PLACENTAL PROGESTERONE AND ITS RECEPTOR IN HIV-ASSOCIATED PRE-ECLAMPSIA

BY

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Submitted in partial fulfilment for the degree of

MASTER OF MEDICAL SCIENCE

in the

Discipline of Optics and Imaging

Doris Duke Medical Research Institute

Collage of Health Sciences

University of KwaZulu-Natal

Durban, South Africa

2022

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, Doris Duke Medical Research Institute, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa under the supervision of Professor T. Naicker and co-supervision of Shoohana Singh.

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DECLARATION

I, **Serisha Azaria Sewnarain** declare that:

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

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

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Signed: _____ **Date:** 18/11/2022

DEDICATION

To El Shaddai

For the nourishment of my soul and the firm foundation I stand upon.

"The Lord is my rock, my fortress, and my saviour" Psalm 18:1-3.

To my parents

Thank you for your unwavering support and infinite love. To my mother, you are my source of inspiration and strength. To my father, your words of encouragement and guidance have propelled me forward.

ACKNOWLEDGEMENTS

The completion of this dissertation would not be possible without:

- My supervisor, Professor Thajasvarie Naicker, for her invaluable guidance, feedback and support. Thank you for always striving for the best, and helping your students reach their full potential.
- My co-supervisor, Ms. Shoohana Singh. Your patience, continuous assistance and motivation is deeply appreciated.
- Mr Braden Pillay, Miss Sayantha Padayachee and Miss Rashmika Madaree, your friendship is my sheltering tree. Thank you for the encouragement throughout this project.
- My grandparents for guiding me with their wisdom and insight.
- The staff and students at the Optics and Imaging Centre.
- My family and friends, you have woven a tapestry of love and support.

FUNDING

This study was funded by the publication award of Professor T. Naicker and student funding was provided by the National Research Foundation.

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LIST OF ABBREVIATIONS

Acquired Immunodeficiency Syndrome	AIDS
Antiretroviral therapy	ART
Combined antiretroviral therapy	cART
Co-regulators	CR
Coronavirus disease	COVID-19
C-reactive protein	CRP
c-Jun N-terminal kinases	JNK
Cyclic adenosine monophosphate	cAMP
Cytochrome P450 family 11 subfamily A member 1	CYP11A1
Diacylglycerol	DAG
Deoxyribonucleic acid	DNA
Early onset pre-eclampsia	EOPE
Extracellular signal related kinases	ERK
Fibrinoid necrosis	FN
GATA-binding factor 2	GATA2
Heat shock protein	HSP
Haemolysis, elevated liver enzyme levels, and low platelet levels	HELLP
Hepatitis E virus	HEV
Highly active antiretroviral therapy	HAART
Human growth hormone	hGH
Human immunodeficiency virus	HIV
Human placental lactogen	hPL
Immunoglobulin G	IgG
Inositol 1,4,5-trisphosphate	IP3
Insulin like growth factor 2	IGF2
Interleukin	IL
Janus kinase	JAK
Killer cell immunoglobulin-like receptor 2DL4	KIR2DL4
Late-onset of Preeclampsia	LOPE
Mitogen-activated protein kinase	MAPK
Membrane progesterone receptor	mPR
Messenger ribonucleic acid	mRNA

Myosin light chain phosphorylation	p-MLC
Natural killer	NK
Neutrophil extracellular traps	NETs
Nuclear progesterone receptor	nPR
Phosphoinositide 3-kinase	P13K
Phospholipase C-γ	PLC-γ
Placental growth factor	PIGF
Placental protein 13	PP13
Pre-eclampsia	PE
Progesterone	P
Progesterone receptor	PR
Progesterone response element	PRE
Protease inhibitor	PI
Protein kinase B	AKT
Proto-oncogene tyrosine-protein kinase Src	Src
Rapidly accelerated fibrosarcoma	Raf
Rat sarcoma	Ras
Ribonucleic acid	RNA
Soluble endoglin	sENG
Soluble fms-like tyrosine kinase 1	sFlt-1
South Africa	SA
Signal transducer and activator of transcription	STAT
StAR Related Lipid Transfer Domain Containing 3	STARD3
Steroid receptor coactivator-2	SRC2
Stress-activated protein kinases	SAPK
Syncytial knot	SK
Syncytiotrophoblast	STB
Tumour necrosis factor	TNF
Vascular endothelial growth factor	VEGF
Vascular endothelial growth factor A	TGF- β1

ABSTRACT

Background: The maintenance of a healthy pregnancy is dependent upon the placental production of progesterone, which interacts with progesterone receptors (PR) to stimulate trophoblast invasion. Pre-eclampsia (PE) is associated with defective trophoblast invasion, and due to the high prevalence of HIV infection and pre-eclampsia in South Africa, this study examined the expression of placental progesterone and PR in HIV-infected women with PE.

Methods: Placental tissue from 180 women were grouped into normotensive (N) (n = 60) and PE (n =120). The PE group was further stratified by gestational age into early-onset pre-eclampsia (EOPE) and late-onset pre-eclampsia (LOPE) (n = 60 per group). Both normotensive and PE groups were stratified by HIV status (HIV positive+ and HIV negative-) into N- (n=30), N+ (n=30), EOPE- (n=30), EOPE+ (n=30), LOPE- (n=30) and LOPE+ (n=30). Immunohistochemistry and morphometric image analysis were used to assess placental progesterone and PR immuno-expression in exchange and conducting villi. The Mann Whitney test was used to compare the effects of pregnancy type (normotensive vs. pre-eclamptic), HIV status (HIV+ vs. HIV-) and PE subtype (EOPE vs LOPE). For comparative analysis across all six study groups, a one-way ANOVA non-parametric Kruskal-Wallis test was used, followed by Dunn's Multiple Comparisons test. A two-way ANOVA was used to compare villi type (exchange vs conducting) and pregnancy type.

Results: Progesterone was immunoexpressed within endothelial, mesenchymal and trophoblast cells within conducting and exchange villi whilst PR was mainly expressed on cytotrophoblasts and syncytiotrophoblasts. Progesterone and PR immuno-expression in exchange villi were significantly lower in the following groups: PE compared to the normotensive group (p = <0.0001 and p = <0.0001, respectively) and EOPE compared to the LOPE group (p = <0.0001 and p = <0.0001). Progesterone immuno-expression in the HIV+ group compared to the HIV- group was significantly lower (p = <0.0001), whilst PR expression was non-significant (p = 0.4291).

Progesterone and PR immuno-expression in conducting villi were downregulated in the following groups: EOPE group compared to the LOPE group (p = <0.0001 and p = <0.0001) and in the HIV+ group compared to the HIV- group (p = <0.0001 and p = 0.0009). Progesterone immuno-expression was higher in the PE group compared to normotensive (p = 0.0326) and PR immuno-expression was non-significant (p = 0.6935).

There was a significant difference in progesterone and PR in exchange vs conducting villi (p = <0.0001 and p = <0.0001, respectively) and villi type accounted for 34.47% and 15.28% of total variance for progesterone and PR, respectively.

Conclusion: This study observed a reduction in progesterone and PR immunoexpression in the exchange villi of pre-eclamptic placenta. Progesterone and PR immuno-expression were also significantly reduced in HIV+ placentas, with the EOPE+ group displaying the lowest immuno-expression. We postulate that HIV infection combined with cART may cause mitochondrial dysfunction that compromises progesterone synthesis. Progesterone deficiency results in minimal binding to PRs, which affects signalling pathways (PI3K/AKT, JAK-STAT, and MAPK cascades) and impairs trophoblast invasion. Notably, the EOPE group has the lowest immuno-expression of progesterone and PRs which links the downregulation of progesterone to defective placentation. This study links HIV infection to reduced progesterone production during pregnancy and associates decreased progesterone and PR immuno-expression with PE.

OKUFINGQIWE

Isendlalelo: Ukugcinwa kokukhulelwa okunempilo kuncike ekukhiqizweni kwe-placenta ye-progesterone, esebenzisana nama-progesterone receptors (PR) ukuze kuvuse ukuhlasela kwe-trophoblast. I-Pre-eclampsia (PE) ihlotshaniswa nokuhlasela kwe-trophoblast enesici, futhi ngenxa yokusabalala okuphezulu kokutheleleka nge-HIV kanye ne-pre-eclampsia eNingizimu Afrika, lolu cwaningo luhlole ukuvezwa kwe-placental progesterone kanye ne-PR kwabesifazane abane-HIV abane-PE.

Izindlela: Izicubu ze-placental ezivela kwabesifazane abangu-180 zahlanganiswa zaba yi-normotensive (n = 60) ne-PE (n = 120). Iqembu le-PE laphinde lahlukaniswa ngeminyaka yokukhulelwa yaba yi-PE yokuqala kanye ne-PE yokufika sekwephuzile (n = 60 iqembu ngalinye). Womabili amaqembu e-normotensive kanye ne-PE ahlukaniswa ngesimo se-HIV (HIV+ ne-HIV negative-) aba yi-N- (n=30), N+ (n=30), EOPE- (n=30), EOPE+ (n=30), LOPE -(n=30) kanye ne-LOPE+ (n=30). I-Immunohistochemistry kanye nokuhlaziywa kwesithombe se-morphometric kwasetshenziselwa ukuhlola i-placenta progesterone kanye ne-PR immuno-expression ekushintsheni nasekuqhubeni i-villi.

Imiphumela: I-progesterone yayingabonakali ngaphakathi kwamaseli e-endothelial, mesenchymal kanye ne-trophoblast ngaphakathi kokuqhuba nokushintshanisa i-villi ngenkathi i-PR iboniswa ikakhulukazi kuma-cytotrophoblasts nama-syncytiotrophoblasts. I-progesterone ne-PR immuno-expression ekushintsheni i-villi yayiphansi kakhulu kumaqembu alandelayo: I-PE uma iqhathaniswa neqembu le-normotensive (p = <0.0001 kanye ne-p = <0.0001, ngokulandelana) kanye ne-EOPE uma kuqhathaniswa neqembu le-LOPE (p = <0.0001 kanye p = <0.0001). I-progesterone immuno-expression eqenjini le-HIV+ uma iqhathaniswa neqembu le-HIV yayiphansi kakhulu (p = <0.0001), kuyilapho inkulumo ye-PR yayingabalulekile (p = 0.4291).

I-progesterone ne-PR immuno-expression ekuqhubeni i-villi yehlisiwe emaqenjini alandelayo: Iqembu le-EOPE uma liqhathaniswa neqembu le-LOPE (p = <0.0001 kanye ne-p = <0.0001) naseqenjini le-HIV+ uma liqhathaniswa neqembu le-HIV (p = <0.0001 futhi p = 0.0009). I-progesterone immuno-expression yayiphezulu eqenjini le-PE uma kuqhathaniswa ne-normotensive (p = 0.0326) kanye ne-PR immuno-expression yayingabalulekile (p = 0.6935).

Kunomehluko omkhulu ku-progesterone ne-PR ekuhwebeni ngokuqhudelana nokuqhuba i-villi (p = <0.0001 kanye ne-p = <0.0001, ngokulandelana) kanye nohlobo lwe-villi lubalelwa ku-34.47% no-15.28% wokuhluka okuphelele kwe-progesterone ne-PR, ngokulandelana.

Isiphetho: Lolu cwaningo lubone ukuncipha kwe-progesterone kanye ne-PR immunoexpression ku-villi yokushintshanisa ye-pre-eclamptic placenta. I-progesterone ne-PR immuno-expression nazo zehliswa kakhulu kuma-placenta e-HIV+, neqembu le-EOPE+ libonisa ukubonakaliswa okuphansi kokuzivikela komzimba. Sibeka umbono wokuthi ukutheleleka nge-HIV kuhlanganiswe ne-cART kungase kubangele ukungasebenzi kahle kwe-mitochondrial okuphazamisa ukwakheka kwe-progesterone. Ukuntuleka kwe-progesterone kubangela ukubophezela okuncane kuma-PRs, okuthinta izindlela zokubonisa (PI3K/AKT, JAK-STAT, kanye ne-MAPK cascades) futhi kulimaze ukuhlasela kwe-trophoblast. Ngokuphawulekayo, iqembu le-EOPE line-immuno-expression ephansi kakhulu ye-progesterone kanye ne-PRs exhumanisa ukulawulwa kwe-progesterone nokuzala okungalungile. Lolu cwaningo luxhumanisa ukutheleleka nge-HIV nokuncipha kokukhiqizwa kwe-progesterone ngesikhathi sokukhulelwa kanye nokuhlotshaniswa nokuncipha kwe-progesterone kanye ne-PR immuno-expression ne-PE.

CHAPTER 1

BACKGROUND AND LITERATURE REVIEW

1.1. Human Immunodeficiency Virus Infection and Acquired Immunodeficiency Syndrome

Human immunodeficiency virus (HIV) infection is a significant public health concern, as approximately 37.7 million people worldwide are infected (UNAIDS, 2021). HIV is an enveloped retrovirus which primarily infects CD4+ T cells of the immune system, leading to a loss of cell-mediated immunity and the development of acquired immunodeficiency syndrome (AIDS). The transmission of HIV occurs through sexual contact with mucosal surfaces, percutaneous inoculation and maternal-infant exposure during pregnancy, childbirth and breastfeeding (Shaw and Hunter, 2012). The initial period of HIV infection is known as acute HIV, and symptoms such as fever, inflammation of lymph nodes and sores around the genitals/mouth are present. The non-specific symptoms of acute HIV, followed by the latency period, result in a large number of undetected HIV infections thus approximately 6.1 million infected individuals are unaware of their HIV-positive status (UNAIDS, 2021).

Women are notably affected by the epidemic, as women and girls constitute 63% of all new HIV infections in sub-Saharan Africa (UNAIDS, 2021). This could be attributed to a larger mucosal surface area and cervical ectopy, which allows for higher exposure of pathogens to target cells in the vaginal cavity (Ramjee and Daniels, 2013). Physiological changes during pregnancy make women more susceptible to HIV; and women who begin antiretroviral therapy (ART) while pregnant are at a greater risk of losing track of their treatment, resulting in difficulty in re-engaging with routine HIV care after birth (Knettel *et al.*, 2018; Rotheram-Borus *et al.*, 2015; Thomson *et al.*, 2018). Furthermore access to health care was compromised during the COVID-19 pandemic (Pant *et al.*, 2020; Pillay *et al.*, 2021).

The 2019 Antenatal HIV Sentinel Survey (ANCHSS) showed an unprecedented 30% HIV prevalence among pregnant women in South Africa (SA) (Woldesenbet *et al.*, 2021). The KwaZulu-Natal (KZN) province has reported the highest HIV prevalence rate in SA for the past five years, with a staggering 40.9% HIV prevalence among pregnant women (Woldesenbet *et al.*, 2021). HIV infection and hypertensive diseases are amongst the leading causes of maternal mortality in SA (StatsSA, 2017). These figures raises concern for the impact of hypertensive disorders such as pre-eclampsia (PE) in HIV-infected pregnant women.

1.2. Pre-eclampsia

1.2.1. Definition and Symptoms

Pre-eclampsia is characterised by a blood pressure of ≥140/90mmHg with or without proteinuria of ≥300 mg, occurring after 20 weeks' gestation (Magee *et al.*, 2022). In the absence of proteinuria, PE

may be diagnosed by the presence of symptoms such as multiple organ dysfunction (renal, hepatic and neurological), thrombocytopenia (less than 100,000/mm^3), pulmonary oedema and utero-placental dysfunction (fetal growth restriction or altered umbilical Doppler ultrasonography) (Ramos *et al.*, 2017; Brown *et al.*, 2018). For patients with pre-existing hypertension, the aforementioned criteria is used to diagnose PE, in conjunction with worsening baseline blood pressure (BP) and proteinuria (Ramos *et al.*, 2017). Unattended PE can lead to seizures (eclampsia), with associated fetal bradycardia, intrauterine growth restriction, low birth weight or placental complications (Gruslin and Lemyre, 2011; Aabidha *et al.*, 2015). The preventative management for PE and eclampsia include aspirin and magnesium sulphate, however the only known treatment for PE is delivery of the neonate and the placenta (Atallah *et al.*, 2017; Smith *et al.*, 2013).

1.2.2. Subtypes of Pre-eclampsia

Pre-eclampsia may be classified into 2 subtypes: early onset pre-eclampsia (EOPE) which presents with clinical symptoms occurring before 34 weeks of gestation whilst late onset pre-eclampsia (LOPE), presents with clinical symptoms after 34 weeks of gestation (Marín *et al.*, 2020). Early onset PE and LOPE share the symptoms of PE, however, they have different prognoses and pathologies (Guo et al., 2021b). Early onset PE is associated with defective placentation that result in inadequate blood supply to meet the demands of the fetus thus causing restricted fetal growth. Late onset PE emanates from an imbalance between fetal metabolic demands and maternal supply (Valensise *et al.*, 2008; Raymond and Peterson, 2011; Marín *et al.*, 2020). EOPE is the rarer subtype, and occurs in 5-20% of PE cases, however it is more severe and is responsible for majority of mother and child related morbidity and mortality (Chappell *et al.*, 2008; Aplin *et al.*, 2020).

1.2.3. Epidemiology

Globally, PE affects approximately 2–8% of pregnancies each year, whilst the overall incidence of hypertensive disorders of pregnancy has increased by 10.92% from 1990 to 2019 (Say *et al.*, 2014; Wang *et al.*, 2021). The World Health Organization (WHO) has reported that the incidence of PE in developing countries is significantly higher compared to developed countries (World Health, 2019; Wang *et al.*, 2021). In sub-Saharan African countries, PE and eclampsia are amongst the leading causes of maternal and fetal morbidity and mortality (Alkema *et al.*, 2016). In South Africa, maternal deaths emanating from hypertensive disorders of pregnancy, in particular, the subtypes PE and eclampsia account for 17% of deaths during the period 2017-2019 (Moodley, 2020). Another South African study with 1547 pre-eclamptic women reported 1% and 21% of maternal and perinatal deaths, respectively (Nathan et al., 2018). Compared to other regions, Sub-Saharan Africa had a higher pooled prevalence of hypertensive disorders of pregnancy, which highlights the importance of PE research (Gemechu et al., 2020).

1.2.4. Risk factors

A study based in sub-Saharan Africa identified several risk factors associated with PE development, such as family or prior history of PE, obesity, advanced age, anaemia and chronic hypertension (Meazaw *et al.*, 2020). Elevated levels of inflammatory markers (CRP, IL-6, IL-8, IL-10 and TNFα) are associated with PE development, which could be due to reduced plasma volume and PE being a manifestation of immune rejection (Black and Horowitz, 2018; Bansal et al., 2018). Additionally, the risk of developing PE is increased by HIV infection and antiretroviral therapy (ART) such as highly active antiretroviral therapy (HAART) (Sansone *et al.*, 2016).

1.2.5. HIV-associated Pre-eclampsia

The association between HIV infection and pre-eclampsia (PE) are conflicting, indicating opposing risk for PE development. The incidence of PE has been reported to be lower in HIV infected people (Kalumba *et al.*, 2013; Sebitloane *et al.*, 2017), whilst others state that HIV infection is a risk factor for hypertensive disorders of pregnancy such as PE (Machado *et al.*, 2014; Sansone *et al.*, 2016). HIV infection aggravates the inflammatory response of the immune system, and results in endothelial dysfunction (Naidoo *et al.*, 2021). Similarly, endothelial dysfunction and an exaggerated immune response causes the maternal syndrome of PE (Rana *et al.*, 2019). Parallels between HIV infection and PE are also observed through the elevation of placental neutrophils and neutrophil extracellular traps (NETs) (Moodley *et al.*, 2020b; Moodley *et al.*, 2020a). However, this event occurs individually as women with both HIV infection and PE have suppressed NETS, which has been attributed to ART usage (Moodley *et al.*, 2020b). The increased risk of PE development in HIV-positive women has been attributed to highly active antiretroviral treatment/therapy (HAART) that re-instates the immune response whilst preventing viral replication and mother-to-child transmission (Sansone *et al.*, 2016). Reports have suggested that HAART increases the risk of PE by immune restoration, or by a direct toxic effect on the liver which impairs the synthesis of retinal-binding proteins, resulting in decreased serum retinol levels in PE (Maharaj *et al.*, 2017; Mawson, 2003). Additionally, the use of ARTs such as protease inhibitor (PI)–based combination antiretroviral therapy (cART) in HIV pregnancies, is associated with decreased progesterone levels, which could induce detrimental placental vascular changes, thus exacerbating PE development (Papp *et al.*, 2014; Mohammadi *et al.*, 2018).

1.2.6. Pathogenesis

The pathogenesis of PE is largely associated with the placenta, as its delivery resolves the disease (Roberts and Hubel, 2009; Phipps *et al.*, 2016). Animal and human studies have indicated that poor trophoblastic invasion with utero-placental ischemia leads to PE development. PE is often considered a two-stage disease (Makris *et al.*, 2007; Gilbert *et al.*, 2007; Palei *et al.*, 2013) where Stage I is characterised by abnormal placentation due to incomplete myometrial spiral artery remodelling and

Stage II is distinguished by excessive release of anti-angiogenic factors from the ischemic placenta into systemic circulation (Figure 1.1) (Phipps *et al.*, 2019).

During the implantation stage of normotensive pregnancies, extravillous trophoblast cells invade the uterus and by adopting an endothelial phenotype induce the transformation of spiral arteries into wide bore conduits. However, in PE, there is shallow interstitial invasion of extravillous trophoblasts and an absence of physiological transformation of myometrial spiral arteries (Rana *et al.*, 2019; Marín *et al.*, 2020). Consequentially, the ischemic placenta releases angiogenic regulators and pro-inflammatory cytokines which cause endothelial injury, inflammation and end-organ damage (Rana et al., 2019). The pathophysiology of PE are also attributed to disorders of hormone homeostasis, as decreased levels of vasodilatory hormones such as progesterone and estradiol promote increased vascular reactivity, resulting in hypertension (Zhorzholadze *et al.*, 2006). Dilation of proximal arteries are mediated through oestrogen and placental growth factor, which are decreased in pre-eclamptic pregnancies, indicative of the hormonal impact on the pathophysiology of PE (Chau *et al.*, 2017; Shu *et al.*, 2021).

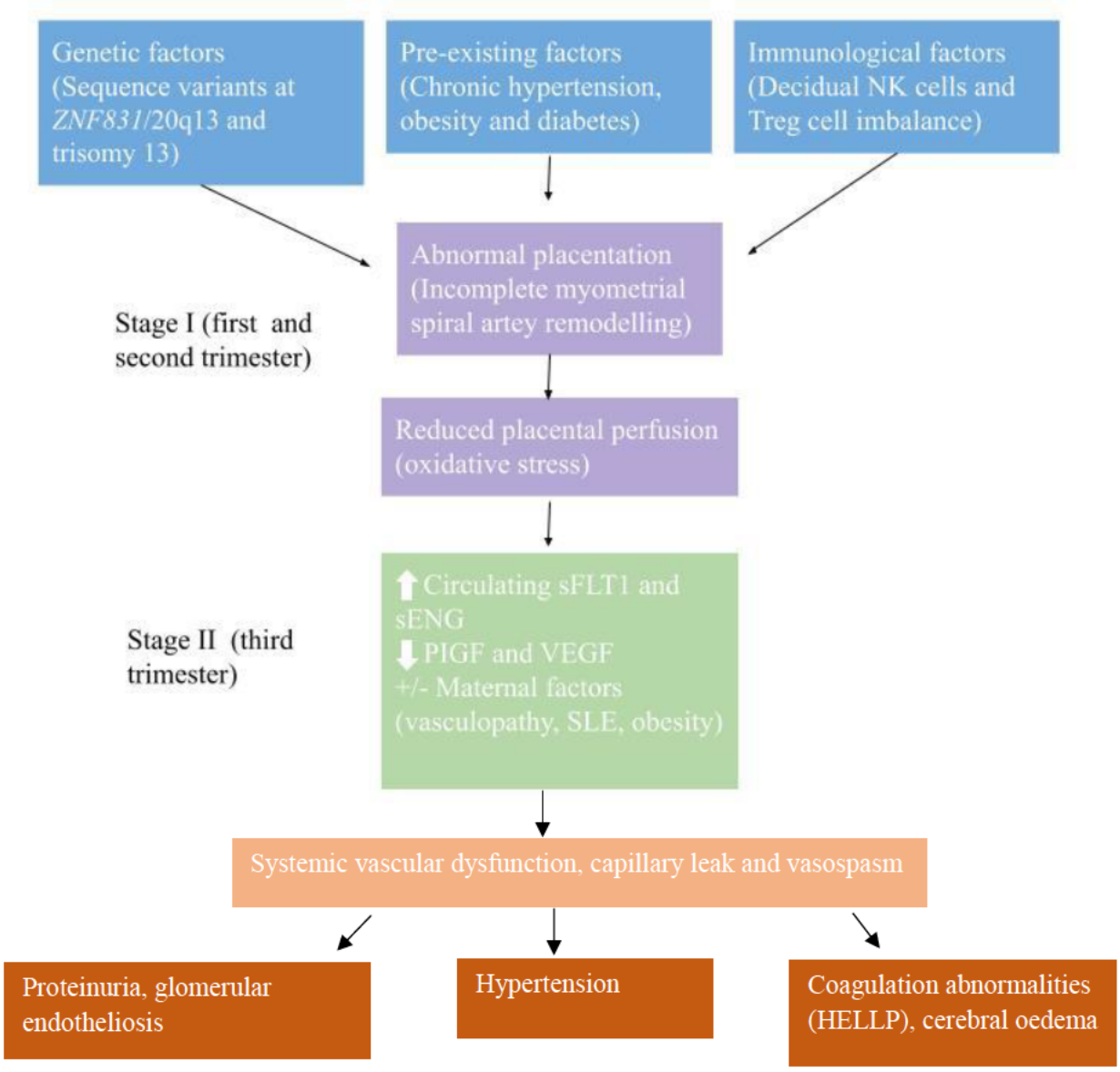


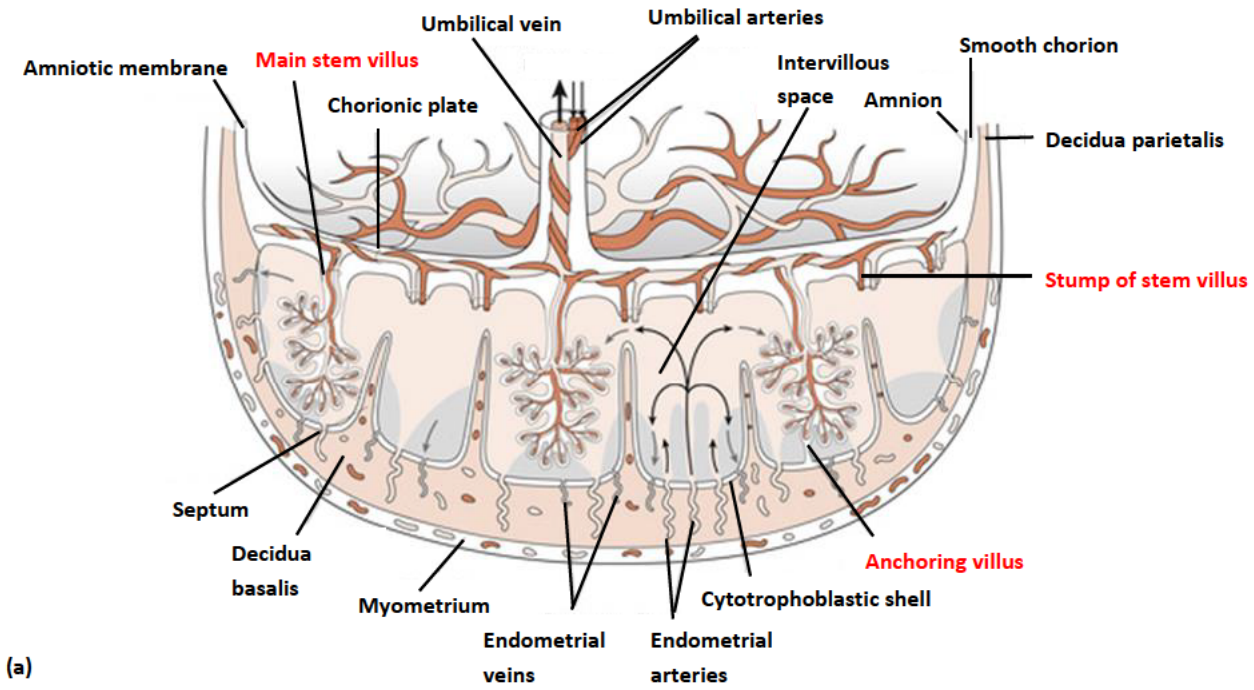
Figure 1.1. Schematic diagram of pre-eclampsia pathogenesis. Adapted from (Phipps *et al.*, 2019). Stage I of PE is caused by genetic factors, pre-existing maternal factors and immunological factors. Stage II occurs after the release of antiangiogenic factors: soluble tyrosine kinase-1 (sFlt-1) and soluble endoglin (sENG). There is a decrease in placental growth factor (PlGF) and vascular endothelial growth factor (VEGF). This may result in haemolysis, elevated liver enzymes and low platelet count (HELLP) syndrome.

1.3. The Placenta

The placenta is the first fetal organ to develop and is connected to the fetus by the umbilical cord. Its many functions include (i) nutrient and gas exchange between the fetus and mother, (ii) providing immunity via the transfer of immunoglobulins from mother to fetus and (iii) the synthesis of hormones for fetal development (Chatuphonprasert *et al.*, 2018; Kapila and Chaudhry, 2021).

1.3.1. Structure of the human placenta

The placenta is a disc-shaped organ that can reach a weight of 500g at birth, with a diameter of 15-20 cm and a width of 2-3 cm (Figure 1.2) (Carlson, 2014). The amniotic membrane on the fetal side of the placenta creates a glossy appearance (Carlson, 2014). The maternal side is sub-divided into 35 lobes which are separated by placental septa. Within each lobe are cotyledons which are comprised of a primary stem villus and its branches. The chorionic villus is the basic structural unit of the placenta, and arises from the chorion (Griffiths and Campbell, 2014). The chorion is a double-layered membrane, consisting of the outer syncytiotrophoblast and inner cytotrophoblast. The villi reach into the intervillous space, and are supplied with maternal blood via spiral arteries. The difference in pressure within the spiral arteries and intervillous space is drastically different, with pressure in the spiral arteries reaching 70 mm Hg, and the intervillous space having a pressure of only 10 mm Hg (Griffiths and Campbell, 2014).



(a)

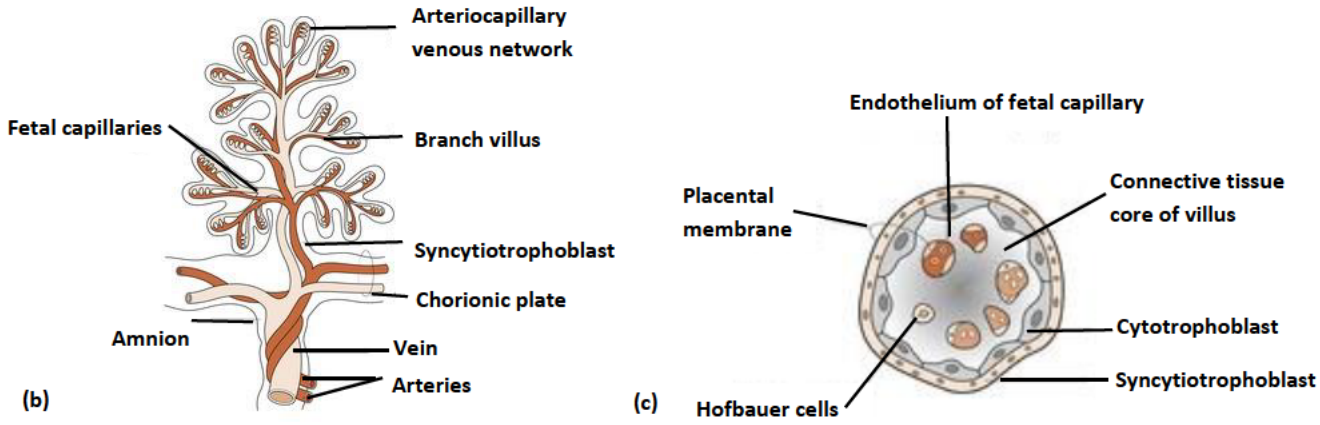


Figure 1.2. Drawings of (a) full term placenta, (b) stem chorionic villus and (c) section of branch villus. Adapted from (BasicmedicalKey, 2016). (a) Transverse section of full-term placenta. Placental septa separates cotyledons, which are comprised of two or more stem villi and their branches. (b) The arteries of the stem chorionic villus carry deoxygenated fetal blood from the fetus, and the vein delivers oxygenated blood to the fetus. (c) The syncytiotrophoblast secretes estrogen and progesterone, and the cytotrophoblast is a proliferative epithelial monolayer.

There two main types of villi are stem and terminal villi. Stem villi are connected to the chorionic plate and consist of condensed fibrous stroma with large vessels (Dunk *et al.*, 2020). During the later stages of gestation, the trophoblastic layer of stem villi are converted to fibrin-type fibrinoid. Stem villi function as support structures of the villous trees, and are connected to terminal villi via intermediate structures (Wang, 2010). The terminal villi are grape-like and are the functional unit of the placenta, where diffusive gas exchange occurs. The terminal villi are smaller with a discontinuous cytotrophoblast layer and 4–6 fetal capillaries, visible in a cross section (Wang, 2010).

1.3.2. Physiology and functions of the placenta

The placenta is solely responsible for gas exchange to the fetus *in utero*. Nutrient and gas exchange occurs due to the perfusion of maternal blood into the intervillous placental space, via active or passive transport (Wright *et al.*, 2011). The placenta also functions as a selective barrier, which metabolises substances and protects the fetus against pathogens. Maternal IgG antibodies cross the placental barrier by pinocytosis and provide passive immunity for the fetus (Palmeira *et al.*, 2012).

A fascinating aspect of the placenta is its endocrine function, which is the synthesis and secretion of peptide and steroid hormones. Important hormones secreted by the placental syncytiotrophoblast include human placental lactogen (hPL) and placental growth hormone (hGH), which collectively stimulate insulin-like growth factor production and promotes gluconeogenesis (Griffiths and Campbell, 2014; Pylypchuk and Pylypchuk, 2021). In addition, hPL stimulates breast development for lactation

(Gude *et al.*, 2004). The glycoprotein hormone, human chorionic gonadotropin (hCG) is the first hormone produced by the placenta, and stimulates the corpus luteum to produce progesterone, until the 8th week of gestation (Theofanakis *et al.*, 2017). Thereafter, the placenta secretes oestrogen and progesterone. Oestrogen stimulates mammary development, promotes uterine growth and increases bloody supply via vasodilation (Griffiths and Campbell, 2014). Progesterone is also essential for preventing uterine contractions and for ensuring pregnancy success.

1.4. Progesterone

1.4.1. Structure and Biosynthesis

Progesterone is an endogenous steroid and sex hormone that is produced by the adrenal cortex, gonads and placenta (Cable and Grider, 2020). Circulating progesterone has a relatively short half-life of five minutes and is bound to cortisol-binding globulin and serum albumin (Taraborrelli, 2015). The structure of progesterone is composed of four carbon rings, with hydrogen and oxygen molecules. Progesterone has a molar mass of 314.469 g/mol (grams per mole), with the molecular formula $C_{21}H_{30}O_2$ (Allen and Wintersteiner, 1934; Ali et al., 2020). The hormone is synthesised from a cholesterol-derived precursor molecule called pregnenolone.

The synthesis of progesterone begins in the mitochondria, where cholesterol is transported from the outer to inner mitochondrial membrane (Miller, 2017). The process is mediated by the steroidogenic acute regulatory (StAR) protein, however, StAR is not present in the placenta. The placenta uses StAR related lipid transfer domain containing 3, (STARD3) and heat shock protein (HSP) to import cholesterol into the mitochondria (Tuckey, 2005; Monreal-Flores *et al.*, 2017). Thereafter, cholesterol is hydroxylated at two positions (C-20 and C-22) and the chain is cleaved off by cytochrome P450scc (CYP11A1) (Slominski *et al.*, 2015). Cholesterol is converted to pregnenolone, the precursor of progesterone, by the rate-limiting enzyme CYP11A1. The conversion of pregnenolone to progesterone is catalysed by type 1 3β-hydroxysteroid dehydrogenase (3β-HSD1) in the mitochondrion (Fig 1.5) (Tuckey, 2005). The expression of 3β-HSD1 is restricted to the placental multinucleated syncytiotrophoblast (STB), and 3β-HSD2 is expressed in the adrenal gland and gonads (Thomas *et al.*, 2015). Progesterone synthesis is regulated by endogenous hormones such as estradiol, insulin, insulin-like growth factor, calcitriol, leptin, and corticotropin-releasing hormone (Costa, 2016).

1.4.2. Functions of Progesterone

Progesterone is an important hormone in the uterine cycle, as it prepares the uterus for implantation by promoting endometrial conversion to the secretory stage (Taraborrelli, 2015). Menstruation occurs when there is no fertilization and there is a decrease in progesterone. However, in the event of fertilization and implantation, the corpus luteum continues to produce progesterone which stimulates the growth of blood vessels supplying the endometrium. Thereafter, the progesterone produced by the

placenta maintains a healthy pregnancy by stimulating the endometrium to secrete nutrients for the embryo, decreasing myometrial contractions and inhibiting lactation (Raghupathy and Szekeres-Bartho, 2022). One of the triggers for lactation is the decrease in progesterone levels after delivery. A decrease in progesterone early on in pregnancy results in increased myometrial contractility, leading to an increased risk of miscarriage (Cable and Grider, 2020; Arab *et al.*, 2019). Progesterone also suppresses the mother's immunological response to fetal antigens throughout pregnancy, preventing the fetus from being rejected.

1.4.3. Mechanism of Action

The steroid nature of progesterone denotes that its mechanism of action is similar to other steroid hormones, such as estrogen and glucocorticoids, which interact with intracellular receptors (Taraborrelli, 2015). Progesterone is lipophilic, and can easily traverse a target cell's membrane and binds to its receptors. This progesterone-receptor complex subsequently travels to the nucleus and binds to DNA, specifically near enhancer regions of genes that contain hormone response elements (Fedotcheva, 2021). Transcription is enhanced or repressed by the attachment of the complex to the promoter, which in turn affects protein synthesis.

1.5. Progesterone Receptor

1.5.1. Structure

Progesterone receptor (PR), or NR3C3, is a nuclear hormone receptor which is activated by the steroid hormone progesterone. It is primarily expressed in female reproductive tissues such as the uterus, ovaries and mammary glands, as well as the central nervous system (Grimm *et al.*, 2016). Upon binding to progesterone, PR regulates the development, differentiation, and proliferation of target tissues and is implicated in endocrine-based cancers (Figure 1.3) (Grimm *et al.*, 2016). PR consists of a central DNA binding domain, a carboxyl-terminal ligand-binding domain and an N-terminal domain which can be modified pro-translationally (Scarpin *et al.*, 2009; Beato *et al.*, 2020). The activation and inhibitory functional elements of PR enhance and repress transcriptional activation with the assistance of co-regulators. The two isoforms of PR, PR-A and PR-B, are encoded by alternative transcription initiation of a single PGR gene, and the latter is considered the complete isoform as PR-A lacks 164 amino acids at the N-terminus (Gadkar-Sable *et al.*, 2005; Scarpin *et al.*, 2009). The third isoform, PR-C is found copiously in myometrial tissue and lacks DNA-binding abilities however, it can bind to its own ligand and dimerize with other receptors (Condon *et al.*, 2006).

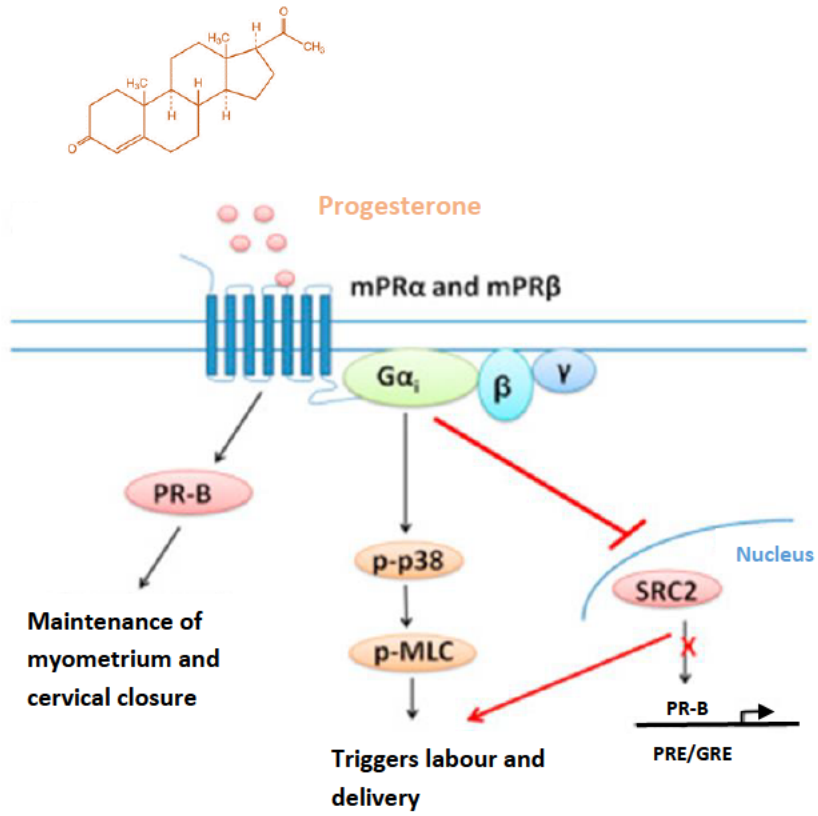


Figure 1.3. Schematic representation of interaction between progesterone and progesterone membrane receptors (mPRs). Adapted from (Valadez-Cosmes *et al.*, 2016). In early pregnancy, progesterone activates mPRα and mPRβ, which induces transactivation of intracellular PR-B, promoting cervical closure and myometrial quiescence. Contrarily, the onsent of labour is associated with increased expression of mPRα, which activates myosin light chain phosphorylation (p-MLC) (through p38 signaling). This results in the downregulation (red lines) of SRC2 (PR-B coactivator), which initiates labor and delivery.

1.5.2. Modes of action

The result of cellular activities, such as direct control of gene expression or activation of signalling cascades, is commonly used to classify progesterone-dependent signalling as genomic or non-genomic.

1.5.2.1. Direct Genomic Signalling

The classical mechanism of progesterone signalling is via direct genomic signalling. The progesterone receptor controls transcription by binding to specific progesterone response elements (PREs) found in the promoter region of target genes. Progestin agonists bind to PR, which induce conformational changes in the receptor structure. The conformational changes promote the dissociation of HSP and allows for receptor dimerization. The receptor-ligand complex enters the nucleus where it binds to the hormone response element DNA site and interacts with coactivator proteins by distinct activation domains (located on the amino- and carboxy-terminal regions of the receptor) (Conneely *et al.*, 2002). The co-activators induce the formation of productive transcription initiation complexes at the receptor

responsive promoter via chromatin remodelling and bridging with general transcription factors. Conversely, the binding of antagonists initiates receptor conformational changes which inhibit binding to co-activators, and alternatively promote the interaction with co-repressor proteins, thus halting transcription of PR (Conneely *et al.*, 2002; Chabbert-Buffet *et al.*, 2005). The PR-A and PR-B receptors have an antagonistic relationship, as PR-A inhibits PR-B-induced DNA transcription (Cable and Grider, 2020). Indirect genomic signalling is ligand independent, and occurs through receptor protein–protein interactions with other sequence-specific transcription factors, rather than by direct receptor binding to hormone response elements.

1.5.2.2. Non-genomic Signalling

When genomic signalling is delayed, the rapid effects of steroid hormones occurs via non-genomic signalling. The quick, non-genomic action of steroids occurs via plasma membrane steroid recognition sites, which modulate second messenger intracellular signalling cascades (Figure 1.4) (Perrotti, 2017). The membrane progesterone receptors (mPRs) are seven-transmembrane proteins belonging to the progestin and adipoQ receptor family, and are located on the cell surfaces of neural, reproductive, and non-reproductive tissues (Thomas *et al.*, 2007; Perrotti, 2017). The signalling pathways utilizing mPRs involve the activation of mitogen-activated protein kinase (MAPK) (Dressing *et al.*, 2011; Rajaram and Brisken, 2012; Zhang *et al.*, 2021).

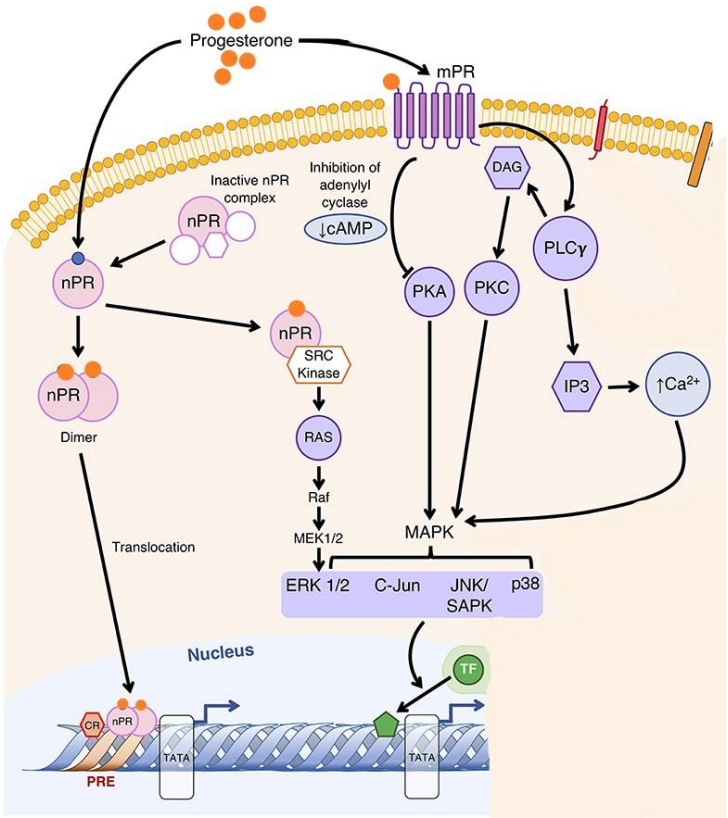


Figure 1.4. Progesterone receptor signalling. Adapted from (Shah *et al.*, 2019). In the classical mechanism (direct genomic signalling), progesterone binds to extranuclear nuclear progesterone receptors (nPR). The dimer is translocated to the nucleus and binds to the progesterone response element (PRE) and activates transcription with the aid of coregulators (CR). In non-genomic signalling, the nPR-progesterone complex activates the MAPK cascade, via Src kinase. Progesterone bound to membrane receptors (mPR) modifies gene transcription controlled by second messengers (cAMP and Ca2+) and protein kinases (PKA and PKC) through the MAPK cascade, resulting in phosphorylation of nuclear transcription factors (TF). Abbreviations: phospholipase Cγ (PLCγ); diacylglycerol (DAG); 1,4,5-trisphosphate (IP3); rapidly accelerated fibrosarcoma (Raf); Mitogen-activated protein kinase kinase (MEK); extracellular signal-regulated kinase (ERK); Stress-activated protein kinases (SAPK)/Jun amino-terminal kinases (JNK).

1.6. Progesterone and Progesterone Receptor in HIV infection

The relationship between HIV and progesterone has been closely linked to progesterone contraceptives. High endogenous progesterone and progestin-only contraceptives have been associated with increased HIV acquisition in women due to an increase in activated HIV targets cells at the cervix (Byrne *et al.*, 2016).

Progestins have also been implicated in the risk of HIV infection by the modulation of chemokine receptors that mediate viral entry (Hall and Klein, 2017). An increased expression of the HIV receptor, CXCR4, in peripheral blood mononuclear cells of healthy and HIV-infected women after progesterone treatment has been reported (Cabrera-Muñoz *et al.*, 2012a). Interestingly, protease inhibitor (PI)-based ARVs given to pregnant HIV-infected women significantly decreases progesterone levels (Powis and Shapiro, 2014; Papp *et al.*, 2014). There is an increase of terminal-villi capillaries in placentas from HIV-positive cART-exposed women, which is inversely correlated to progesterone levels. Low progesterone has been associated with adverse birth outcomes, and it is shown that progesterone supplementation after ARV exposure reduces placenta vascular changes, resulting in more favourable birth outcomes (Mohammadi *et al.*, 2018). It is suggested that progesterone could have a protective function against HIV infection, as the hormone influences the immune system and can act directly on the virus (Cabrera-Muñoz *et al.*, 2012b). Additionally, Muñoz *et al.*, (2007) demonstrated that progesterone reduces autocrine tumour necrosis factor (TNF) levels, thus inhibiting HIV-1 viral amplification. Progesterone interacts with the immune system and the virus however, the effect of progesterone is dependent on its concentration or the phase of HIV infection (Cabrera-Muñoz et al., 2012b).

It is also plausible that mutations in the PR gene influence the activity of the immune system, as it has been shown to play a protective role against hepatitis E virus (HEV) in HIV-infected patients (Lhomme *et al.*, 2016; López-López *et al.*, 2019). However, a more recent study has identified a mutation in the

progesterone receptor (PR) named PROGINS, which is associated with HEV infection in HIV-positive patients due to altered serum levels of IL-10 and decreased T-cell stimulation (Debes *et al.*, 2018). Studies investigating the relationship between HIV and progesterone receptors in pregnancy are limited, warranting further research.

1.7. Progesterone and Progesterone Receptor in HIV associated Pre-Eclampsia

Progesterone produced by the placenta is crucial for maintaining a healthy pregnancy. The level of progesterone and/or its receptor levels in PE is debatable and controversial (Açıkgöz *et al.*, 2013; Wan *et al.*, 2018; Moon *et al.*, 2014). It is postulated that the level of pregnenolone and progesterone is increased in PE, due to increased expression of the rate-limiting enzyme CYP11A1 (Moon *et al.*, 2014). The upregulation of CYP11A1 occurs at mRNA and protein levels in pre-eclamptic placenta, and is speculated to contribute to the pathogenesis of PE (He *et al.*, 2013). In contrast, several studies have indicated that the serum levels of progesterone in pre-eclamptic pregnancies are significantly lower than normotensive pregnancies (Iou *et al.*, 2005; Wan *et al.*, 2018; Chowdhury *et al.*, 2020). A study by Chowdhury *et al.*, (2020) indicated that although progesterone in pre-eclamptic placental explants cultures were lower than normotensive cultures, there is no difference in serum levels of progesterone between EOPE and LOPE. Moreover, the supplementation of progesterone has been suggested as a therapeutic tool for PE (Zhu *et al.*, 2013; Pei *et al.*, 2022). The use of progesterone as a preventative measure for PE is attributed to its ability to increase the expression of HLA-G protein, which increases maternal immune tolerance, in placental cytotrophoblast cells (Yie *et al.*, 2005).

Progesterone signalling pathways have been implicated in placental vascularization and PE, however, there is a paucity of data on the expression of placental PRs. An *in vitro* study by Park *et al.*, revealed that PR expression levels were elevated in the placenta of pre-eclamptic women (Park *et al.*, 2018). Furthermore a genetic variation in the PRs of pre-eclamptic women may exist as PR is connected with a defective decidualization fingerprint, and PR-B gene expression is disrupted in PE (Pretscher *et al.*, 2021; Garrido-Gomez *et al.*, 2021).

The implication of progesterone in the disease progression of HIV infection is closely linked to HAART, however research investigating the effect of progesterone and PR in HIV-associated pre-eclampsia is limited. HIV infection and hypertension are the leading causes of maternal death in South Africa, and the pathogenesis of PE, particularly in HIV-infected pregnant women, and its associated death is unknown (StatsSA, 2017). The KwaZulu-Natal province has the highest HIV prevalence rate in SA, making it an ideal location to study the relationship between progesterone, PR and HIV-associated pre-eclampsia (Woldesenbet *et al.*, 2021).

1.6. Aim and objectives of the study

1.6.1. Aim

To investigate the expression of placental progesterone and progesterone receptors (PR) in HIV associated pre-eclampsia.

1.6.2. Objectives

1. To quantify and compare the expression of placental progesterone and PR in normotensive pregnant *vs* preeclamptic women regardless of HIV status, using Immunohistochemistry (IHC) interfaced with morphometric image analysis.
2. To quantify and compare the expression of placental progesterone and PR by gestational age in preeclamptic women (early *vs* late onset PE) using IHC interfaced with morphometric image analysis.
3. To quantify and compare the expression of placental progesterone and PR between HIV infected *vs* uninfected women regardless of pregnancy type using IHC interfaced with morphometric image analysis.
4. To quantify and compare the expression of placental progesterone and PR based on villi type (exchange villi *vs* conducting villi) across all groups using IHC interfaced with morphometric image analysis.
5. To correlate patient demographics with progesterone and PR levels across all groups.

CHAPTER 2

Research article: Placental progesterone (P) and its receptor (PR) in HIV-infected pre-eclamptic women

This chapter investigates the immuno-expression of placental progesterone (P) and progesterone receptor (PR) in pre-eclamptic versus normotensive pregnancies with HIV infection. This chapter follows the manuscript format of a DoHET accredited peer-reviewed journal.

Citation: Sewnarain SA., Singh S., and Naicker T. (2022). Placental progesterone (P) and its receptor (PR) in HIV-infected pre-eclamptic women. **Submitted** to Systems Biology in Reproductive Medicine, Manuscript ID: 222515046. Impact factor: 3.061

Taylor & Francis
Taylor & Francis Group

Submission ID	222515046
Manuscript Title	Placental progesterone (P) and its receptor (PR) in HIV-infected pre-eclamptic women
Journal	Systems Biology in Reproductive Medicine
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Keywords	preeclampsia, human immunodeficiency virus, progesterone, progesterone receptor, pregnancy

Research article

Placental progesterone (P) and its receptor (PR) in HIV-infected pre-eclamptic women

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Abstract

Given the high prevalence of HIV infection and pre-eclampsia (PE) in South Africa, this study evaluated and compared the placental immunostaining of progesterone (P) and progesterone receptors (PR) in the synergy of HIV infected PE compared to normotensive pregnant women using immunohistochemistry interfaced with morphometric image analysis. Progesterone immunostaining was expressed widely across exchange and conducting villi within mesenchymal, endothelial and trophoblast cells. In contrast, PR was expressed within syncytiotrophoblasts and was absent within endothelial cells. In exchange villi, P and PR immuno-expression was significantly lower in PE compared to the normotensive group (p = <0.0001 and p = <0.0001 respectively) and within the EOPE compared to the LOPE group (p = <0.0001 and p = <0.0001 respectively). Progesterone immuno-expression was significantly lower in the HIV+ compared to the HIV- group (p = <0.0001), whilst PR was non-significant. In conducting villi, P and PR immuno-expression was significantly lower in the EOPE compared to the LOPE group (p = <0.0001 and p = <0.0001 respectively) and in the HIV+ compared to the HIV- group (p = <0.0001 and p = 0.0009 respectively). Progesterone immuno-expression was slightly higher in the PE compared to normotensive group and PR immuno-expression was non-significant. There was a significant difference between P and PR within exchange vs. conducting villi regardless of pregnancy type, with villi type accounting for 34.47% and 15.28% of total variance for P and PR, respectively. Placental P and PR immuno-expression was downregulated in the duality of PE and HIV+ infection. The use of cART may result in defective P synthesis, which causes insufficient binding to its receptors. Consequently, PI3K/AKT, JAK-STAT, and MAPK signalling pathways may be affected, impairing trophoblast invasion and leading to pre-eclampsia development. Notably, the decrease in P and PR immuno-expression in EOPE validates their effect on placentation.

Keywords: preeclampsia, human immunodeficiency virus, progesterone, progesterone receptor, pregnancy

Abbreviations: P: progesterone; PR: progesterone receptor; PE: pre-eclampsia; EOPE: early onset pre-eclampsia; LOPE: late onset pre-eclampsia; HIV: human immunodeficiency virus; cART: combined antiretroviral therapy; PI3K/AKT: phosphoinositide 3-kinase/protein kinase B; JAK/STAT: Janus kinase/signal transducers and activators of transcription; MAPK: mitogen-activated protein kinase

Introduction

South Africa is the epicentre of the human immunodeficiency virus (HIV) pandemic. Moreover, the 40% prevalence rate of HIV infection amongst pregnant women is unacceptably high (Woldesenbet et al., 2021). This is alarming and remains a grave public health challenge because HIV infection and hypertensive diseases of pregnancy, such as pre-eclampsia (PE), are the primary causes of maternal mortality in South Africa (StatsSA, 2017).

Preeclampsia is a placental condition that manifests after 20 weeks' gestation and is defined by hypertension (blood pressure of ≥140/90mmHg) with or without proteinuria (≥300 mg) (Magee et al., 2022). It is classified according to gestational age, as early onset pre-eclampsia (EOPE) and late onset pre-eclampsia (LOPE), with the former being the more severe subtype (Steegers et al., 2010; Gomathy et al., 2018). Pre-eclampsia is a two-stage condition, characterized in Stage 1 by aberrant placentation with incomplete myometrial spiral artery remodelling. The resultant hypoxic environment predisposes the excessive release of anti-angiogenic substances into circulation causing maternal signs and symptoms (Stage 2) (Roberts and Hubel, 2009). The definitive treatment of PE is delivery of the neonate and placenta (Uzan et al., 2011).

The placenta is chiefly responsible for the production of the steroid hormone, progesterone (P), during pregnancy (Fox and Sebire, 2007). Progesterone is a lipophilic, four carbon-ring molecule that is derived from a cholesterol-derived molecule, pregnenolone (Taraborrelli, 2015). It ensures pregnancy success by stimulating uterine vascularization, inhibiting lactation and decreasing myometrial contractions (Raghupathy and Szekeres-Bartho, 2022). A decline in P levels early in pregnancy causes increased myometrial contractions, which could lead to rejection of the fetus (Mesiano, 2007).

Progesterone exerts its action on target cells by binding to progesterone receptors (PR) on the cell membrane or in the cytoplasm (Szekeres-Bartho et al., 2009). With the aid of co-regulators, the activating and inhibiting functional components of PR promote and repress transcriptional activity, respectively (Rekawiecki et al., 2020). Progesterone receptors contain an N-terminal domain which allows for post-translational modification (Scarpin et al., 2009). There are three isoforms of PR, and the PR-C isoform is expressed abundantly in the syncytiotrophoblast of placental villi (Taylor et al., 2006). In mammalian cells, PRs take part in cytoplasmic or membrane-associated signalling complexes that trigger the Src/Ras/Raf/ mitogen-activated protein kinase (MAPK) signalling cascade to regulate cell proliferation (Mani and Oyola, 2012).

Studies of P regulation in PE are conflicting. Whilst some studies report increased P production in PE (Moon et al., 2014; Park et al., 2018), others indicate that serum and placental immuno-expression of P and PR are decreased (Wan et al., 2018; Garrido-Gomez et al., 2021). Nonetheless, it is implied that

dysregulation of steroidogenesis occurs in PE, leading to altered P immuno-expression (Berkane et al., 2018).

HIV infection is associated with decreased P levels, emanating from the use of protease inhibitors (Papp et al., 2015). Women receiving combined antiretroviral therapy (cART) for HIV prophylaxis are at an increased risk for PE development (Sansone *et al.*, 2016; Sikhosana *et al.*, 2022). Progesterone is crucial in uterine vessel dilation before the tenth week of pregnancy, which helps to lower systemic blood pressure along with the reducing vascular resistance (Maliqueo et al., 2016). It is postulated that a decrease in P levels could contribute to the pathophysiology of hypertensive disorders of pregnancy (Powis and Shapiro, 2014), however there is a scarcity of data in this area.

In an attempt to understand the role of P and PR in the duality of HIV infection comorbid with pre-eclampsia, this study aims to morphometrically evaluate the immuno-expression of P and its receptor by pregnancy type, HIV status, gestational age (early and late onset PE) and villi type (exchange and conducting villi) across all study groups.

Materials and Methods

Study population

The placental samples were collected from primigravid and multigravid pre-eclamptic and normotensive Black South African pregnant women who attended the antenatal clinic of a regional hospital in Umlazi, eThekwini, KwaZulu-Natal, South Africa. A sample size of 180 pregnant women is required to detect a moderate effect size of 0.45 and 0.42 between normotensive (N) vs. pre-eclamptic women and HIV negative (-) vs. HIV positive (+) women, respectively. In order to compare six groups: normotensive (HIV- vs. HIV+), EOPE (HIV- vs. HIV+) and LOPE (HIV- vs. HIV+), a sample size of 30 in each group would be needed to detect a moderate effect size of 0.75 (Cohen, 1988). All calculations are with 80% power and 95% probability and were done using G*Power statistical software.

Inclusion criteria

Pregnant women who are ≥ 18 years of age with known HIV status and diagnosed with PE were included in the study. PE was defined as sustained systolic blood pressure ≥140/90mmHg taken twice at least 6 hours apart, with/without proteinuria (≥300mg in a 24 hour urine sample or +2 on the urine dipstick analysis) and multiorgan dysfunction (Magee et al., 2022).

Exclusion criteria

The following criteria excluded women from this study: unknown HIV status, abruption placentae or intra-uterine death, chorioamnionitis, chronic hypertension, gestational diabetes and diabetes mellitus, epilepsy, heart failure, chronic renal disease, connective tissue disease, systemic lupus erythematosus, sickle cell disease, anti-phospholipid antibody syndrome, thyroid disease, history of smoking and substance abuse, treatment with: aspirin, warfarin, non-steroidal anti-inflammatory drugs, lipid lowering antibiotics or anti-hypertensive drugs and asthma medication.

Immunostaining

Placental samples were previously fixed in 10% buffered formaldehyde and embedded into paraffin wax blocks in accordance to standard laboratory practice (Burton et al., 2014). The placental wax embedded tissue blocks were cut into 3µm sections using a rotary microtome (Leica Microsystems, Germany) and mounted onto adherent slides. Sections were deparaffinized with xylene and rehydrated with decreasing concentrations of ethanol. Slides were immersed in antigen retrieval and endogenous peroxidase was used as a blocking agent. Non-specific binding was prevented by using a protein block, thereafter the tissue was incubated overnight in a humidity chamber at 4 °C with the primary antibodies; monoclonal (mouse IgG1 κ) progesterone antibody (1:200, Novus Biologicals, United States) and monoclonal (mouse IgG1 κ) anti-progesterone antibody (1.5 µg/ml, Abcam, Cambridge, United Kingdom). After washing, the placental sections were incubated for 10 minutes at room temperature with the secondary antibody, biotinylated goat anti-mouse (IgG) from a mouse specific diamino-benzidine (DAB) detection immunohistochemistry (IHC) kit (Abcam, Cambridge, United Kingdom). Visualisation was enabled via the DAB chromogen followed by haematoxylin as a counterstain. Sections were then dehydrated and mounted with dibutylpthalate polystyrene xylene (DPX). Method controls involved substitution of primary antibody with diluent (DAKO REAL diluent).

Morphometric analysis

Placental sections were viewed with the Axioscope A1 microscope (Carl Zeiss, Germany). Four fields of view per slide were selected and images were captured at 20x objective magnification using AxioVision software (Carl Zeiss, Germany; version 4.8.3). The percentage of immunostaining specific to P and PR antibody expression was quantified using colour deconvolution on Fiji ImageJ software (Jensen, 2013; Crowe and Yue, 2019). Colour deconvolution involves separating colours of an image into three channels, red, green, and blue. For this investigation, the red channel represents DAB staining and blue represents haematoxylin staining. The percentage of P and PR expression was determined by dividing the percentage of DAB staining by the total tissue area.

Statistical analysis

Statistical analysis was performed using GraphPad Prism™ (San Diego, CA, USA). In order to compare the effects of pregnancy type (normotensive vs. pre-eclamptic), HIV status (HIV+ vs. HIV-) and PE subtype (EOPE vs LOPE), the Mann Whitney test was employed. A one-way ANOVA non-parametric Kruskal-Wallis test was utilized, followed by Dunn's Multiple Comparisons test for comparative analysis across all 6 study groups. A two-way ANOVA was used to compare villi types (exchange vs conducting) and pregnancy types. The data was summarized using descriptive statistics, median and interquartile range (IQR). A p value of < 0.05 determined statistical significance.

Ethical approval

This was a retrospective cross-sectional study that utilized archived wax embedded placental samples. Institutional ethics consent for use of the samples was obtained (BCA338/17). Informed consent was obtained from all participants in the primary study and the anonymity of participants was maintained.

Results

Clinical characteristics

Maternal age (p = <0.0001), systolic blood pressure (p = <0.0001), diastolic blood pressure (p = <0.0001) and parity (p = 0.0023) differed significantly across the groups (Table 1).

Table 1. Maternal demographics across the study population

Parameters	**Normotensive**		**EOPE**		**LOPE**		
	HIV- (n = 30)	HIV+ (n = 30)	HIV- (n = 30)	HIV+ (n = 30)	HIV- (n = 30)	HIV+ (n = 30)	***p* value**
Maternal age (years)	24.5 (21.75-29.25)	27 (24.75-32)	23 (20-30.5)	32 (27-37)	22.5 (19-26)	27 (25-33)	<0.0001 ****
Maternal weight (kg)	73 (61.93-96.12)	75 (55.35-87.43)	67.58 (59.75-95.25)	79 (68.48-87.55)	69.85 (55.75-81)	85 (69.8-94.5)	0.1086
Systolic BP (mmHg)	110.5 (105.5-116.3)	111 (104.3-117.3)	151 (143-160)	148 (136-161)	153 (146-173)	145 (142-153.5)	<0.0001 ****
Diastolic BP (mmHg)	71 (65.5-79)	70.5 (63.5-76.5)	95.5 (90.75-104.3)	92 (90-98)	101 (94-108.8)	96 (91-98.5)	<0.0001 ****
Parity	2	2	2	2	1	2	0.0023**

	(2-3)	(2-3)	(1-3.25)	(2-4)	(1-2)	(2-3)

Level of significance: *($p < 0.05$), **($p < 0.01$), ***($p < 0.0001$)

Immuno-localization of placental progesterone and its receptor

Progesterone was immunostained within endothelial, mesenchymal and trophoblast cells within conducting (stem) and exchange (intermediate and terminal) villi. Progesterone receptor immuno-expression was restricted to trophoblast cell types (cytotrophoblast and syncytiotrophoblast), with minimal immunostaining within the mesenchymal core (Fig.2 and 4).

Morphometric image analysis

Exchange Villi

Progesterone

The immunoexpression of P was significantly lower in PE compared to the normotensive (N) group, regardless of HIV status [25.42% (24.07-27.04) *vs.* 23.82% (21.69-26.33); p = <0.0001]. Also, irrespective of HIV status, P was lower in EOPE compared to the LOPE group [22.76% (20.93-25.22) *vs.* 24.88% (22.85-27.07); p = <0.0001]. Regardless of pregnancy type, a lower percentage of P was immunoexpressed in HIV+ compared to HIV- participants [25.70% (23.58-27.32) *vs* 23.55% (21.52-25.52); p = <0.0001].

Progesterone receptor

Irrespective of HIV status, PR immuno-expression was lower in PE compared to the N group [13.17% (11.89-14.84) *vs*. 11.60% (9.91-12.95); p = <0.0001]. Based on gestational age and regardless of HIV status, PR immuno-expression was lower in EOPE compared to LOPE groups [10.88% (9.31-12.20) *vs*. 12.17% (10.74-13.83); p = <0.0001]. The percentage of PR expressed was not significant between HIV+ compared to HIV- groups [11.99% (10.27-13.72) *vs*. 12.14% (10.66-13.85); p = 0.4291].

Conducting Villi

Progesterone

A slightly higher immuno-expression of P within the conducting villi was observed in PE compared to the normotensive group, regardless of HIV status [18.98% (17.03-20.74) *vs.* 19.42% (17.64-22.27); p

= 0.0326]. The percentage of P immuno-expression was lower in EOPE in comparison to LOPE, irrespective of HIV status [21.60% (18.16-24.17) *vs.* 18.58% (17.50-20.01); *p* = <0.0001]. Regardless of pregnancy type, P was significantly lower in the HIV+ group compared to the HIV- group [20.07% (17.91-23.32) *vs*. 18.74% (17.03-20.38); *p* = <0.0001].

Progesterone receptor

The immuno-expression of PR in PE was not significant in comparison to N groups, irrespective of HIV status [10.14% (8.90-11.41) *vs.* 9.99% (8.79-11.38); *p* = 0.6935]. Regardless of HIV status, PR immuno-expression was lower in the EOPE group compared to the LOPE group [9.33% (8.18-10.69) *vs* 10.89% (9.76-11.96); *p* = <0.0001]. Irrespective of pregnancy type, the percentage of PR immuno-expression in the HIV+ group was lower than the HIV- group [10.54% (9.07-11.70) *vs* 9.81% (8.54-11.10); *p* = 0.0009].

Immuno-expression across all groups

The comparative analysis of all 6 study groups yielded significant results in both exchange and conducting villi (p = <0.0001) and is outlined in table 2. The EOPE+ group demonstrated the lowest immuno-expression of both P and PR.

Table 2. Immuno-expression of placental progesterone and PR across all 6 study groups.

	Exchange Villi		**Conducting Villi**	
	Progesterone	**PR**	**Progesterone**	**PR**
	p = <0.0001****	*p* = <0.0001****	*p* = <0.0001****	*p*= <0.0001****
N-	26.62 (25.10-28.66)	13.72 (12.12-15.52)	19.16 (16.79-21.62)	10.40 (9.06-11.59)
N+	24.47 (23.29-25.95)	12.68 (11.72-14.05)	18.91 (17.66-20.30)	10.04 (8.86-11.22)
EOPE-	24.88 (22.76-26.63	10.93 (9.29-12.22)	23.81 (21.14-26.30)	9.61 (8.40-11.05)
EOPE+	21.17 (18.80-22.82)	10.79 (9.13-12.14)	18.78 (15.84-21.62)	8.96 (7.73-9.92)
LOPE-	25.18 (23.14-27.32)	11.60 (10.30-12.99)	18.58 (17.60-20.10)	11.20 (10.08-12.82)
LOPE+	24.74 (22.66-26.70)	12.71 (11.50-14.87)	18.61 (17.11-20.01)	10.44 (9.65-11.55)

Immuno-expression between villi types

Progesterone

A two-way ANOVA was utilized to assess the effect of villi type (exchange *vs.* conducting) on the results. Villi type accounted for 34.47% of the total variance (F = 594.29, p = <0.0001), whilst pregnancy type accounted for 10.25% of the total variance (F = 35.34, p = <0.0001). The Bonferroni post hoc test was utilized to determine significance between the exchange and conducting villi of each group (N-: p = <0.0001, N+: p = <0.0001, EOPE-: p = <0.0001, EOPE+: p = <0.0001, LOPE-: p = <0.0001, LOPE+: p = <0.0001).

Progesterone receptor

Villi type accounted for 15.28% of the total variance for PR (F = 193.75, p = <0.0001) whilst pregnancy type accounted for 9.55% of the total variance (F = 24.22, p = <0.0001). The Bonferroni post hoc test was utilized to determine significance between the exchange and conducting villi of each group (N-: p = <0.0001, N+: p = <0.0001, EOPE-: p = <0.0001, EOPE+: p = <0.0001, LOPE-: p = <0.0001, LOPE+: p = <0.0001).

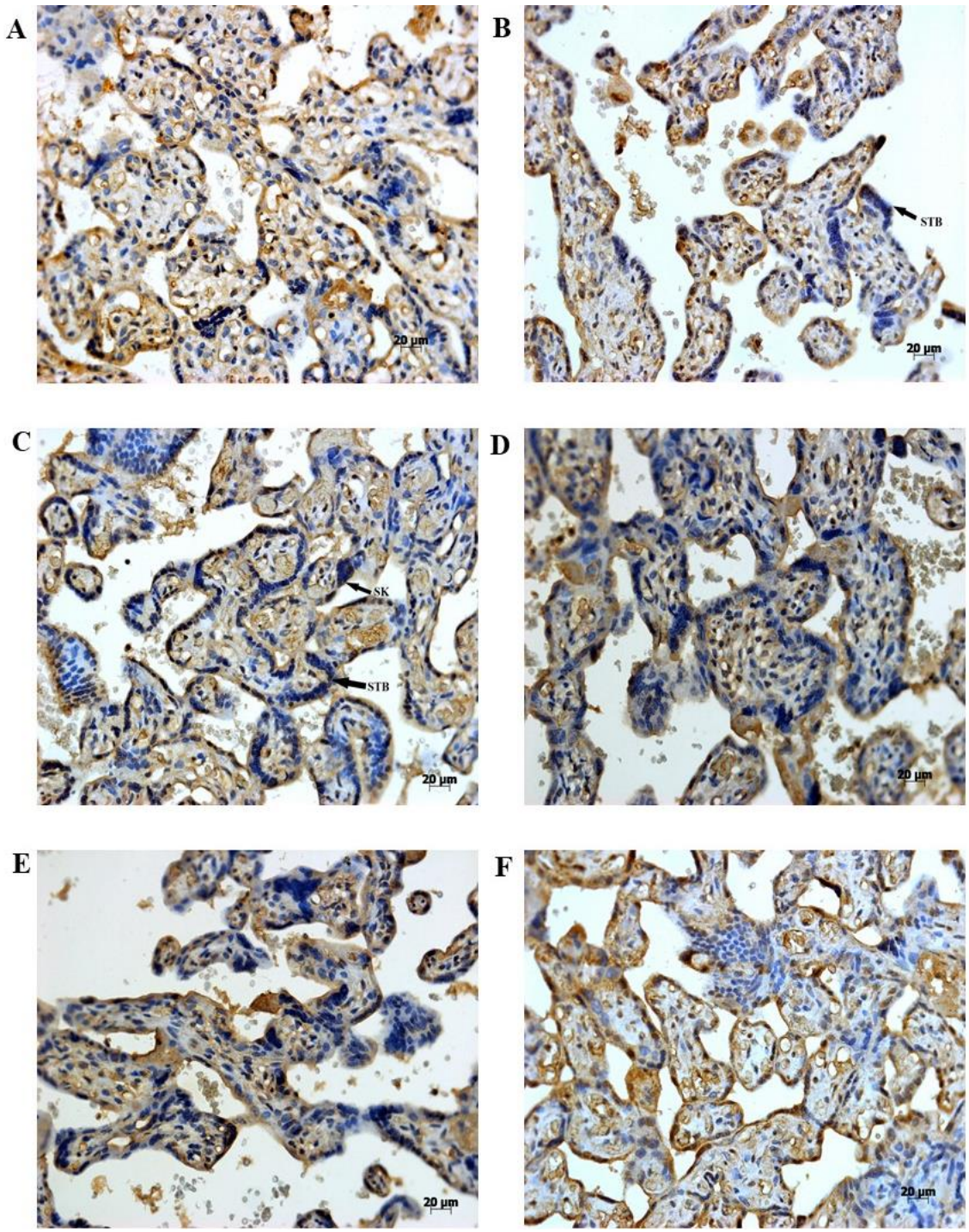

Figure 1. Progesterone immuno-localization in exchange villi. (A) HIV negative, normotensive healthy controls (N-); (B) HIV positive, normotensive (N+); (C) HIV negative, EOPE (EOPE-); (D) HIV positive, EOPE (EOPE+); (E) HIV negative, LOPE (LOPE-) and (F) HIV positive LOPE (LOPE+). Magnification: x40. Morphological observations: SK = Syncytial knot; STB = syncytiotrophoblast.

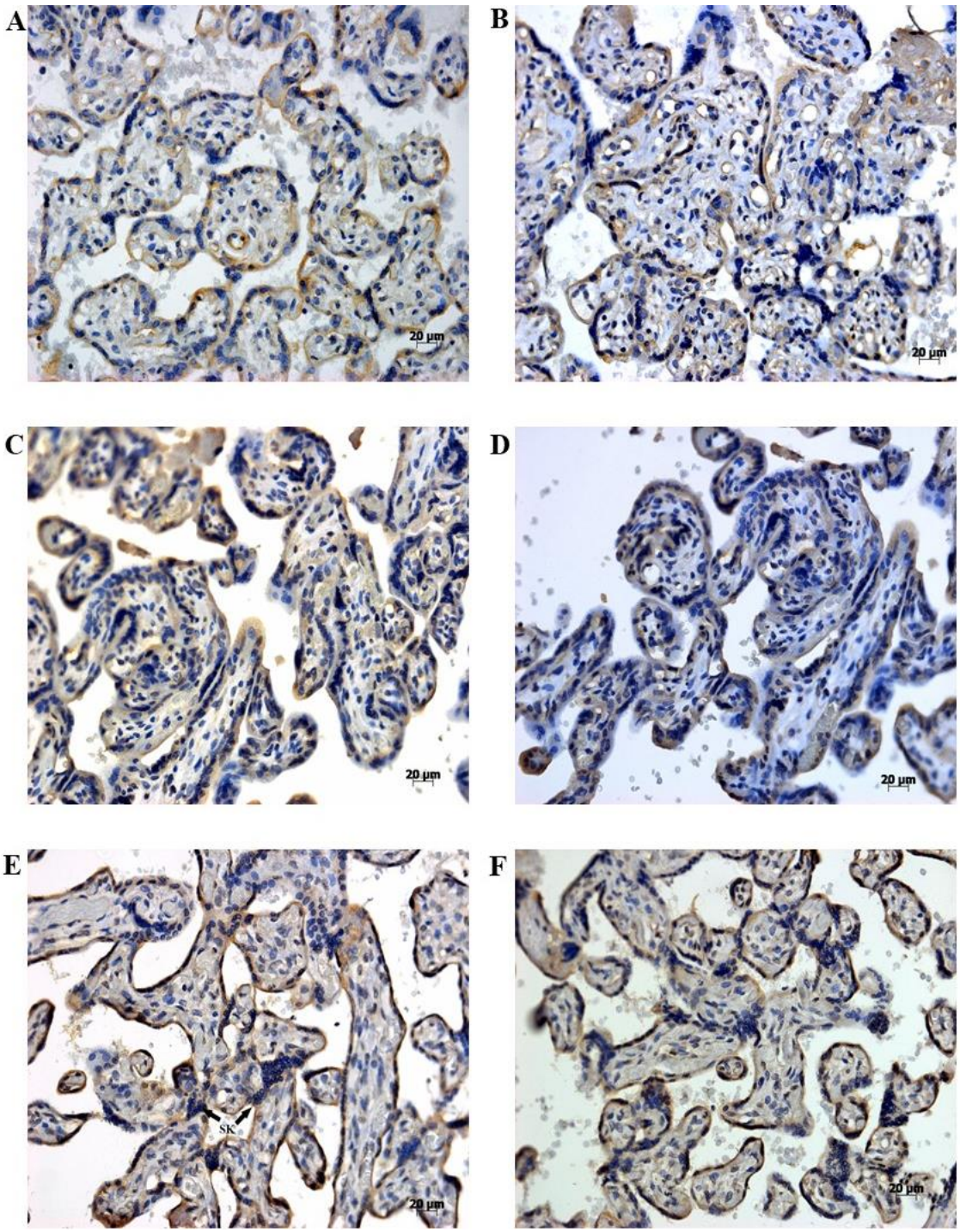

Figure 2. Progesterone receptor immuno-localization in exchange villi. (A) HIV negative, normotensive healthy controls (N-); (B) HIV positive, normotensive (N+); (C) HIV negative, EOPE (EOPE-); (D) HIV positive, EOPE (EOPE+); (E) HIV negative, LOPE (LOPE-) and (F) HIV positive LOPE (LOPE+). Magnification: x40. Morphological observations: SK = Syncytial knot; STB = syncytiotrophoblast.

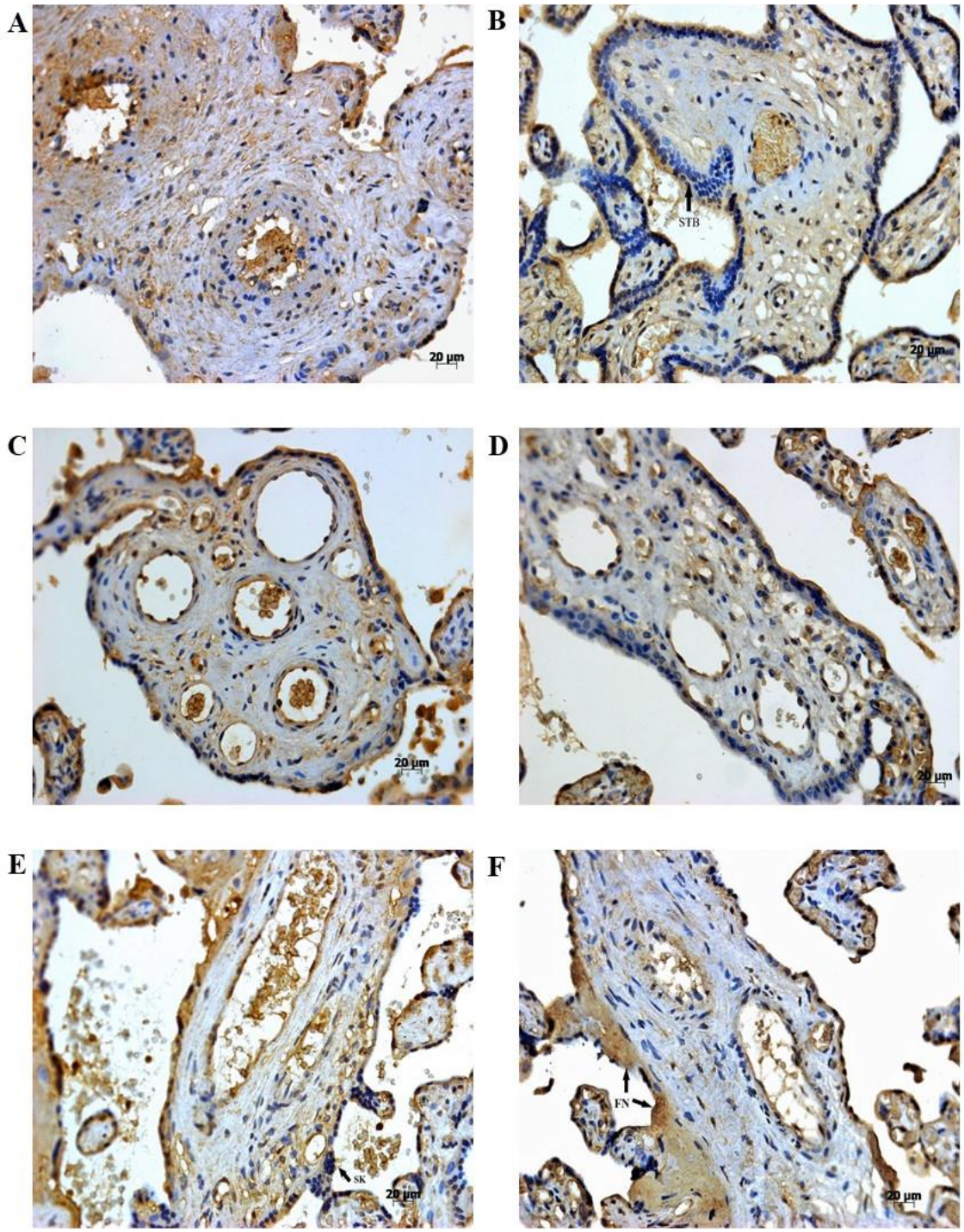

Figure 3. Progesterone immuno-localization in conducting villi. (A) HIV negative, normotensive healthy controls (N-); (B) HIV positive, normotensive (N+); (C) HIV negative, EOPE (EOPE-); (D) HIV positive, EOPE (EOPE+); (E) HIV negative, LOPE (LOPE-) and (F) HIV positive LOPE (LOPE+). Magnification: x40. Morphological observations: SK = Syncytial knot; STB = Syncytiotrophoblast; FN = Fibrinoid necrosis

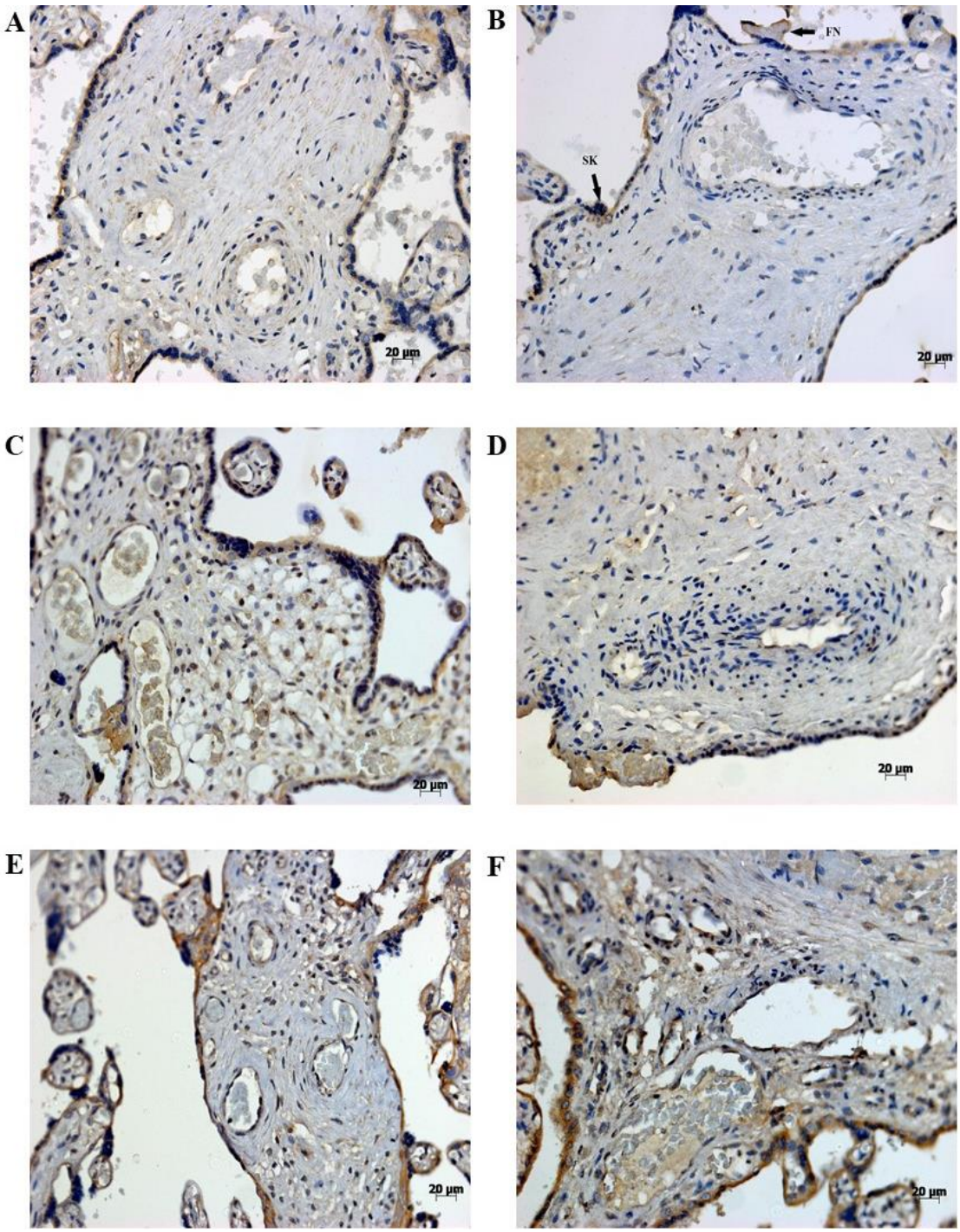

Figure 4. Progesterone receptor immuno-localization in conducting villi. (A) HIV negative, normotensive healthy controls (N-); (B) HIV positive, normotensive (N+); (C) HIV negative, EOPE (EOPE-); (D) HIV positive, EOPE (EOPE+); (E) HIV negative, LOPE (LOPE-) and (F) HIV positive LOPE (LOPE+). Magnification: x40. Morphological observations: SK = Syncytial knot; FN = Fibrinoid necrosis

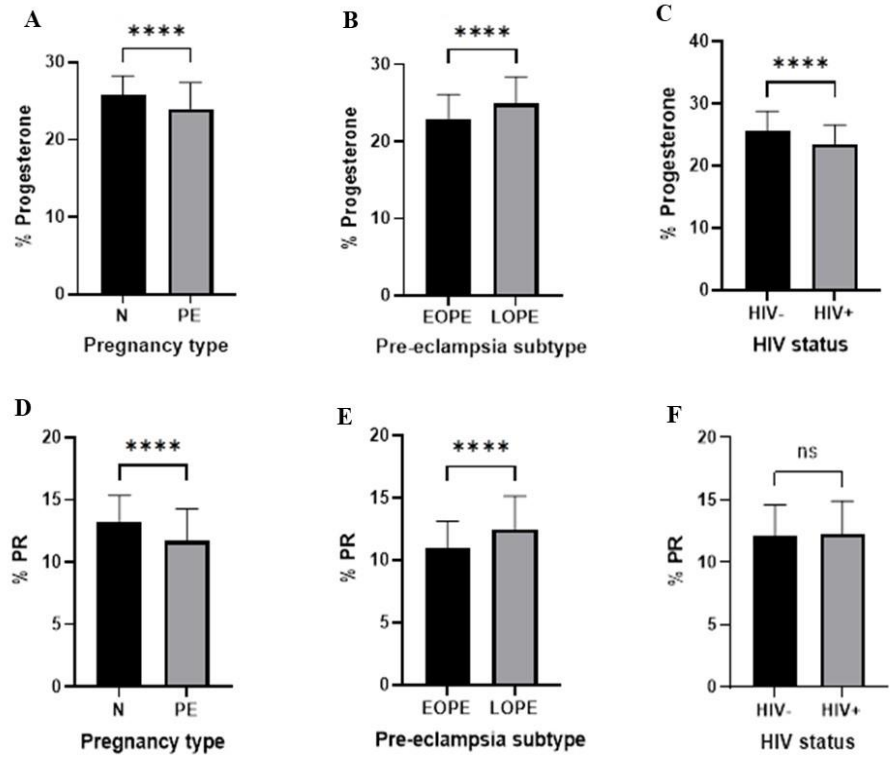

Figure 5. Percentage progesterone and PR immuno-expression in the exchange villi. The % progesterone within the exchange villi was quantified and compared between (A) pregnancy type, (B) PE subtype and (C) HIV status. The % PR immuno-expression was also compared between (D) pregnancy type, (E) PE subtype and (F) HIV status.

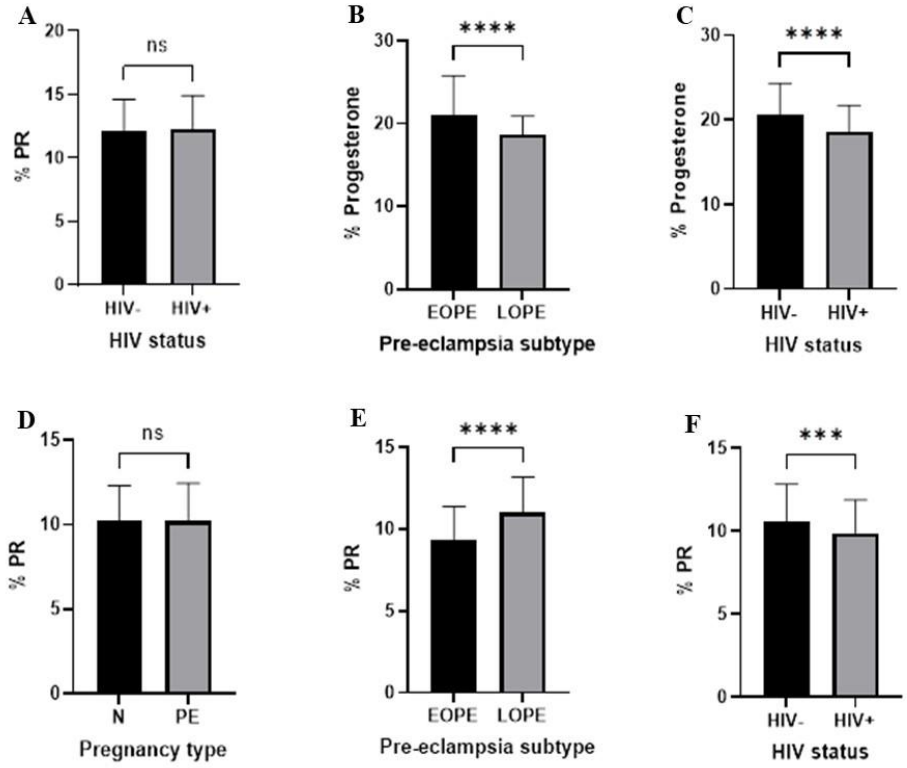

Figure 6. Percentage progesterone and PR immuno-expression in the conducting villi. The % progesterone within the exchange villi was quantified and compared between (A) pregnancy type, (B) PE subtype and (C) HIV status. The % PR immuno-expression was also compared between (D) pregnancy type, (E) PE subtype and (F) HIV status.

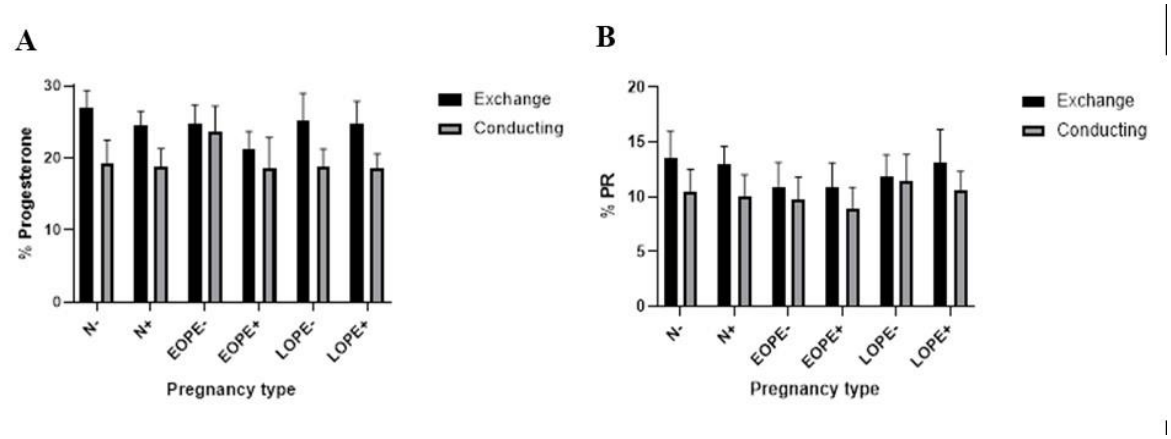

Figure 7. Progesterone and PR immuno-expression based on villi type across all groups. (A) Comparison of progesterone immuno-expression based on villi types across all groups and (B) comparison of PR immuno-expression based on villi types across all groups.

Correlation of patient demographics with progesterone and PR levels

There was largely no significance between the correlation of patient demographics (systolic and diastolic blood pressure) with progesterone and PR levels (Table 3 and 4). However, there was a significant correlation (p = 0.034* and r_s= 0.426) between the expression of PRs and systolic blood pressure in the conducting villi of the LOPE+ group (Table 4).

Table 3. Spearman correlation of patient demographics (systolic and diastolic blood pressure) with progesterone and PR immuno-expression in exchange villi

	Systolic blood pressure					
	N-	**N+**	**EOPE-**	**EOPE+**	**LOPE-**	**LOPE+**
P	-0.204	0.124	0.150	0.131	0.133	0.099
	p = 0.281	p = 0.512	p = 0.430	p = 0.516	p = 0.482	p = 0.638
PR	-0.017	-0.241	0.282	-0.106	0.043	0.235
	p = 0.930	p = 0.200	p = 0.131	p = 0.598	p = 0.822	p = 0.258
	Diastolic blood pressure					
P	-0.131	0.267	-0.048	0.014	0.009	-0.091
	p = 0.489	p = 0.153	p = 0.801	p = 0.944	p = 0.961	p = 0.666
PR	0.190	-0.020	0.180	-0.260	0.020	-0.090
	p = 0.313	p = 0.911	p = 0.335	p = 0.194	p = 0.904	p = 0.668

Table 4. Spearman correlation of patient demographics (systolic and diastolic blood pressure) with progesterone and PR immuno-expression in conducting villi

	Systolic blood pressure					
	N-	**N+**	**EOPE-**	**EOPE+**	**LOPE-**	**LOPE+**
P	-0.027	0.246	0.034	0.131	0.025	-0.167
	p = 0.887	p = 0.190	p = 0.859	p = 0.514	p = 0.897	p = 0.424
PR	-0.019	0.106	-0.088	-0.253	0.084	0.426
	p = 0.920	p = 0.576	p = 0.644	p = 0.202	p = 0.660	p = 0.034*

	Diastolic blood pressure					
P	0.130	0.340	0.260	0.050	-0.200	0.170
	$p = 0.502$	$p = 0.065$	$p = 0.157$	$p = 0.821$	$p = 0.292$	$p = 0.412$
PR	0.017	-0.289	-0.097	0.041	-0.168	0.316
	$p = 0.927$	$p = 0.121$	$p = 0.609$	$p = 0.838$	$p = 0.375$	$p = 0.124$

Level of significance: *(p < 0.05), **(p < 0.01), ***(p < 0.0001)

Discussion

The findings of this study demonstrate a significant decrease of P and PRs immunostaining in the exchange villi of pre-eclamptic compared to normotensive placentas, regardless of HIV status. Progesterone is primarily produced by the placenta after the 8th week of gestation (Pylypchuk and Pylypchuk, 2021) and the abundant immuno-expression of P and PRs in normotensive placental tissue is expected. Notably the decline in P in our study is corroborated by several studies that noted lower circulating levels of P in PE (Iou et al., 2005; Kiprono et al., 2013; Wan et al., 2018; Chowdhury et al., 2020), suggesting a hormonal deficiency in PE.

Target tissues can be affected by P through membrane-initiated PR signalling, which activates phosphoinositide 3-kinase (PI3K)/AKT, JAK-STAT, and MAPK cascades. These signalling pathways are involved in trophoblast invasion and differentiation (Gupta et al., 2016). It is widely accepted that PE is characterised by defective interstitial invasion of extravillous trophoblasts and the lack of a physiological transformation of myometrial spiral arteries (Rana et al., 2019). Progesterone promotes trophoblast invasion and prevents the apoptosis of trophoblasts, with the possibility of alleviating PE symptoms (Pei et al., 2022). PE is associated with elevated apoptosis of trophoblast cells (Naicker et al., 2013). In our study, the decreased immuno-expression of progesterone receptors in trophoblasts of PE placentas limits the binding of P to its receptors, which inhibits the activation of cell signalling pathways such as MAPK, PI3K/AKT and JAK-STAT and thereby impairing trophoblast invasion. This reinforces the implication of impaired P production and receptor signalling in the pathogenesis of PE.

A previous study reported that P mitigates hypertension in response to placental ischemia in a reduced uterine perfusion pressure rat model (Kiprono et al., 2013). Progesterone has a protective effect on vascularization (by increasing endometrial vascularity and blood flow) (Wen et al., 2009), and the decreased immuno-expression of P and PRs observed in our study could negatively impact vascularization and blood flow to the fetus.

The findings of our study report lower immuno-expression of P and PRs based on gestational age; been downregulated in the EOPE compared to the LOPE group, regardless of HIV status. A recent study observed disrupted estrogen receptor 1 and progesterone receptor B gene expression in severe PE (Garrido-Gomez et al., 2021), which correlates with our findings of decreased PR expression in the EOPE group, as EOPE is associated with greater severity and adverse outcomes for both the mother and the baby (Gomathy et al., 2018). Progesterone synthesis by the placenta is dependent upon the mitochondria, and a review (Marín et al., 2020) described the altered mitochondrial structure and function within syncytiotrophoblast which effect oxidative stress and cell apoptosis in EOPE and LOPE. Notably, mitochondrial dysfunction promotes impairment of P synthesis, leading to increased trophoblast apoptosis associated with EOPE development (Marín et al., 2020).

Currently, the subtypes of PE are classified by gestational age where EOPE is associated with defective placentation of placental origin and LOPE is associated with maternal metabolic defects of maternal origin (Burton et al., 2019). Furthermore, recent studies have investigated potential biomarkers distinguishing EOPE and LOPE, such as miRNAs and differentially expressed genes of placental and peripheral blood (Lykoudi et al., 2018; Guo et al., 2021a). The progesterone/estrogen ratio varies in PE, according to the disease sub-type, phenotype and severity (Kale et al., 2020). In our study, the downregulation of P and PRs immuno-expression in EOPE compared to LOPE placentae presents the possibility of P and PR as a potential biomarker for subtyping PE.

The findings of our study also report a decreased immuno-expression of P and PR in the HIV+ compared to HIV- group, regardless of pregnancy type. These findings align with a previous study (Zhou et al., 2018) which observed lower plasma P levels in HIV+ women during the first and second trimesters of pregnancy. It is plausible that this downregulation may be attributed to protease inhibitor-based cART or directly from HIV infection alone. Protease inhibitors used in HIV management has been associated with mitochondrial dysfunction and impairment of villous trophoblast differentiation and P synthesis (Fraichard et al., 2021). Lower P levels in women receiving protease inhibitor- based cART have been reported, and are associated with adverse birth outcomes such as fetal growth restriction (Papp et al., 2015).

Regardless of study group, our findings report that P and PR immuno-expression is significantly higher in exchange *vs.* conducting villi. Furthermore, regardless of villi type, a significant difference was observed between groups. It is noted that in the conducting villi, P was marginally higher in the PE group compared to normotensive, and a non-significant difference was observed in the immuno-expression of PRs. The differences in P and PR immuno-expression may be attributed to surface area of the villi as (Sankar et al., 2013) indicated that the villous surface area and diameter were reduced in placentas with PE, however, the terminal villi density was greater in placentas with PE than in controls. (Mohammadi et al., 2018) noted that changes to placental vascular formation have also been noted in

HIV infection with cART exposure, and it is suggested that progesterone supplementation could be used for enhanced placental function The decline in P and PRs immuno-expression in our study prompts the need for further research into the outcomes of progesterone as a supplement during PE and HIV+ pregnancies.

Across all 6 groups in our study, the EOPE+ group had the lowest immuno-expression of P and PRs. In a South African case-control study, (Sikhosana et al., 2022) observed that untreated HIV infection has a protective effect against PE, however this protective effect is negated with the use of ARVs resulting in a greater risk of women developing PE. In contrast, (Conde-Agudelo et al., 2008) noted that PE develops regardless of HIV treatment. Whilst the susceptibility of PE in HIV infection remains under debate, our study suggests that the combination of PE and HIV infection can synergistically impact progesterone synthesis and the expression of receptors.

A limitation of this study would be that the different types of PRs (PR-A and PR-B) were not specified. The immuno-expression of the different PR receptors could vary across the placental tissue and could be distinctively impacted by PE and HIV infection. An additional limitation would be that the duration of cART treatment received by the HIV+ participants was not specified, and the type of treatment received and duration could impact the results.

In conclusion, this study reports a downregulation of P and PR immuno-expression in the exchange villi of pre-eclamptic placenta. Similarly, P and PR immuno-expression were also significantly downregulated in HIV+ placentas, with the EOPE+ group exhibiting the lowest immuno-expression. We suggest that PI-based cART can result in mitochondrial dysfunction which impairs progesterone synthesis. Progesterone deficiency causes insufficient binding to PRs, affecting signalling pathways such as the PI3K/AKT, JAK-STAT, and MAPK cascades, which affects trophoblast invasion. Of note, the EOPE group has a lower immuno-expression of P and PRs compared to LOPE, directly linking the decline to its placental origin, defective placentation, the severity of PE, and to adverse outcomes of both mother and baby. Progesterone supplementation in HIV-infected women with PE should be considered in a large study cohort.

Acknowledgements

The authors wish to thank Prof T Naicker and the National Research Foundation (MND210517601903) for funding.

Funding

We wish to acknowledge the funding of this project by Prof T Naicker (University of KwaZulu-Natal).

Declaration of interests

No potential conflict of interest was reported by the authors.

Author contributions

Conceptualised study design and provided samples for analysis: TN, SS; methodological aspects of the study: SAS, SS; writing main draft of manuscript: SAS; editing and proofreading manuscript: TN, SS.

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CHAPTER 3

SYNTHESIS

Women are disproportionately affected by the HIV epidemic in South Africa (SA) (Gray *et al.*, 2021; Palanee-Phillips *et al.*, 2022). The prevalence of HIV among pregnant women is an alarming 40.9% in the KwaZulu-Natal province (Woldesenbet *et al.*, 2021). In addition, hypertensive disorders of pregnancy (particularly pre-eclampsia and eclampsia) is the main direct cause of maternal mortality in SA (StatsSA, 2017). The hormonal impact on the pathophysiology of PE have been contentious thus far, as studies reporting progesterone levels in PE are conflicting (Moon *et al.*, 2014; Zheng *et al.*, 2016; Wan *et al.*, 2018). Furthermore, HIV infection is associated with altered progesterone levels during pregnancy, however there is a paucity of data regarding the expression of progesterone receptors (PR) in HIV-positive pre-eclamptic pregnancies (Papp *et al.*, 2016; Papp *et al.*, 2015). In this study, we investigated the expression of progesterone and PR in the synergy of HIV-infection and PE.

We observed a significant decrease in the immuno-expression of placental progesterone and PR in the exchange villi of pre-eclamptic compared to normotensive women, regardless of HIV status. These findings corroborate the findings of several other studies that also demonstrate decreased progesterone levels in the serum and placenta of pre-eclamptic women (Uddin *et al.*, 2015; Kiprono *et al.*, 2013; Zhang *et al.*, 2021). The downregulation of progesterone and PR immune-expression in PE placenta may be attributed to the dysregulation of steroidogenesis and cAMP signalling pathways (Feng *et al.*, 2022). The steroidogenesis pathway was found to be compromised by differential metabolites (CYP21A2, HSD3B1 and HSD3B2) and altered gene expression in PE placenta (Feng *et al.*, 2022). Notably, an alteration in the steroidogenesis pathway would directly impact the biosynthesis of progesterone, which is cholesterol-derived.

Progesterone is of paramount in the maintenance of a healthy pregnancy, and is responsible for reducing myometrial contractility, promoting immune tolerance to antigens such as trophoblasts and improving utero-placental circulation (Czajkowski *et al.*, 2007; Cable and Grider, 2020). Progesterone can exert its effect on target tissues via membrane-initiated PR signalling. The interaction of mPR with Src activates Ras/Raf/MEK/MAPK and PI3K/AKT kinase cascades (Mauvais-Jarvis *et al.*, 2021). The MAPK-dependent phosphorylation of PRs regulates posttranslational modifications required for successful protein trafficking and receptor turnover, in addition to influencing PR translocation and transcriptional activity (Mauvais-Jarvis *et al.*, 2021). The downregulation of PRs observed in our study may emanate from abnormal MAPK-dependent phosphorylation of PRs, which is detrimental to PR promotor selection and gene expression. Moreover, the reduced expression of PRs could negatively impact progesterone biosynthesis through a feedback loop.

The cross talk of cell signalling pathways such as MAPK, phosphoinositide 3-kinase (PI3K)/AKT and JAK-STAT are also implicated in trophoblast invasion and syncytialization (Gupta *et al.*, 2016; Nadeau

and Charron, 2014). A decrease in progesterone and disruption of these cell signalling pathways could hinder trophoblast invasion and differentiation. The impact of progesterone on trophoblast invasion during gestation is two-fold; progesterone promotes trophoblast invasion and prevents apoptosis of trophoblasts (Pei *et al.*, 2022). However, the shallow interstitial invasion of trophoblasts and defective spiral artery transformation has been linked to an elevation of trophoblast cells and is synonymous with PE (Naicker *et al.*, 2013), thus a decline in the progesterone and PRs observed in our study may be implicated in PE pathogenesis.

Our study also reports that the PR immuno-expression was significantly lower in the EOPE group compared to the LOPE indicating the effect of gestational age on progesterone and its receptor, regardless of HIV status. Although the incident rate of EOPE is lower than LOPE, studies have indicated that EOPE is associated with increased maternal and perinatal complications (Gomathy *et al.*, 2018; Vaddamani *et al.*, 2017). The adverse maternal and perinatal outcomes described in EOPE cases could be attributed to the significantly decreased placental expression of progesterone and PR. Whilst LOPE is considered a maternal condition associated with a genetic predisposition to cardiovascular disease, the EOPE subtype of pre-eclampsia is of placental origin, as it stems from poor placentation with deficient trophoblast invasion and reduced transformation of uterine spiral arteries (Burton *et al.*, 2019). This non physiological conversion results in arteries with smaller lumens hence blood flow is reduced causing uteroplacental hypoxia with increased oxidative stress and placental apoptosis (Marín *et al.*, 2020). The increased oxidative stress observed in EOPE may be attributed to mitochondrial dysfunction, as the mitochondria controls cell metabolism and produces reactive oxygen species (ROS) (Murphy and Hartley, 2018; Marín *et al.*, 2020). Additionally, placental progesterone is synthesized by the mitochondria of syncytiotrophoblast cells, thus mitochondrial dysfunction would result in impaired progesterone synthesis fuelling elevation of apoptosis and ROS in PE.

The distinct clinical manifestations and disease progression of EOPE and LOPE indicate the possibility of biomarkers that could distinguish between subtypes. A study by Guo *et al.* (2021) observed that classical biomarkers of PE (vascular endothelial growth factor receptor 1, leptin and Pregnancy-associated plasma protein A2), were upregulated in EOPE and expressed in extravillous trophoblasts and syncytiotrophoblasts, whilst LOPE has altered gene expression in peripheral blood. (Guo et al., 2021a). Additionally, novel biomarkers for EOPE and LOPE such as Insulin Like Growth Factor 2 (IGF2), GATA-binding factor 2 (GATA2) and Killer cell immunoglobulin-like receptor 2DL4 (KIR2DL4) were associated with differentially expressed genes of the placenta (Guo et al., 2021a). Placenta derived substances such as placental growth factor (PlGF) and placental protein 13 (PP-13) have been considered as predictive markers for PE, however the ability of these biomarkers to differentiate between EOPE and LOPE are unknown (Wu *et al.*, 2015). The downregulation of progesterone and PR in the EOPE group compared to the LOPE group in our study highlights the

potential predictor test value of progesterone and PR as biomarkers to distinguish between the two subtypes.

Our results also demonstrate a lower immuno-expression of progesterone and PR in the HIV+ group compared to the HIV- group, irrespective of pregnancy type. These results are congruent with a study by Zhou *et al.* (2018) which reported reduced plasma progesterone levels in HIV+ women in the first and second trimesters of pregnancy (Zhou *et al.*, 2018). Other studies which report lower progesterone levels in HIV+ pregnancies and linked to the use of protease inhibitor-based combined antiretroviral therapy (cART) (Papp *et al.*, 2015; Fraichard *et al.*, 2021). All women in our study received standard cART for pregnant women as prophylaxis for HIV management. Both villous trophoblast differentiation and progesterone production are impaired by the protease inhibitor, lopinavir, possibly through mitochondrial fusion and unfolded protein response (UPR) pathway activation (Fraichard *et al.*, 2021). Nonetheless, there is a growing body of evidence that suggests that protease inhibitor-based cART therapy induces morphological changes in the placenta, which may impact the organ's endocrine function thereby impairing progesterone production (Papp *et al.*, 2014; Mohammadi *et al.*, 2018; Yampolsky *et al.*, 2021).

Studies have indicated that untreated HIV-infection may be protective against PE development, however the use of cART negates this and increases the risk of developing PE (Sikhosana *et al.*, 2022; Tooke *et al.*, 2016; Phoswa *et al.*, 2019). The use of cART containing zidovudine has been associated with increased mitochondrial oxidative stress, defective VEGFR-2 signalling and increased trophoblast apoptosis, which further augments susceptibility to PE development (Hernández *et al.*, 2017). The changes in placental morphology, mitochondrial dysfunction and decrease in progesterone and PRs caused by cART could contribute to the development of PE in HIV-infected women, which is outlined in Figure 3.1.

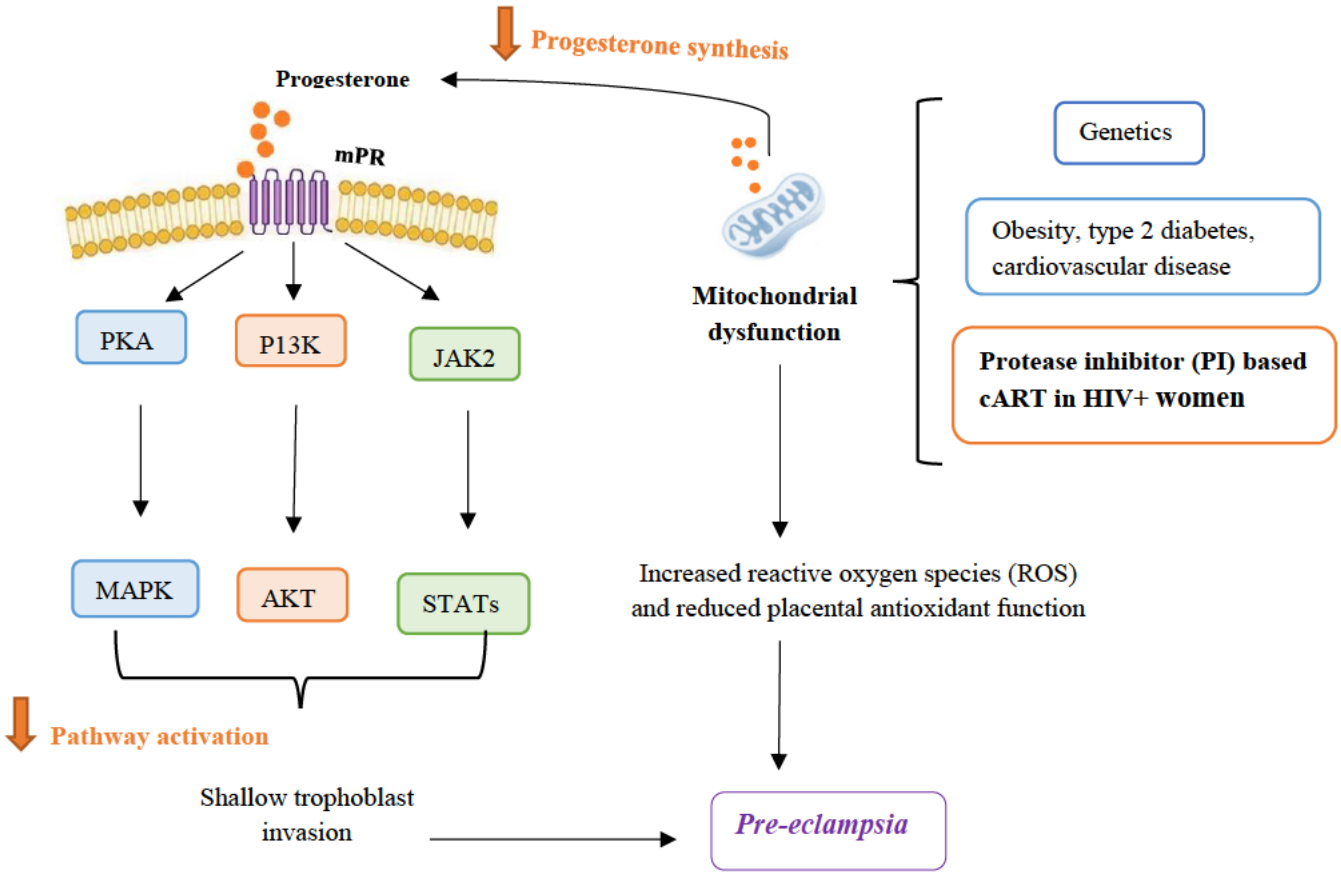

Figure 3.1. Schematic representation of pre-eclampsia pathogenesis. It is postulated that the use of PI-based cART in HIV+ pregnancies among other factors, contribute to mitochondrial dysfunction which impairs progesterone synthesis. This results in reduced binding of progesterone to PRs and decreased activation of MAPK, PI3K/AKT and JAK-STAT signalling pathways, which influence trophoblast invasion and differentiation. This contributes to the pathogenesis of pre-eclampsia.

The findings of our study indicate that villi type (exchange vs conducting) affects progesterone and PR immuno-expression, and that progesterone and PR immuno-expression are significantly higher in exchange compared to conducting villi. The exchange and conducting villi have different functions and surface area, which may attribute to the differences in progesterone and PR immuno-expression observed between the villi types. The conducting/stem villi emanate from the chorionic plate and consist of large vessels (arteries and veins) and microvessels (arterioles and venules), surrounded by a condensed fibrous stroma (Wang, 2010). As gestation progresses, fibrinoid partially replaces the trophoblast layer of the stem villi. (Wang, 2010). Although our study found no statistically significant

difference in PR immuno-expression in pre-eclamptic versus normotensive conducting villi, the immuno-expression of PRs observed in our study was limited to syncytiotrophoblast layer, of conducting villi at term may reflect higher fibrin levels with the aging placenta.

The exchange/terminal villi are grape-like structures with dense capillarization and highly dilated sinusoids. A thin basement membrane separates the fetal capillary vessels and syncytiotrophoblasts of the terminal villi. The terminal/exchange villi have smaller diameters and greater surface area than stem villi and encompass 60% of villous cross sections (Castellucci and Kaufmann, 2006). Therefore, the higher percentage of progesterone and PR immunostaining observed in exchange compared to conducting villi could be attributed to the greater surface area of exchange villi. It is notable that pregnancy type can affect villous diameter, as PE placentas are associated with reduced villous surface area and diameter (Sankar *et al.*, 2013). Additionally, Mohammadi *et al.* (2018) reported that HIV infection with cART exposure increases the number of terminal-villi capillaries and decreases progesterone levels (Mohammadi *et al.*, 2018).

A striking observation from our study is that the EOPE+ group had the lowest immuno-expression of progesterone and PRs out of the six groups reflecting effect on the deficient placentation and the effect cART. While the direct effect of HIV on progesterone remains elusive, studies have demonstrated that PI-based cART downregulates progesterone production in HIV+ pregnant women (Papp *et al.*, 2015; Fraichard *et al.*, 2021). The downregulation of progesterone could impact the development and disease progression of PE.

Limitations

One limitation of this study is that the different types of PRs (PR-A and PR-B) were excluded. The immuno-expression of PR-A and PR-B may vary across placental tissue and may be affected differently by HIV infection and PE. Another limitation would be the lack of information regarding the duration of the cART treatment received by the HIV+ participants, which could influence the outcomes.

Conclusion

This study demonstrates the downregulation of progesterone and PR immuno-expression in the exchange villi of pre-eclamptic compared to normotensive placenta. Furthermore, immuno-expression of progesterone and PR is significantly lower in HIV+ placentas, with the EOPE+ group having the lowest immuno-expression. Therefore, the presence of HIV may hinder progesterone production and signalling. This decrease in progesterone leads to insufficient binding to PRs thereby impacting signalling pathways such as the PI3K/AKT, JAK-STAT and MAPK cascades. These pathways play a crucial role in trophoblast invasion, and disruption of this signalling augments defective trophoblast invasion in EOPE. Notably, the EOPE group has lower immuno-expression of progesterone and PRs than the LOPE group, which thus links the downregulation of progesterone and PR to defective

placentation and the severity of PE. The results of this study warrants further investigation into the use of progesterone supplementation to prevent PE in HIV+ pregnancies.

Future recommendations

We recommend the investigation of progesterone and PRs as potential biomarkers for differentiating between EOPE and LOPE. Future studies could specify between PR-A and PR-B to determine the effect of HIV and PE on the different PR types in the placenta. Additionally, large-scale cohort studies could be utilised to determine the effect of progesterone supplementation on PE development in HIV+ pregnancies.

CHAPTER 4

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APPENDIX

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

28 June 2022

Miss Serisha Azaria Sewnarain (217055224)
School of Lab Med & Medical Sc
Medical School

Dear Miss Sewnarain,

Protocol reference number: BREC/00004394/2022
Project title: Placental Progesterone and its Receptor in HIV-associated Pre-eclampsia
Degree: MMedSc

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval as a **sub-study BCA338/17** and may begin as from 28 June 2022. Please ensure that any outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is valid for one year from **28 June 2022**. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

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

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2020) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

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

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 12 July 2022.

Yours sincerely,

[redacted]

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Appendix 1: Ethical Approval (BCA338/17)