consequential vasodilation. *In vitro* studies highlight the inhibitory role of sEng on endothelial function, whilst *in vivo* studies on pregnant rats allude to an adenovirus assisted elevation in both sFlt1 and sEng, which function in concert to enhance the effects of either molecule resulting in preeclampsia [22].

In our study, circulating VEGF levels were below the lowest standard concentration of the commercially available ELISA utilized (31.2pg/mL). Our findings support previous reports of undetectable circulating VEGF levels in pregnant women [27-29].

Although our findings were not significantly different for PlGF, a trend was noted (**Figure 1C**). Normotensive pregnancies when compared to preeclamptic pregnancies in both cohorts indicated increased concentrations of PlGF. Both VEGF and PlGF are proangiogenic factors essential for the maintenance of reproductive health inclusive of blood vessel remodelling and development [30]. Studies have shown that the circulating levels of VEGF in normal pregnancies increase quickly following fertilization [31]. Subsequent studies have therefore focused on proangiogenic/antiangiogenic factors in the second trimester of normotensive and preeclamptic pregnancies [8, 18, 30, 32, 33], whilst our study involved third trimester pregnancies.

Placental mRNA expression of both sFlt1 and sEng in our study were higher in the preeclamptic (HIV negative and positive) compared to the normotensive (HIV negative and positive) pregnancies (**Figure 4A-B**). The sFlt1expression was slightly lower in the P- compared to N- groups. This may be ascribed to our placental collection method. Recently, syncytial aggregates were shown as rich sources of sFlt1 and sEng, suggesting that they may have been lost in our RNA*later* solution [34]. Nevertheless the trend observed for sEng corresponds to previous data [18, 33, 35]. No correlation was noted between placental and serum levels for sFlt1 and sEng (**Figure 5A-B**).

Whilst biological variances in the placenta were considered, other limitations include the "spot measurement" post delivery, gestational age, placental storage in RNA*later* as well as small sample size. Preeclampsia is associated with defective trophoblast invasion occurring early in pregnancy hence a major limitation of this study is that it reflects a spot measurement obtained post-delivery. Albeit difficult, the influence of these growth factors on placentation requires investigation. Unexpectedly, placental weight was greater in our preeclamptic cohorts.

Recent epidemiological studies suggest that placental weight may not be a risk indicator for placental dysfunction in preeclampsia [36]. Whilst the placenta is a rich angiogenic contributor, the maternal circulatory angiogenic imbalance evident in preeclampsia may be ascribed to hypoxia.

Moreover, the standard of care for HIV positive patients include HAART for the mothers own health or dual antiretroviral therapy for the prevention of mother to child transmission. These treatment modalities may tip the balance in favor of angiogenic restoration in HIV compromised preeclampsia. Additionally, HAART is proposed to restore the immune response of HIV positive preeclamptic women [9]. A concise anti-retroviral treatment history was unavailable in our study. In conclusion our study supports the hypothesis that preeclampsia is characterized by an imbalance between pro and antiangiogenic factors. Irrespective of the HIV status, our study report elevations in circulating sFlt1 and sEng levels. Whether the pregnancy is complicated by HIV infection or not, does not affect the role of the antiangiogenic factors in preeclampsia development. Our study supports the use of sFlt1 and sEng as discriminatory prognostic indicators for preeclampsia at term. To our knowledge, this is the first study exploring the relationship of pro and antiangiogenic factors in preeclampsia compromised by HIV infection.

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Conflict of Interest

The authors report no conflict of interest.

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Table 1: Clinical Profile

	Normotensive Pregnant Women		Preeclamptic Pregnant Women		
	HIV negative (N-)	**HIV positive (N+)**	**HIV negative (P-)**	**HIV positive (P+)**	**P value**
n	20	21	19	16	
Maternal Age (yrs)	24(21-26)	27(24-30)	30(20-32)	31(25-33)	p=0.014*
Gestational age(wks)	38(38-39)	39(38-40)	39(37-40)	38(36-38)	p=0.046*
Birth weight (kg)	3.2(3.1-3.5)	3.4(2.9-3.8)	3.3(2.8-3.9)	2.8(2.7-3.5)	n/s
Parity	0 (1)	1 (1)	1 (2)	1 (1)	p=0.05
Maternal weight(kg)	70 (65-75)	75 (72-84)	73.7 (65.7-101.4)	75.5 (62.5-102.5)	n/s
Placental weight (g)	360(320-400)	400(345-475)	460(360-500)	460(350-510)	p=0.018*
Systolic BP(mmHg)	110 (107.3-119.8)	112 (107.5-124.5)	154 (151-162)	148 (145-154.5)	p<0.0001*
Diastolic BP (mmHg)	70(66.2-73.7)	70(67-75)	93(88-106)	95(85-98)	p<0.0001*

Summary statistics are presented as median and interquartile range, n=76

*p<0.05

Wks: weeks; kg: kilograms; g: grams

BP: blood pressure

Table 2

Table II: Comparison of pro and antiangiogenic factors of maternal serum across study groups

	Normotensive Pregnant Women		Preeclamptic Pregnant Women		
	HIV Negative (N-)	HIV Positive (N+)	HIV Negative (P-)	HIV Positive (P+)	P value
n	20	21	19	16	
sFlt-1(pg/ml)	12354(8261-14902)	8414(5490-14330)	15774(10328-21156)	11134(7848-17411)	p=0.02*
#sEng(ng/ml)	12.80(10.13-18.18)	9.68(5.78-15.65)	24.84 (11.12-30.75)	14.57(11.75-34.40)	p=0.001*
#PlGF(pg/ml)	334.2(181-651)	252.8(111-447.4)	171(22.35-382.8)	265(69.68-615.9)	ns

Summary statistics are presented as median and interquartile range, n=76

, n=76

Actual data shown and not log transformed data

*p<0.05

Nanograms (ng)

Picograms(pg)

Non significant (ns)

Figure 1

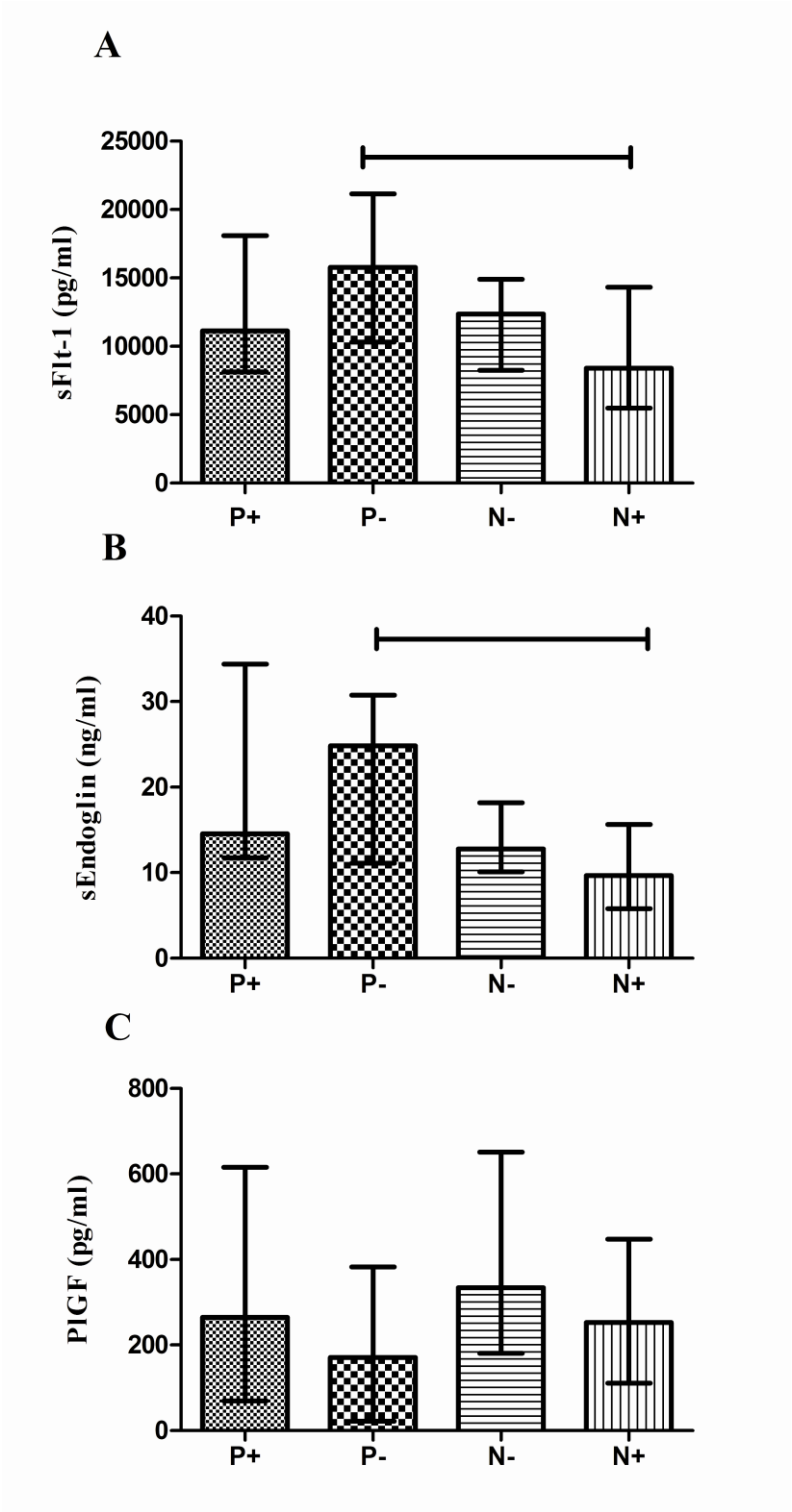

Fig.1 Anti- and proangiogenic serum concentrations (A-C, median and inter-quartile range): (A) sFlt1 (pg/ml) (B), sEng (ng/ml) and (C) PlGF (pg/ml) in HIV positive preeclamptic (P+); HIV negative preeclamptic (P-); HIV negative normotensive pregnant (N-) and HIV positive pregnant women (N+).

Figure 2

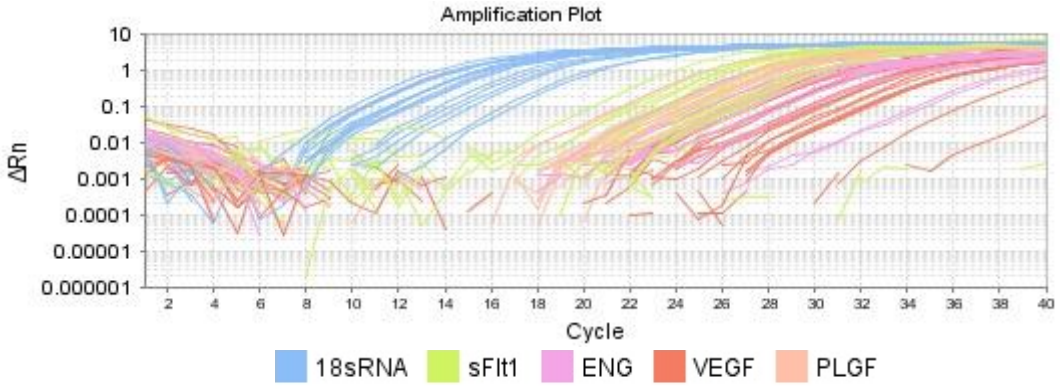

Fig.2 Representative Image of real time PCR showing the relationship between 18sRNA *vs* the proangiogenic and antiangiogenic factors

Figure 3

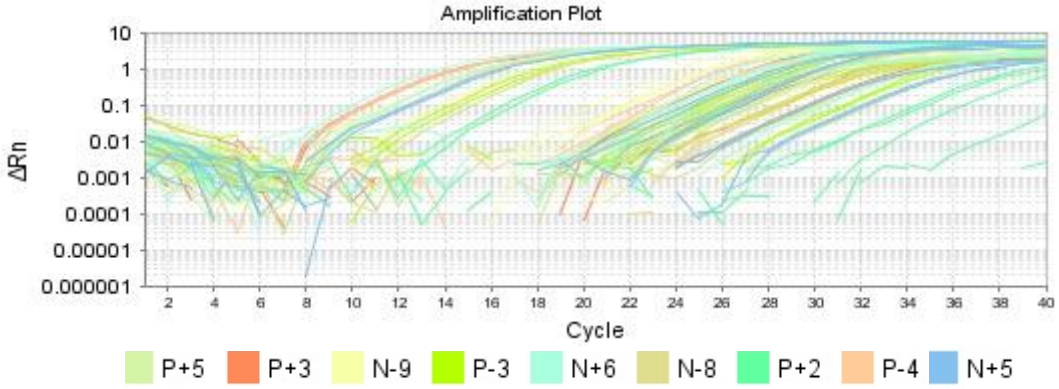

Fig.3 Representative Image of real time PCR showing the relationship between 18sRNA *vs* the proangiogenic and antiangiogenic factors between study groups

Figure 4

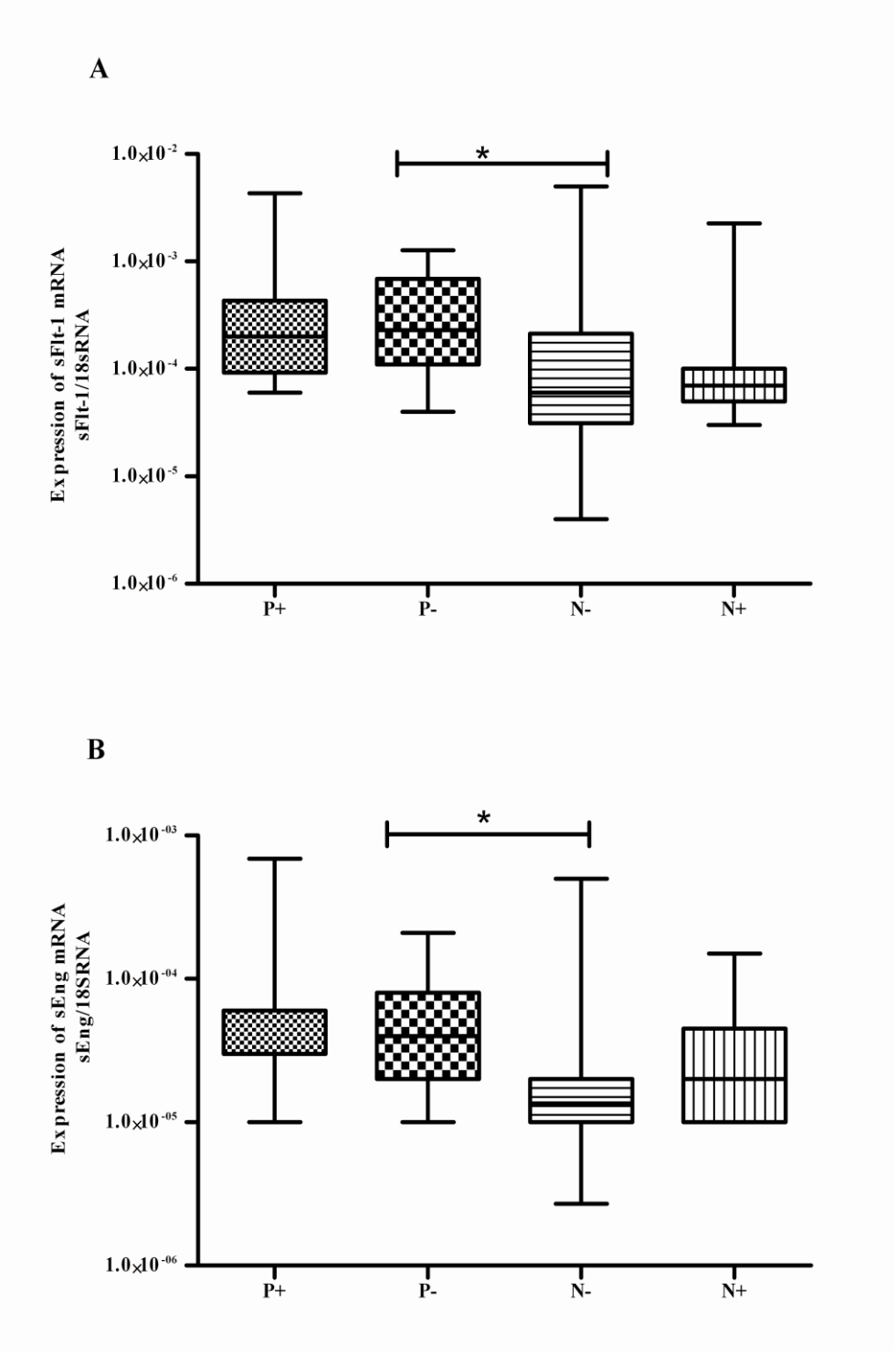

Fig.4 Anti-angiogenic placental concentrations (A-B, median and interquartile range): (A) sFlt-1/18S mRNA and (B) sEng /18S mRNA in HIV positive preeclamptic (P+); HIV negative preeclamptic (P-); HIV negative normotensive pregnant (N-) and HIV positive pregnant women (N+).

Figure 5

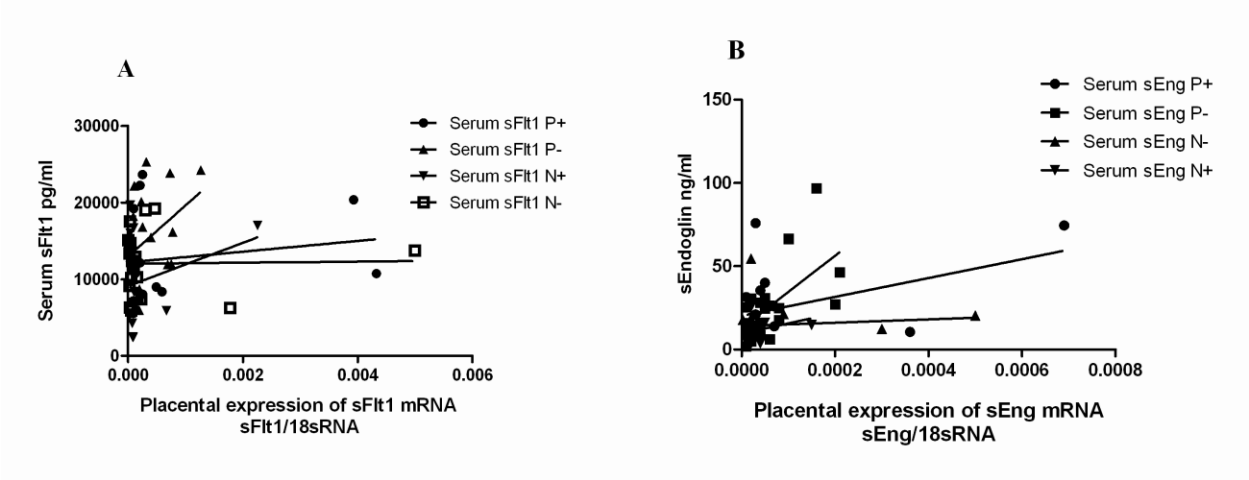

Fig.5 Correlation analyses (A-B) between serum and placental expression of (A) sFlt1 and (B) sEng within HIV positive preeclamptic (P+); HIV negative preeclamptic (P-); HIV negative normotensive (N-) and HIV positive normotensive (N+) pregnancies. Data has been log transformed to ensure normality between the groups.