THE ROLE OF SOLUBLE E-SELECTIN AND THROMBOSPONDIN-2

IN

HIV ASSOCIATED PREECLAMPSIA

By

GIRIJA NAIDOO

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PREFACE

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

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

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Girija Naidoo   **Professor Thajasvarie Naicker**

(Student number: 215028434)   (Supervisor)

DECLARATION

I, **Girija Naidoo** declare that:

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DEDICATION

To my mother Meryl for her continuous love, support, motivation and selfless sacrifice

To my father Karun for all his patience, love, care and constant encouragement

To my Most Revered Sri Venkateswara Swamy of Tirumala Hills, Tirupati, without whom, this thesis would not be possible.

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LIST OF ABBREVIATIONS

Cell Adhesion Molecules	CAMs
Early onset Preeclampsia	EOPE
Haemolysis, Elevated Liver enzymes, Low Platelet count	HELLP
Highly Active Antiretroviral Therapy	HAART
Human Immunodeficiency Virus	HIV
Interleukin-1	IL-1
Messenger RNA	mRNA
MicroRNAs	miRNAs
Placental Growth Factor	PlGF
Preeclampsia	PE
Soluble Endoglin	sEng
Soluble E-selectin	sE-selectin
Soluble Fms-like Tyrosine Kinase 1	sFlt1
South Africa	SA
Thrombospondin	TSP
Thrombospondin-2	TSP-2
Tumour Necrosis Factor-α	TNF-α
Vascular Endothelial Growth Factor	VEGF

ABSTRACT

Objective: HIV infection and hypertensive disorders of pregnancy are common causes of maternal mortality in South Africa. Preeclampsia (PE) is a pregnancy-specific disorder that contributes to the majority of maternal deaths caused by hypertension in pregnancy. Reduced placentation, endothelial dysfunction of multiple organs and inflammation occur during PE. Endothelial cells express the adhesion molecule soluble E-selectin (sE-selectin) in response to inflammation. This molecule facilitates the cohesion of leukocytes to endothelial cells.

In PE, endothelial cell activation and dysfunction cause endothelial cells to secrete the glycoprotein thrombospondin-2 (TSP-2). TSP-2 affects cellular functions, plays a regulatory role in the extracellular matrix and is an inhibitor of angiogenesis. In PE, an imbalance of angiogenic and anti-angiogenic factors result in dysregulation of angiogenesis.

Considering the high rate of maternal mortality in South Africa due to HIV infection and PE, the aim of this study was to investigate the role of sE-selectin and TSP-2 in HIV-associated preeclamptic and normotensive pregnancies.

Method: The study population ($n = 72$) comprised of normotensive pregnant ($n = 36$) and preeclamptic ($n = 36$) groups. These groups were further stratified by HIV status (negative *vs*. positive). The Bio-Plex immunoassay technique was used to measure serum concentrations of sE-selectin and TSP-2.

Results: There was a statistical difference observed in gestational age, systolic blood pressure, diastolic blood pressure and baby weight across the study groups ($p < 0.0001$).

sE-selectin: Based on pregnancy type and HIV status, levels of serum sE-selectin were significantly increased in preeclamptic HIV-negative compared to normotensive HIV-negative groups ($p = 0.0070$).

TSP-2: Regardless of HIV status and based on pregnancy type, TSP-2 levels were significantly elevated ($p = 0.0429$) in preeclamptic compared to normotensive groups. Based on HIV status, a significant upregulation ($p = 0.0095$) of TSP-2 was noted in HIV-positive compared to HIV-negative groups. Furthermore, based on pregnancy type and HIV status, levels of TSP-2 were statistically significant across all study groups ($p = 0.0229$).

Conclusion: This study highlights the role of sE-selectin and TSP-2 in preeclamptic women compromised by HIV infection and demonstrates the potential biomarker value of sE-selectin and TSP-2 in the early diagnosis of preeclampsia.

ABSTRACT (ISIZULU)

Inhloso: Ukungenwa yigcinwane leSandulela-ngculazi nezinkinga zempilo ezihlobene nephika kubantu abakhulelwe yizona eziyimbangela evamile yokushona kwabesifazane uma bebeletha eNingizimu Afrika. I-Preeclampsia (PE) wuhlobo lokugula oluhambisana nokukhulelwa okuyilona oluvame ukuba yimbangela yokushona kwababelethayo okudalwa yiphika ngesikhathi bekhulelwe. Uma umuntu ene-PE inhliziyo ishaya kancane, amaphaphu akhe nezinye izingxenye ezingaphakathi emzimbeni zingasebenzi kahle futhi abe nezinhlungu. Ngenxa yezinhlungu, izicubu zamaphaphu azibe zisavuma ukudonsa kalula umoya emaphashini okuyisimo semolekhyuli esibizwa nge-*sE-selectin*. Le molekhyuli isiza ukudluliseleni umoya onempilo ezicutshini zamphaphu.

Uma une-PE, ukusebenza nokungasebenzi kwezicubu zamaphaphu kwenza ukuthi lezi zicubu zikhiqize uketshezi olulimaza amaphrotheni i-*thrombospondin-2* (TSP-2). I-*TSP-2* iphazamisa ukusebenza kwezicubu zamaphaphu, idlale indima ekuphazamisekeni kokwakheka kwezicubu zamaphaphu futhi icindezela uketshezi olusiza ukuvuselela izicubu zamaphaphu, i-*angiogenesis*. Uma une-PE, ukungahambisani ngendlela kokwakhela nokungakheki kwezicubu ezintsha emaphashini kuholela ekutheni i-*angiogenesis* ingabe isasebenza ngendlela.

Ngokubona amazinga aphezulu okushona kwabantu bebeletha eNingizimu Afrika ngenxa yokuhaqwa yiHIV nePE, inhloso yalolu cwaningo bekungukuphenya iqhaza le-*sE-selectin* ne-*TSP-2* kubantu abakhulelwe abanomfutho wegazi ophezulu nabangenawo umfutho wegazi ophezulu.

Uhlelo Olulandeliwe: Abantu ababambe iqhaza ocwaningweni ($n = 72$) bahlanganise amaqoqo abantu abakhulelwe abangenawo umfutho wegazi ophezulu ($n = 36$) nabanomfutho wegazi ophezulu ($n = 36$). Lawa maqoqo aphinde ahlukaniswa ngesimo sawo seHIV (abahaqekile nabangahaqekile). Kusetshenziswe uhlelo lokubheka isibalo samasosha omzimba lwe-Bio-Plex ukuze kubhekwe ubungako be-*sE-selectin* ne-*TSP-2* egazini kwababambe iqhaza.

Imiphumela: Ngokweminininingwane eqoqiwe ubonakele umehluko uma kuqhathaniswa izikhathi zokukhulelwa, umfutho wegazi odalwa yizicubu nalowo odalwa wukusebenza kwemithambo kanjalo nesisindo sengane kuwona wonke amaqoqo abeyingxenye yocwaningo ($p < 0.0001$).

I-*sE-selectin*: Ngokubheka uhlobo lokukhulelwa nesimo somuntu seHIV, amazinga oketshezi lwe-*sE-selectin* abephezulu kubantu abanomfutho wegazi ophezulu abangenayo i-HIV uma kuqhathaniswa nabantu abangenawo umfutho wegazi ophezulu abangenayo iHIV (p = 0.0070).

I-*TSP-2*: Ngaphandle kokubheka isimo somuntu seHIV kodwa ngokubuka isimo sokukhulelwa, amazinga e-*TSP-2* abephezulu kubantu abanenkinga yomfutho wegazi ophezulu uma kuqhathaniswa nabangenayo le nkinga (p = 0.0429). Uma kubhekwa ngokwesimo seHIV, kugqame amazinga phezulu impela e-*TSP-2* (p = 0.0095) kubantu abaneHIV uma kuqhathaniswa nabangenayo i-HIV. Ngaphezu kwalokho, ngokubheka isimo sokukhulelwa nesimo seHIV kanye kanye, amazinga e-*TSP-2* abephezulu kuwona wonke amaqoqo (p = 0.0229).

Isiphetho: Lolu cwaningo lugqamisa iqhaza le-*sE-selectin* ne-*TSP-2* kwabesifazane abanenkinga yomfutho wegazi ophezulu abaphinde bahaqwe yiSandulela-ngculazi futhi luveza namathuba okuthi kube nendlela eqondile yokuthola ubungako be-*sE-selectin* ne-*TSP-2* ekuhlolweni kwe-PE uma isaqala.

CHAPTER 1

INTRODUCTION

1.1 Problem statement

Currently in South Africa, there is a high prevalence of maternal deaths due to HIV infection and hypertensive disorders in pregnancy. Additionally, it has also been suggested that immunosuppressive conditions such as HIV infection may impact on certain pregnancy related complications (Kalumba *et al.*, 2013). Preeclampsia (PE) is a pregnancy specific condition that results in endothelial dysfunction, excessive inflammation and dysfunction of multiple organs (Gupte and Wagh, 2014). The only effective treatment for PE is expulsion of the fetus and placenta via delivery (Pillay *et al.*, 2019). Therefore, research into the duality of HIV infection and PE is of immense importance and warrants immediate attention in view of the high incidence of maternal deaths in South Africa.

1.2 Human Immunodeficiency Virus (HIV)

HIV is a global health concern with approximately 36.9 million people living with HIV infection. Africa is one of the more severely affected regions in the world with an estimated 25.7 million people being infected (WHO, 2018). In South Africa (SA) the prevalence of HIV infection is exceedingly high with 7.97 million people being infected (StatsSA, 2019). Furthermore, a serious dilemma to gynaecologists is the fact that in 2019, one-fifth of women in the reproductive ages of 15 to 49 years are HIV positive (StatsSA, 2019). In pregnancy, HIV infection and hypertensive disorders are the commonest sources of maternal mortality and morbidity (Kalumba *et al.*, 2013).

The Human Immunodeficiency Virus (HIV) utilizes a variety of mechanisms to counteract and evade the immune system thereby developing into a later stage of disease known as the acquired immune deficiency syndrome (AIDS) (Naif, 2013). Immune cells are specifically targeted and attacked by HIV within the immune system; thereby impairing their function (WHO, 2018). HIV infects and destroys T cells by binding to CD4+ proteins on the cell surface (Naif, 2013). The resulting immunodeficiency weakens the immune system and increases susceptibility to infections, diseases and certain cancers (WHO, 2018).

1.3 Preeclampsia

1.3.1 Preeclampsia Classification

Preeclampsia is a hypertensive disorder of pregnancy (Padayachee *et al.*, 2019). The clinical stages of this disorder are characterised by new-onset high blood pressure (systolic blood pressure ≥140mmHg or diastolic blood pressure ≥90mmHg) and proteinuria (urinary protein ≥300mg per 24 hours) with such characteristics developing at or after 20 weeks of gestation (Brown, 2018). Preeclampsia may be classified by gestational age into two main subtypes known as early-onset preeclampsia (EOPE) and late-onset preeclampsia, with early-onset preeclampsia being a major cause of maternal and neonatal mortality and morbidity (Gathiram and Moodley, 2016).

1.3.2 Epidemiology

Preeclampsia is a primary obstetric concern affecting approximately 2-8% of pregnancies globally (Salimi *et al.*, 2019). In South Africa, maternal deaths due to hypertension in pregnancy are 14.8%, of which 83% are attributed to preeclampsia (NCCEMD, 2018). Specifically, the prevalence of preeclampsia in KwaZulu-Natal is 12% (NCCEMD, 2018).

1.3.3 Aetiology of preeclampsia

During normal, uncomplicated pregnancies, cytotrophoblasts invade the myometrium of the uterus and remodel the spiral arteries (Uzan *et al.*, 2011). Spiral arteries become modified into low-resistance, high capacity vessels due to the replacement of the media muscle fibers with a fibrinoid type material (Uzan *et al.*, 2011). These flaccid, large-bore vessels then decrease resistance to maternal blood flow thus allowing the efficient supply of blood (containing oxygen and nutrients) to the fetus, during a process referred to as placentation (Naicker *et al.*, 2013; Geldenhuys *et al.*, 2018; Armaly *et al.*, 2018).

The placenta creates an appropriate fetal environment (Salimi *et al.*, 2019) which is crucial for growth and development of the fetus within the uterus (Peixoto *et al.*, 2018). During pregnancy, the maternal blood supply is utilised by the placenta to enable the growth of the fetus by facilitating gaseous exchange, uptake of nutrients and elimination of waste (Belkacemi *et al.*, 2015).

Abnormal placentation or impairment of placentation may result in restriction of fetal growth, as well as preeclampsia (Peixoto *et al.*, 2018). In preeclampsia, cytotrophoblast invasion is decreased and the physiological transformations of spiral arteries are limited to the decidua (Naicker *et al.*, 2003). Early-onset preeclampsia in particular, adversely affects both the mother and fetus where growth within the uterus is restricted due to a dysfunctional placenta (Peixoto *et al.*, 2018). Mechanisms such as spiral artery thrombosis and oxidative stress within the placenta are also suggested to be involved in the pathogenesis of preeclampsia (Salimi *et al.*, 2019).

1.3.4 Pathogenesis of preeclampsia

In EOPE, cytotrophoblasts are unable to invade the lining of the uterus (Geldenhuys *et al.*, 2018) and the remodelling of myometrial spiral arteries is defective (Gathiram and Moodley, 2016). Late-onset preeclampsia arises due to placental ischaemia with the resultant increase in oxidative stress (Geldenhuys *et al.*, 2018). The ischaemic placenta causes increased trophoblast microparticle release, resulting in enhanced pro-inflammatory cytokine induction (Raghupathy, 2013). The 'systemic' stage of preeclampsia ensues as a result of endothelial dysfunction (Figure 1) (Raghupathy, 2013).
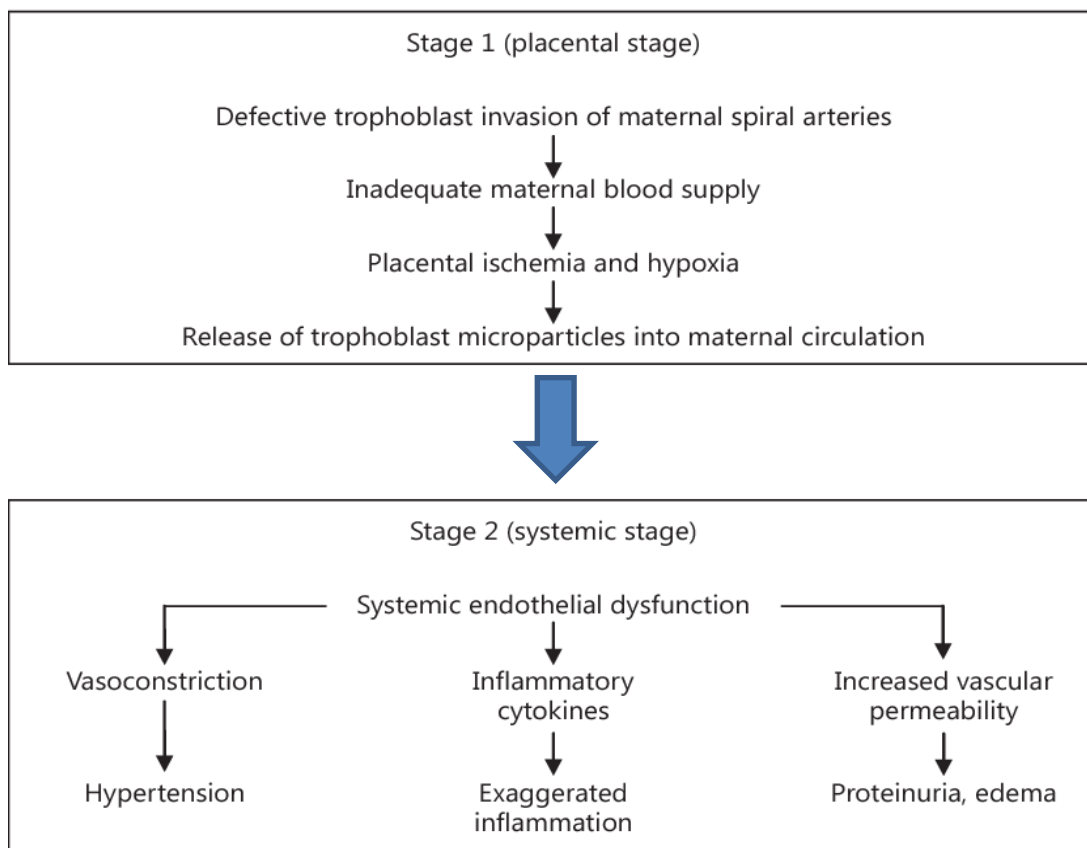
Figure 1: The stages of progression in preeclampsia. Adapted from (Raghupathy, 2013).

1.3.5 Immune maladaptation

Paternal, fetal, as well as placental antigens, are foreign to the immune system of the mother during pregnancy. Therefore, the regulation of immunological processes prevents fetal rejection and also defends the mother against infection (Geldenhuys *et al.*, 2018). The immune system of the mother permits fetal exposure to cellular as well as humoral components, thus playing an important role during pregnancy (Lokki *et al.*, 2018).

Usually Th2 polarisation occurs during pregnancy, where the balance between Th1 and Th2 phenotypes shift toward a Th2 immune response (Rana *et al.*, 2019). However, during preeclamptic pregnancies, there is an abnormal shift from the Th2 phenotype to Th1 (Rana *et al.*, 2019). Since downregulation of this Th1 immune response does not occur, there is an increase in Th1 pro-inflammatory cytokines such as interleukin-18 and tumour necrosis factor-alpha (TNF-α) (Geldenhuys *et al.*, 2018). These cytokines cause trophoblast cells to undergo apoptosis which leads to inadequate invasion of trophoblast cells (Geldenhuys *et al.*,

2018; Rana *et al.*, 2019). This pro-inflammatory state affects immune tolerance and leads to dysregulation of the immune system in preeclampsia (Geldenhuys *et al.*, 2018).

The complement system plays a vital role in maintaining immune tolerance;this involves activation as well as regulation of proteins which enable differentiation between self and foreign cells (Lokki *et al.*, 2018). The complement system is also involved in the elimination of pathogens and removal of debris by facilitating phagocytosis however, activation of this system can result in destruction of tissues, cell death and inflammation (Figure 2) (Lokki *et al.*, 2018).
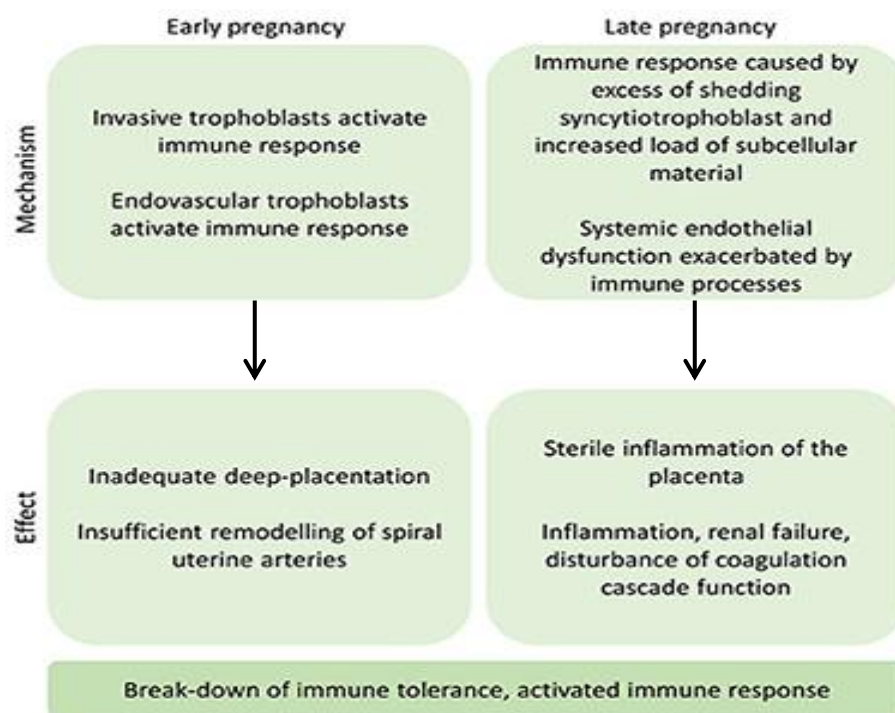
Figure 2: Mechanisms of the immune system in preeclampsia. In preeclampsia, mechanisms of the immune system play a role in both the initial and advanced pregnancy stages. Trophoblast cell invasion occurs at the bed of the placenta or within spiral arteries of the uterus. The inability of the complement system to identify these trophoblasts can result in shallow placentation and also compromise remodelling of maternal spiral arteries. During advanced stages of pregnancy, activating the complement system enables clearing of additional debris from syncytiotrophoblasts by phagocytosis

and may also lead to placental inflammation being induced. Activation of the immune system can also worsen dysfunction of the endothelium, along with a disturbance in the function of coagulation, thus causing a shift from Th2- to Th1- cells. Adapted from (Lokki *et al.*, 2018).

1.3.6 Angiogenesis

During a healthy pregnancy, angiogenesis plays a vital role in meeting the increasing fetal metabolic requirements as well as supporting growth and overall health of the fetus (Murthi *et al.*, 2014). The process of angiogenesis involves modification of differentiated endothelial cells leading to the formation of new blood vessels from pre-existing vessels (Figure 3) (Belkacemi *et al.*, 2015). The process of angiogenesis is controlled by pathways in which the expression of genes and proteins occur; dysregulation of this expression is suggested to be associated with preeclampsia development (Peixoto *et al.*, 2018).
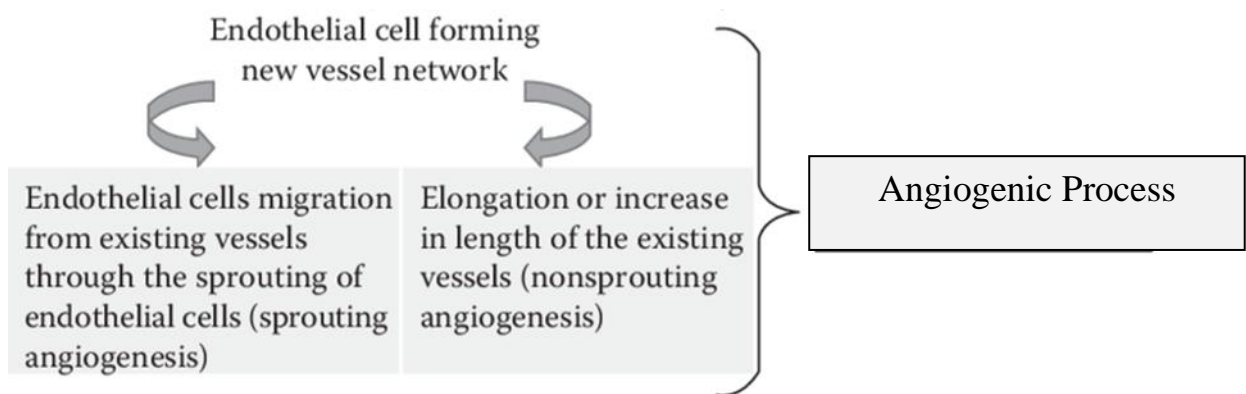
Figure 3: The process of angiogenesis. Adapted from (Belkacemi *et al.*, 2015).

1.3.7 Gene expression and preeclampsia

MicroRNAs (miRNAs) are regulatory molecules that have the ability to suppress and upregulate gene expression within various gene networks (Nejad *et al.*, 2019). These small non-coding molecules of RNA bind to a target via incomplete attachment which is complementary (Salimi *et al.*, 2019) (Nejad *et al.*, 2019). The miRNAs target messenger RNA (mRNA) in order to regulate the expression of genes (Salimi *et al.*, 2019), where each mRNA is targeted by a number of different miRNAs (Nejad *et al.*, 2019). Due to their regulation of

gene expression, miRNAs play a vital role during the process of inflammation, angiogenesis and apoptosis, which are dysfunctional in preeclampsia (Salimi *et al.*, 2019).

Placental processes, like the invasion of trophoblast cells, as well as immune activation, are regulated by miRNAs (Salimi *et al.*, 2019). During the gestation period, the placenta expresses miRNAs. The expression of these miRNAs is altered during the different pregnancy stages (Salimi *et al.*, 2019). Modified miRNA levels have also been detected within the placenta of preeclamptic individuals (Salimi *et al.*, 2019). Preeclampsia is a multi-gene, polygenic disorder of pregnancy (Peixoto *et al.*, 2018). Hence, the pathogenesis of preeclampsia may be affected by genetic alterations (Salimi *et al.*, 2019).

1.3.8 Preeclampsia risk factors

There are multiple predisposing risk factors which increase the possibility of developing preeclampsia (Table 1) (Armaly *et al.*, 2018). Conditions like antiphospholipid syndrome, chronic renal disease and previous preeclampsia are some of the risk factors associated with preeclampsia (Armaly *et al.*, 2018). Another factor is obesity which results in inflammation, insulin resistance and increased risk of developing preeclampsia (Lokki *et al.*, 2018). Furthermore, studies have shown that the onset of preeclampsia is higher in women that have fathers born from preeclamptic pregnancies (Salimi *et al.*, 2019).

Table 1: Predisposing risk factors associated with preeclampsia development. CI = confidence interval; OR = odds ratio; RR = relative risk. Adapted from (Armaly *et al.*, 2018).

Risk factor	OR or RR (95% CI)
Antiphospholipid antibody syndrome	9.7 (4.3–21.7)
Renal disease	7.8 (2.2–28.2)
Prior preeclampsia	7.2 (5.8–8.8)
Systemic lupus erythmatosis	5.7 (2.0–16.2)
Nulliparity	5.4 (2.8–10.3)
HIV+ HAART treatment	5.6 (1.7–18.1)
HIV positive (untreated)	4.9 (2.4–10.1)
Chronic hypertension	3.8 (3.4–4.3)
Diabetes Mellitus	3.6 (2.5–5.0)
Multiple Gestation	3.5 (3.0–4.2)
Strong family history of cardiovascular disease (heart disease or stroke in $\geq$2 first degree relatives)	3.2 (1.4–7.7)
Obesity	2.5 (1.7–3.7)
Family history of preeclampsia in first degree relative	2.3–2.6 (1.8–3.6)
Advanced maternal age ($>$40) for multips	1.96 (1.34–2.87)
Advanced maternal age ($>$40) for nulliparas	1.68 (1.23–2.29)

1.3.9 Complications of preeclampsia

Common maternal complications associated with severe cases of preeclampsia include placental abruption, acute kidney injury, pulmonary edema and the HELLP (haemolysis, elevated liver enzymes, low platelet count) syndrome (Machado *et al.*, 2012). The HELLP syndrome is also suggested to have similar mechanisms to preeclampsia, such as a decrease of pro-angiogenic factors, as well as an elevation of anti-angiogenic factors (Machado *et al.*, 2012). Restriction of fetal growth, neural damage caused by hypoxia and neonatal death are some of the fetal complications associated with severe cases of preeclampsia (Machado *et al.*, 2012). The management of preeclampsia includes discontinuance of pregnancy ($<$ 24 weeks), expectant management (24-32 weeks) or delivery ($\geq$ 32 weeks) (Figure 4) (Machado *et al.*, 2012).
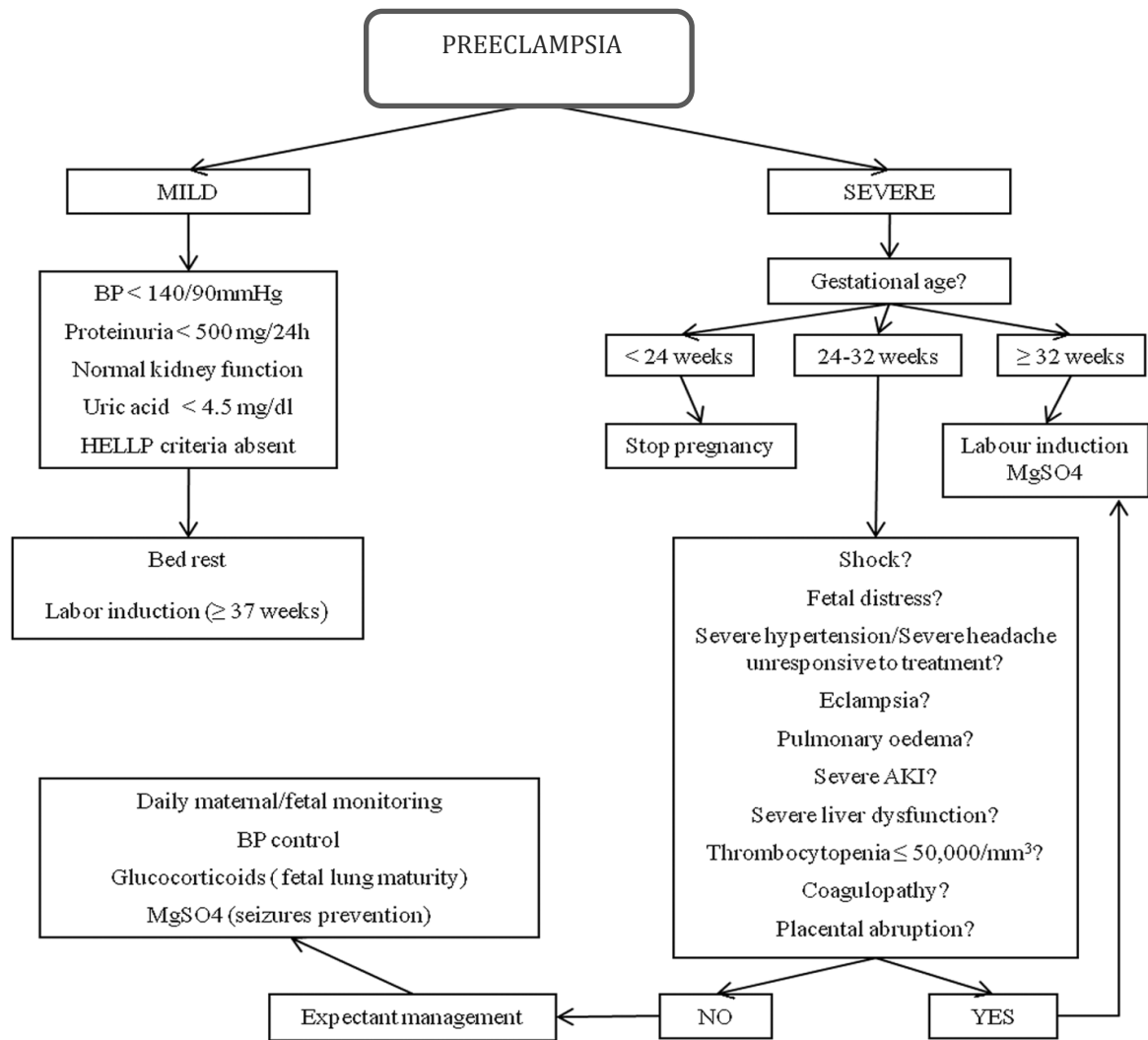
Figure 4: Proposed algorithm for the management of preeclampsia. AKI = acute kidney injury; BP = blood pressure; HELLP = haemolysis, elevated liver enzymes and low platelet count; MgSO4 = magnesium sulphate. Adapted from (Machado *et al.*, 2012).

1.4 HIV-associated preeclampsia

Both HIV infection and preeclampsia are common causes of maternal mortality (Moodley *et al.*, 2013). During normal pregnancy, an altered immune sensitivity arises, thus permitting foetal tolerance as well as maternal resistance to infection, while preeclampsia exhibits an

immune response which is exaggerated (Thakoordeen *et al.*, 2017). This exaggerated immune response is presumed to be neutralised by the decreased immune activity and lowered immune response associated with HIV infection (Thakoordeen *et al.*, 2017).

The correct use of antiretroviral therapy inhibits replication of HIV, improves the function of the immune system and decreases the risk of developing complications associated with AIDS (Deeks *et al.*, 2013). It is a standard of care practice in SA to receive highly active antiretroviral therapy (HAART) to treat HIV infection during pregnancy (Phoswa *et al.*, 2018). HAART has the ability to decrease replication of HIV in the mother, along with reducing HIV transmission from mother to offspring (Pillay *et al.*, 2019).

The link between HIV infection, the effect of HAART and the development of preeclampsia is conflicting (Pillay *et al.*, 2019). The administration of HAART induces reconstitution of the immune system, restoring the maternal immune response, which as a result, makes women more susceptible to developing preeclampsia (Moodley *et al.*, 2013). The risk of developing preeclampsia is supposedly higher in HIV-infected women compared to women that do not have HIV (Phoswa *et al.*, 2018). Preeclampsia is also purported to be more prevalent in HIV-infected patients on HAART compared to untreated HIV-infected patients due to reconstitution of the immune system initiated by HAART (Phoswa *et al.*, 2018).

1.5 Selectins

Selectins are a family of calcium-dependent glycoproteins which bind to a carbohydrate ligand and facilitate the movement of leukocytes from blood vessels into tissue (Feng, 2017). The selectin family consists of L-selectin, E-selectin and P-selectin (Vestweber, 1999). L-selectin is expressed by leukocytes and plays an essential role in the rolling of lymphocytes, while E-selectin and P-selectin are expressed by activated platelets and endothelial cells, respectively (Feng, 2017). The shared structural components of these selectins include a lectin-like domain at the NH2-terminus, an epidermal growth factor-like domain (EGF), various short consensus repeat domains (CRs), a single transmembrane region and a C-terminal cytoplasmic domain (Figure 5) (Feng, 2017) (Silva *et al.*, 2018). Soluble E-selectin (sE-selectin) in specific, is a cleavage form of E-selectin that is membrane-bound, hence the transmembrane domain and cytoplasmic domain are absent (Oh, 2007).
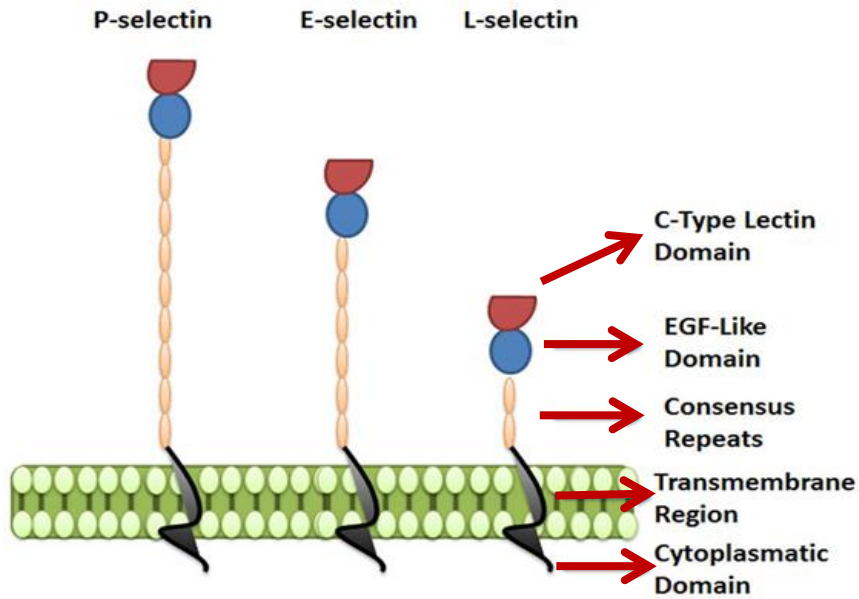
Figure 5: Structural components of selectin family members. The structural domains include a C-type lectin domain, an epidermal growth factor-like domain (EGF), a varying number of short consensus repeats, a transmembrane region and a cytoplasmic domain. Adapted from (Silva *et al.*, 2018).

1.6 The function of soluble E-selectin

The function of adhesion molecules includes the regulation of leukocyte migration into the perivascular tissue as well as the attachment of leukocytes to endothelial cells (Carty *et al.*, 2012). Interaction between leukocytes and the endothelium is facilitated by sE-selectin during leukocyte recruitment (Figure 6) (Hoffman *et al.*, 2018; Silva *et al.*, 2018).
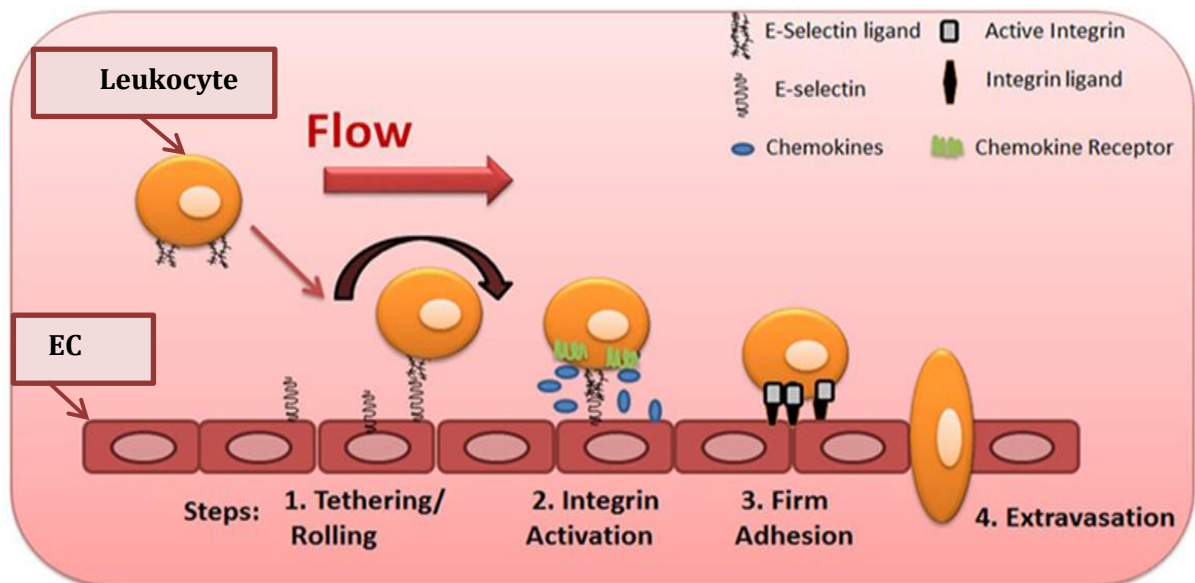
Figure 6: Leukocyte recruitment and migration. E-selectin expressed on the endothelial cell surface binds to the E-selectin ligand on the surface of leukocytes (Step 1- Tethering/Rolling). Chemokines (released by macrophages) bind to chemokine receptors found on the surface of leukocytes, resulting in the activation of integrins (Step 2- Integrin Activation). Active integrins bind to integrin ligands on the surface of leukocytes which results in adhesion of the leukocyte to the endothelium (Step 3- Firm Adhesion). The leukocyte then migrates across the endothelial barrier towards surrounding tissue (Step 4- Extravasation). EC-endothelial cell. Adapted from (Silva *et al.*, 2018).

The endothelium also regulates the activation and adhesion of neutrophils via cell adhesion molecule expression. One of the cell adhesion molecules (CAMs) that facilitate the adhesion of neutrophils to the endothelium is sE-selectin (Lyall and Greer, 1996). Neutrophil activation in many diseases including preeclampsia is associated with elevated CAM expression (Lyall and Greer, 1996). It is therefore plausible that CAMs may play a role in the activation of neutrophils which arises during preeclampsia. Since sE-selectin is an adhesion molecule it is predictable that its concentration would be higher in preeclamptic women compared to non-pregnant women (Lyall and Greer, 1996).

Cytokines such as interleukin-1 (IL-1) or TNF-α, as well as lipopolysaccharide, trigger the expression of sE-selectin (Vestweber, 1999), resulting in increased levels of this glycoprotein possibly due to the activation and damage of endothelial cells (Carty *et al.*, 2012). The anti-inflammatory cytokine interleukin-10 suppresses pro-inflammatory cytokines like TNF-α (Raghupathy, 2013). The expression of sE-selection also can be inhibited by interleukin-4

while transforming growth factor-β and glucocorticoids have the ability to counteract sE-selectin expression induced by cytokines (Vestweber, 1999).

1.7 Thrombospondins

The thrombospondin (TSP) family are a group of extracellular glycoproteins which include TSP-1, -2, -3, -4 and -5 (Lawler, 2000). These proteins aid in intercellular communication as well as communication between cells and the extracellular matrix (Lawler, 2000). They also play a role in the remodelling of vasculature, as well as cardiac remodelling (Mirochnik *et al.*, 2008). Thrombospondin-1 and 2 have similar structural domains (Mirochnik *et al.*, 2008) that comprise of an N-terminal domain (THBS-N), an oligomerization domain, a von Willebrand Factor type C (VWC) domain, three thrombospondin repeats (TSRs), and a signature domain comprising three epidermal growth factor (EGF)-like repeats, a calcium-binding wire and a lectin-like C-terminal globe (Figure 7) (Carlson *et al.*, 2008).
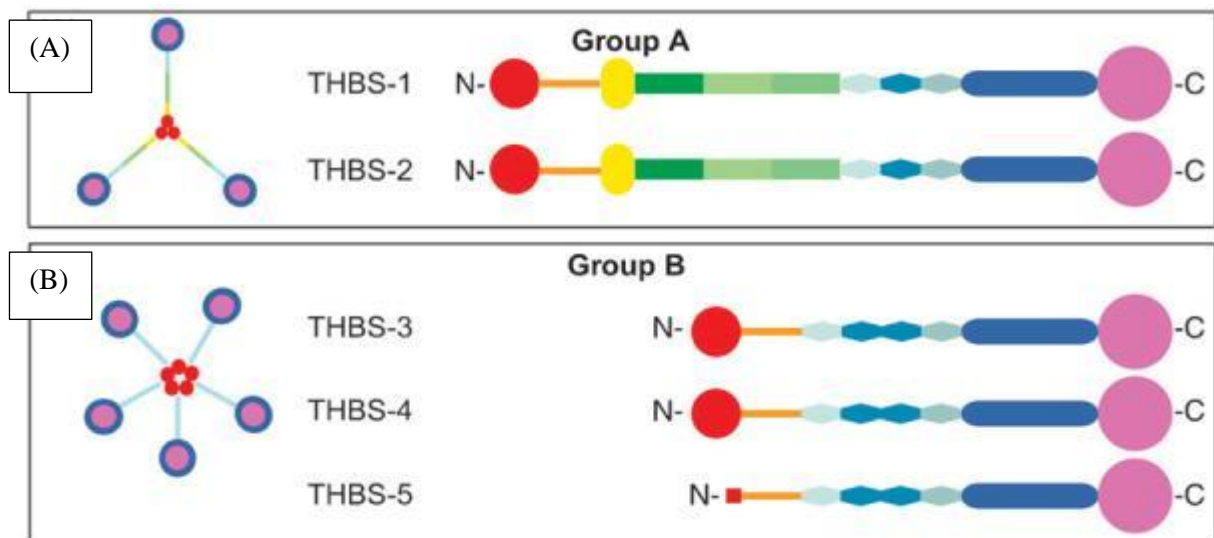
Figure 7: Modular structures of Thrombospondin (THBS) family members. THBS-1 and -2 comprise Group A and form trimers. THBS-3, -4, and -5 comprise Group B and form pentamers. The modules are coloured as follows: THBS-N (red), oligomerization coiled-coil (orange), VWC (yellow), TSR1–3 (shades of green), EGF-like repeats (shades of blue-green, with predicted or known calcium-binding repeats coloured in the darkest shade), calcium-binding wire (blue), lectin-like module (purple). The N- and C-termini are labelled. Adapted from (Carlson *et al.*, 2008).

1.7.1 Thrombospondin-1 and -2

Thrombospondin-1, which is located in the extracellular space stimulates vascular smooth muscle cell migration while also suppressing endothelial cell motility as well as chemotaxis (Mirochnik *et al.*, 2008). Thrombospondin-1 promotes apoptosis in T cells and endothelial cells (Mirochnik *et al.*, 2008). This glycoprotein also has the ability to interact and bind to receptors including CD47, CD36 and certain integrins (Krady *et al.*, 2008).

In addition to TSP-1 and -2 sharing structural similarities, they also have functional homology (Mirochnik *et al.*, 2008). One such function is the ability of both TSP-1 and -2 to interact with the low-density lipoprotein receptor, which leads to the inhibition of microvascular endothelial cell division (Krady *et al.*, 2008). Thrombospondin-1 was first recognised as an angiogenesis inhibitor; however, TSP-2 also inhibits angiogenesis (Krady *et al.*, 2008) by affecting proliferation and causing endothelial cells to undergo apoptosis (Stenczer *et al.*, 2011). While TSP-1 and -2 display anti-angiogenic properties which aid in the regulation of angiogenesis, studies have suggested that these proteins also regulate tumour angiogenesis (Krady *et al.*, 2008).

1.7.2 The function of Thrombospondin-2

Thrombospondin-2 is secreted mainly by endothelial cells. This glycoprotein plays a regulatory role in the extracellular matrix and the process of angiogenesis (Mirochnik *et al.*, 2008). Angiogenesis facilitates placental development by enhancing circulation and flow of blood within the placenta, which contributes to effective placentation (Geldenhuys *et al.*, 2018).

However, in preeclampsia, an imbalance of angiogenic and anti-angiogenic placental factors exists. More specifically, an increase in anti-angiogenic proteins such as soluble Fms-like Tyrosine Kinase 1 (sFlt1) and soluble Endoglin (sEng) is evident with a concomitant downregulation of vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) (Govender *et al.*, 2015; Ngene *et al.*, 2019).

The expression of sFlt1 occurs on endothelial, inflammatory and trophoblast cells (Lokki *et al.*, 2018). Cells of the placenta also secrete sFlt1 (Geldenhuys *et al.*, 2018). In order to fulfil its anti-angiogenic role, the sFlt1protein blocks PlGF and VEGF signalling, therefore dysregulating angiogenesis. Adapted from (Nejad *et al.*, 2019).

Although TSP-2 plays a role in angiogenesis regulation, healing of wounds and haemostasis, its anti-angiogenic properties affect platelet aggregation, stimulate endothelial cell apoptosis and restrict proliferation (Stenczer *et al.*, 2011; Krady *et al.*, 2008). Thrombospondin-2 also displays anti-inflammatory properties by stimulating anti-inflammatory regulatory T-cells which aid in the suppression of inflammation (Nakao and Morita, 2019).

1.8 Aim and objectives of this study

HIV infection and PE have a substantial impact on the high prevalence rate of maternal mortality and morbidity in South Africa. There is a dire paucity of data on the effect of sE-selectin and TSP-2 in the duality of HIV infection and PE hence the aim of this study was to determine the role of soluble E-selectin and thrombospondin-2 in the duality of HIV associated preeclampsia.

Objectives of the study include:

-To determine the effect of pregnancy type (normotensive versus preeclampsia) on the concentration of sE-selectin, irrespective of HIV status.

-To determine the effect of pregnancy type (Normotensive versus preeclampsia) on the concentration of thrombospondin-2, irrespective of HIV status.

-To determine the effect of HIV status (HIV positive versus HIV negative) on the concentration of sE-selectin, irrespective of pregnancy type.

-To determine the effect of HIV status (HIV positive versus HIV negative) on the concentration of thrombospondin-2, irrespective of pregnancy type.

-To compare and contrast the concentration of sE-selectin across the study population.

-To compare and contrast the concentration of thrombospondin-2 across the study population.

-To correlate maternal clinical and demographic findings with sE-selectin and thrombospondin-2 across the study population.

CHAPTER 2

Original Article: The role of thrombospondin-2 (TSP-2) in HIV-associated preeclampsia

Chapter 2 is an original article submitted to European Journal of Obstetrics & Gynecology and Reproductive Biology, which is a DOHET accredited international journal. It investigates the role of thrombospondin-2 (TSP-2) in HIV-associated preeclampsia. This study revealed a significant elevation of TSP-2 in preeclamptic and HIV positive pregnancies. Also, a significant upregulation of TSP-2 was found in HIV-associated preeclampsia.

Citation: Naidoo G., Nandlal L., Naicker T. (2019). The role of thrombospondin-2 (TSP-2) in HIV-associated preeclampsia. **Submitted to** *European Journal of Obstetrics & Gynecology and Reproductive Biology*, Manuscript ID: EJOGRB-19-20740

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Title: The role of thrombospondin-2 (TSP-2) in HIV-associated
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Article Type: Full Length Article

Section/Category: Obstetrics

Keywords: HIV; preeclampsia; TSP-2

Corresponding Author: Miss Girija Naidoo,

Corresponding Author's Institution:

First Author: Girija Naidoo

Order of Authors: Girija Naidoo; Louansha Nandlal; Thajasvarie Naicker

Abstract: Objective: TSP-2 is a glycoprotein that influences cellular
activities such as proliferation, motility, and apoptosis. Additionally,
it is a regulator of the extracellular matrix, inflammation and
angiogenesis. In preeclampsia, angiogenesis is dysregulated due to an
imbalance of angiogenic and anti-angiogenic placental factors. In light
of the high rate of HIV infection in South Africa, this study determined
the serum concentration of thrombospondin-2 (TSP-2) in HIV-associated
preeclampsia.

Study design: This study utilised retrospectively collected serum samples
from normotensive pregnant (n = 36) and pre-eclamptic (n = 36) groups.
The latter groups were subdivided into HIV positive and HIV negative
women. TSP-2 levels were measured using the Bio-Plex immunoassay
technique.

Results: A statistical difference was noted across the study groups for
gestational age, systolic blood pressure, diastolic blood pressure and
baby weight ($p < 0.0001$). Based on pregnancy type, a significant
elevation ($p = 0.0429$) of TSP-2 was observed in preeclamptic (median =
25.35 ng/ml; 95% CI: 34.88-28.47) compared to normotensive pregnant
(median = 24.80 ng/ml; 95% CI: 27.36-23.83) women. Regardless of
pregnancy type and based on HIV status, a significant increase of TSP-2
levels ($p = 0.0095$) was observed in HIV positive (median = 28.99 ng/ml;
95% CI: 37.41-26.98) compared to HIV negative (median = 24.80 ng/ml; 95%
CI: 28.88-21.26) women. Additionally, based on pregnancy type and HIV
status, TSP-2 levels were statistically significant across all groups ($p
= 0.0229$).

Conclusion: Our findings demonstrate a significant elevation of TSP-2
levels in preeclamptic compared to normotensive pregnancies, regardless
of HIV status. This upregulation may account for the defective
trophoblast cell invasion in preeclampsia. Furthermore, based on HIV
status, a significant upregulation of TSP-2 levels was noted and this may

be attributed to the action of tat protein. TSP-2 may be utilised as a biomarker for the early detection of preeclampsia.

Suggested Reviewers: N C Ngene
University of the Witwatersrand, Johannesburg
ngenenc@gmail.com
Research expertise lie in the field of obstetrics and gynecology.

Magaret Alese
Ekiti State University
margaret.alese@eksu.edu.ng
Basic scientist in preeclampsia research.

Opposed Reviewers: Jagidesa Moodley
jmog@ukzn.ac.za
Conflict of interest.

The role of thrombospondin-2 (TSP-2) in HIV-associated preeclampsia

Girija Naidoo[a] Louansha Nandlal[a] and Thajasvarie Naicker[a]

Affiliation

[a]Optics and Imaging Centre, Doris Duke Medical Research Institute, College of Health Sciences, University of KwaZulu-Natal, Durban, KwaZulu-Natal, South Africa

Corresponding author:

Girija Naidoo

Postal address: Private Bag 7, Congella, 4013, South Africa

Telephone No: +27 (0)31 260 4435

Fax No: +27 (0)31 260 4311

Email address: 215028434@stu.ukzn.ac.za; naickera@ukzn.ac.za

ABSTRACT

Objective: TSP-2 is a glycoprotein that influences cellular activities such as proliferation, motility, and apoptosis. Additionally, it is a regulator of the extracellular matrix, inflammation and angiogenesis. In preeclampsia, angiogenesis is dysregulated due to an imbalance of angiogenic and anti-angiogenic placental factors. In light of the high rate of HIV infection in South Africa, this study determined the serum concentration of thrombospondin-2 (TSP-2) in HIV-associated preeclampsia.

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Results: A statistical difference was noted across the study groups for gestational age, systolic blood pressure, diastolic blood pressure and baby weight ($p < 0.0001$). Based on pregnancy type, a significant elevation ($p = 0.0429$) of TSP-2 was observed in preeclamptic (median = 25.35 ng/ml; 95% CI: 34.88-28.47) compared to normotensive pregnant (median = 24.80 ng/ml; 95% CI: 27.36-23.83) women. Regardless of pregnancy type and based on HIV status, a significant increase of TSP-2 levels ($p = 0.0095$) was observed in HIV positive (median = 28.99 ng/ml; 95% CI: 37.41-26.98) compared to HIV negative (median = 24.80 ng/ml; 95% CI: 28.88-21.26) women. Additionally, based on pregnancy type and HIV status, TSP-2 levels were statistically significant across all groups ($p = 0.0229$).

Conclusion: Our findings reveal a significant elevation of TSP-2 levels in preeclamptic compared to normotensive pregnancies, regardless of HIV status. This upregulation may account for the defective trophoblast cell invasion in preeclampsia. Furthermore, based on HIV status, a significant upregulation of TSP-2 levels was noted, and this may be attributed to the action of *tat* protein. TSP-2 may be utilised as a biomarker for the early detection of preeclampsia.

KEYWORDS

HIV; preeclampsia; TSP-2

INTRODUCTION

Maternal mortality in South Africa is high due to HIV and other related infections, haemorrhage and hypertension in pregnancy (1). HIV infection affects 13.5% of the total South African population, 20% of whom are in their reproductive age (2). Preeclampsia accounts for the majority of deaths emanating from hypertension in pregnancy (1). Due to the high prevalence of these medical conditions, research into this duality is imperative.

The systemic endothelial cell activation and dysfunction in preeclampsia, causes endothelial cells to secrete thrombospondin-2 (TSP-2) (3, 4). The thrombospondin family are a group of calcium-binding glycoproteins which include TSP-1, -2, -3, -4 and -5 (5). Each isomer has distinct roles emanating from the regulation of its genetic transcription (6). Thrombospondins are a major component of platelets and the extracellular matrix (6, 7). TSP 1 and 2 have similar cohesive functions in apoptosis, platelet aggregation and inflammation (7). TSP-2 is also involved in the assembly of connective tissue components (8). Moreover, TSP-2 is highly expressed in developing blood vessels (9). TSP-2 is an inhibitor of angiogenesis which may arise from its TGF-β independent activity localised on its properdin type 1 modules (9). Angiogenesis is essential for effective placentation during pregnancy (10). However, in preeclampsia, an imbalance of angiogenic factors is evident with a decrease in pro-angiogenic factors and a concomitant elevation of anti-angiogenic factors (11). In HIV infection angiogenesis is dysregulated (12). Therefore, in light of the anti-angiogenic role of TSP-2, this study aimed to investigate, the role of TSP-2 in HIV-associated preeclamptic versus normotensive pregnancies.

METHODS AND MATERIALS

Ethical approval- Institutional ethical permission for this study (BCA338/17) was obtained from the Biomedical Research Ethics Committee, University of KwaZulu-Natal.

Study population- Post informed consent, the study population consisted of normotensive pregnant ($n = 36$) and preeclamptic ($n = 36$) participants. Both groups were further stratified by HIV status into normotensive HIV negative (n = 14), normotensive HIV positive (n = 22) , preeclamptic HIV negative (n = 18) and preeclamptic HIV positive (n = 18) pregnant women. Preeclampsia was defined as a new-onset high blood pressure (systolic blood pressure ≥140mmHg or diastolic blood pressure ≥90mmHg) together with one or more of the following conditions: proteinuria (urinary protein ≥300mg per 24 hours), maternal organ dysfunction or uteroplacental dysfunction, with such characteristics developing at or after 20 weeks of gestation (13). The exclusion criteria for the latter group was polycystic ovarian syndrome, eclampsia, chronic hypertension, intrauterine death, abruption placentae, pre-gestational or gestational diabetes, chronic diabetes, systemic lupus erythematosus, chronic renal disease, sickle cell disease, thyroid disease, antiphospholipid antibody syndrome, cardiac disease, pre-existing seizure disorders, active asthma that required medication during the gestation period, unknown HIV status as well as patients that were not booked into the hospital.

Sample type- This study utilised retrospectively collected serum samples from women who attended a large regional hospital in Durban, South Africa.

Milliplex multiplex method- The concentration of TSP-2 was quantified using the MilliPlex Human Angiogenesis Magnetic Bead Panel 2 kit according to the manufacturer's instructions (MILLIPLEX® MAP Human Angiogenesis Panel 2, catalogue no: HANG2MAG-12K). Assay buffer (200μl) was added to a 96-well plate. Thereafter, 25μl of standards, controls, assay buffer, serum matrix solution, serum samples and antibody-immobilized beads were added to the appropriate wells. The plate was then incubated with agitation at 2-8℃ overnight. After incubation, 200μl of wash buffer was used to wash the plate 3 times; detection antibodies were dispensed into each well, followed by incubation with agitation at room temperature for 1 hour. The reporter conjugate Streptavidin-Phycoerythrin was added to each well and incubated with agitation at room temperature for 30 minutes. Lastly, the plate was washed with wash buffer three times and Sheath fluid was added to each well. The Bio-Plex® MAGPIX™ Multiplex Reader (Bio-Rad Laboratories Inc., USA) was used to read the plate and the Bio-Plex Manager™ analysis software version 4.1 was used to analyse the data.

Statistical analysis- All statistical analysis was completed using GraphPad Prism version 5.00 (GraphPad Software, San Diego, California, USA). The Kolmogorov Smirnov normality test was used to determine that the data were non-parametrically distributed. A Mann-Whitney *U* test was to determine significance based on pregnancy type (normotensive *vs* preeclamptic) and HIV status (negative *vs* positive). One-way ANOVA analysis of variance test along with Dunn's *post hoc* test (for multiple comparisons) was used to determine statistical significance across all study groups. The non-parametric data were presented as median and interquartile range. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Patient demographic and clinical characteristics- Table 1 displays the demographical data and clinical characteristics of patients across the study groups. Gestational age, systolic blood pressure, diastolic blood pressure and baby weight were statistically different across the study groups ($p < 0.0001$). Gestational age was lower in preeclamptic women than in normotensive women. The systolic and diastolic blood pressures were higher in the preeclamptic group compared to the normotensive group. Baby weight was lower in the preeclamptic group compared to the normotensive group. Maternal age, maternal weight, parity and gravidity did not exhibit any statistical difference. Maternal age varied from 18 to 43 years. Maternal weight was higher in the preeclamptic HIV negative group compared to the rest of the study groups. The preeclamptic HIV positive group consisted of a twin pregnancy.

Serum concentrations of TSP-2

Pregnancy type- As shown in Figure 1A, regardless of HIV status and based on pregnancy type (normotensive *vs*. preeclamptic) a significant difference was observed in the levels of TSP-2 ($p = 0.0429$). An upregulation trend in the concentration of TSP-2 was noted in the preeclamptic (median = 25.35 ng/ml; 95% CI: 34.88-28.47) compared to the normotensive group (median = 24.80 ng/ml; 95% CI: 27.36-23.83).

HIV status- Regardless of pregnancy type, a significant difference was observed in TSP-2 ($p = 0.0095$) levels based on HIV status (negative *vs*. positive). A significant increase in the concentration of TSP-2 was noted in the HIV positive group (median = 28.99 ng/ml; 95% CI: 37.41-26.98) compared to the HIV negative group (median = 24.80 ng/ml; 95% CI: 28.88-21.26; Figure 1B).

Across all groups- Based on pregnancy type and HIV status, TSP-2 levels were statistically significant across all groups ($p = 0.0229$). The concentration of TSP-2 was higher in the preeclamptic HIV positive group (median = 37.49 ng/ml; 95% CI: 47.19-28.83) compared to the preeclamptic HIV negative group (median = 25.34 ng/ml; 95% CI: 31.72-18.96) (Table 2 and Figure 1C). The concentration of TSP-2 was also elevated in the normotensive HIV positive group (median = 26.38 ng/ml; 95% CI: 30.88-21.89) compared to the normotensive HIV negative group (median = 24.80 ng/ml; 95% CI: 29.54-20.06). Additionally, the concentration of TSP-2 was statistically significant between the normotensive HIV negative group and the preeclamptic HIV positive group ($p = 0.0290$). The concentration of TSP-2 was also statistically significant between the preeclamptic HIV negative group and the preeclamptic HIV positive group ($p = 0.0094$).

Table 1. Patient demographic and clinical characteristics across study groups ($n = 72$).

	Normotensive HIV negative (n=14)	Normotensive HIV positive (n=22)	Preeclamptic HIV negative (n=18)	Preeclamptic HIV positive (n=18)	p-Value
Maternal age (years)	24.50 (29.25-21)	24.50 (29-22)	27.50 (36.50-21.00)	27 (38.50-22)	0.5869
Maternal weight (kg)	70.10 (76-54.83)	70.35 (79.45-60.75)	87 (95-65)	74 (86.70-66.50)	0.0784
Gestational age(weeks)	38 (40.25-37)	37.50 (38.25-37)	30 (33.50-27.25)	30.50(32.25-28)	< 0.0001***
Parity	1 (1.250-0)	1 (2-0)	1 (2.250-1)	1 (2-0)	0.4408
Gravidity	2 (2.250-1)	2 (3-1)	2 (3.250-2)	2 (3-1)	0.4317
Systolic blood pressure (mmHg)	105.5 (115.5-102)	108.5 (115-100.8)	154 (165-148.5)	157.5 (169.3-146.5)	< 0.0001***
Diastolic blood pressure (mmHg)	68.50 (74.75-62.50)	71.50 (75-64.75)	102 (109-95.25)	99.50 (106.5-97)	< 0.0001***
Baby weight (kg)	3.340 (3.870-3.000)	3.00 (3.730-3.038)	1.880 (2.908-1.478)	2.175 (2.693-1.335)	< 0.0001***

Data represented as median (interquartile range); *** $p < 0.001$.

Table 2. Serum concentrations (ng/ml) of TSP-2 across all study groups.

	Normotensive HIV negative (n=14)	Normotensive HIV positive (n=22)	Preeclamptic HIV negative (n=18)	Preeclamptic HIV positive (n=18)	p-Value
TSP-2	24.80 (27.36-18.55)	26.38 (30.88-19.46)	25.34 (29.11-16.74)	37.49 (39.56-23.99)	0.0229*

Data represented as median (interquartile range); * $p < 0.05$.

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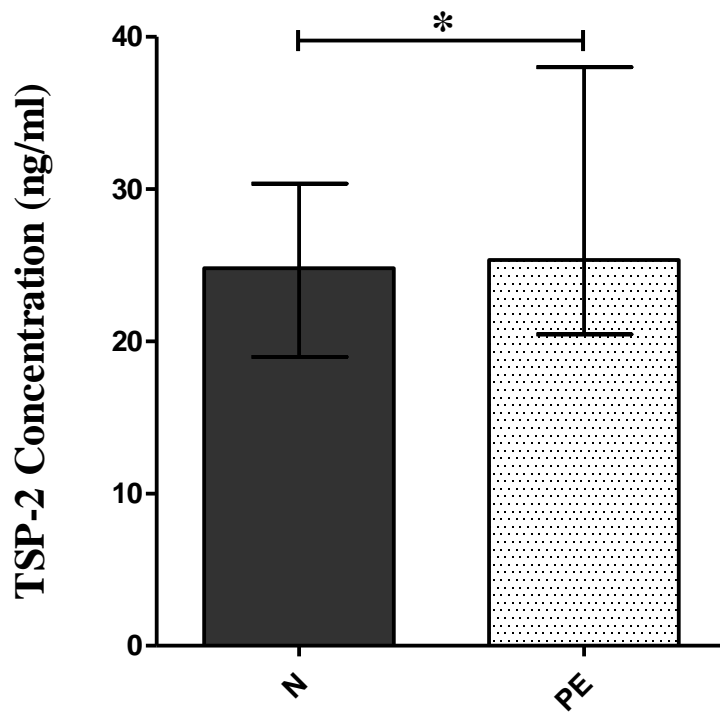
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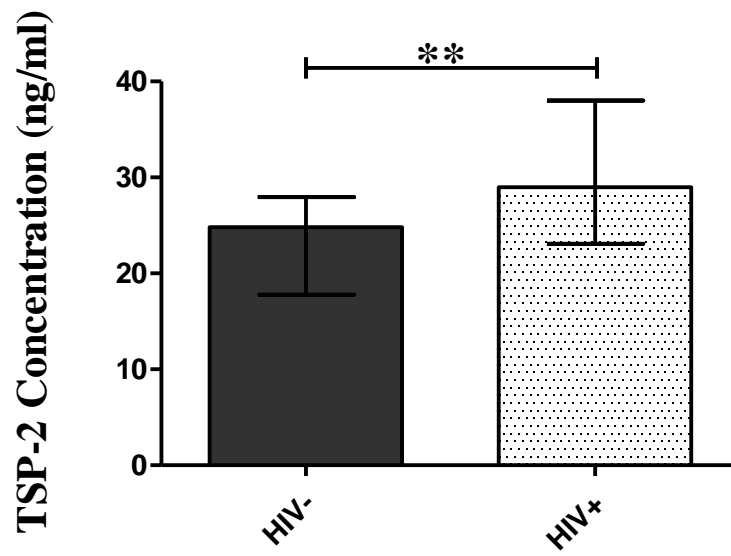
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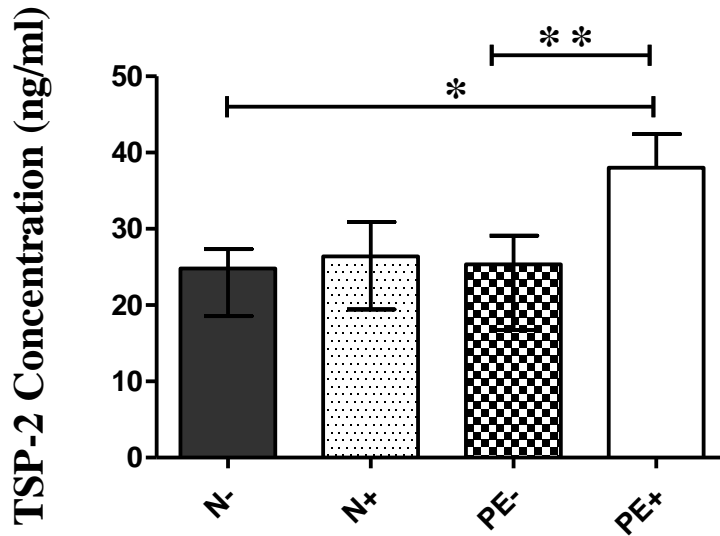
Figure 1. Serum concentrations of TSP-2 (ng/ml): **(A)** Normotensive (N) *vs* Preeclamptic (PE) groups. *Serum concentrations of TSP-2 are statistically different between the normotensive and preeclamptic group, $p = 0.0429$. **(B)** HIV-negative (HIV-) *vs* HIV-positive (HIV+) groups. **Serum concentrations of TSP-2 are statistically different between the HIV-negative and HIV-positive group, $p = 0.0095$. **(C)** Normotensive HIV-negative (N-); Normotensive HIV-positive (N+); Preeclamptic HIV-negative (PE-) and Preeclamptic HIV-positive (PE+) groups. *Serum concentrations of TSP-2 are statistically different between the normotensive HIV-negative group and the preeclamptic HIV-positive group, $p = 0.0290$. **Serum concentrations of TSP-2 are statistically different between the preeclamptic HIV-negative group and the preeclamptic HIV-positive group, $p = 0.0094$.

DISCUSSION

To the best of our knowledge, this is the first study to report upregulation of serum concentration of TSP-2 in an HIV positive preeclamptic cohort. We report a significant difference in serum TSP-2 concentration based on pregnancy type, irrespective of HIV status. TSP-2 is a calcium-binding glycoprotein that is involved in cellular activities such as apoptosis, angiogenesis and aids in communication between cells and the extracellular matrix, affecting cellular functions such as proliferation and invasion (4, 14, 15). Additionally, TSP-2 also plays a role in the remodelling of the vasculature (3, 5, 16). It is therefore plausible that TSP-2 may account for the dysfunctional invasion of trophoblast cells in preeclampsia. Preeclampsia is associated with defective trophoblast cell invasion that culminates in inhibition of myometrial spiral artery remodelling (17). Since TSP-2 regulates the bioavailability of proteases, it would regulate cell migration as well as the physiological transformation of the spiral artery (8, 18).

Notably, integrins have a vital role in regulating cell behaviour, proliferation and migration (19). Specifically, $\alpha_4\beta_1$ integrins are receptors for TSP-2 (20) therefore, the dysregulation of TSP-2 noted in our study may emanate from an aberrant integrin expression that influences trophoblast cell invasion (21).

Moreover, microRNAs play an essential role in regulating migration and invasion of trophoblasts. Recently, the overexpression of miR-221-3p was shown to stimulate the growth of trophoblasts, migration as well as invasion. In contrast, miR-221-3p knockout has an opposing effect (22). This study reported that the mRNA expression of TSP-2 was elevated in preeclampsia and was negatively correlated with miR-221-3p. These findings corroborate our findings of an up-regulation of serum TSP-2 in preeclampsia and highlight the role of TSP-2 in trophoblast cell migration.

The expression of TSP-2 is modulated by hypoxia (3). Verma *et al*. (2018) reported elevated expression of HIF-1α in preeclampsia compared to normotensive pregnancies (23). Therefore, the ischaemic microenvironment of preeclampsia could contribute to the upregulation of TSP-2 in preeclampsia, as observed in our study.

Preeclampsia is characterised by a dysfunctional endothelium and endotheliosis (24). It is also widely accepted that in preeclampsia the anti-angiogenic proteins, sFlt1 and sEng are upregulated with a concurrent decline in VEGF and PlGF (25, 26).

A study conducted by Govender *et al.* (2013) reported that the levels of anti-angiogenic soluble Fms-like Tyrosine Kinase 1 (sFlt1) are higher in HIV negative pregnancies (preeclamptic and normotensive) than in HIV positive pregnancies and suggests neutralisation of the exaggerated immune response in preeclampsia (27). sFlt1 works by blocking pro-angiogenic proteins such as placental growth factor and vascular endothelial growth factor, thus dysregulating angiogenesis (28).

TSP-2 has been demonstrated to be anti-angiogenic, pro-apoptotic and immunomodulatory (3). In our study, the elevated serum TSP-2 levels may be due to the systemic endothelial damage emanating from its angiogenic inhibitory role. The anti-angiogenic function of TSP-2 is reliant on its interaction with the CD36 receptor (29). Structurally, TSP-2 contains type 1 repeats which bind to CD36 on the membrane of endothelial cells; this binding inhibits angiogenesis by inducing endothelial cell apoptosis (30, 31).

Subsequent to the damage of tissue, thrombospondins regulate remodelling and inflammation (32). The expression of TSP-2 is elevated during the remodelling of tissue which is associated with inflammation (33). Park *et al*. (2004) showed that during an autoimmune disease such as rheumatoid arthritis, TSP-2, a constituent of the synovial microenvironment, regulates tissue inflammation (34). TSP-2 causes suppression of inflammation by activating regulatory anti-inflammatory T-cells (35). The elevated TSP-2 observed in our study reflects the hyper-inflammatory environment of preeclampsia.

In this study, TSP-2 was significantly different by HIV status with an increasing level in HIV positive women. The antiviral property of TSP-2 observed in our study is corroborated by a number of other studies (36, 37). The mechanism of action involves the binding of *tat* protein (the transactivator of HIV-1) to TSP-2 (37). *Tat* is a potent angiogenic factor due to its similar arginine and lysine-rich sequence to VEGF (38). Therefore, in our study, it is plausible that the binding of the *tat* protein to TSP-2 promotes an antagonistic angiogenic activity in HIV infection.

Additionally, the upregulation of TSP-2 observed in the HIV positive group in our study may be attributed to the binding affinity of TSP-2 to gp120 of HIV-1 via CD36 (36). More specifically the conserved regions flanking the V3 loop of gp120 provide the antiviral property for direct HIV-1 inhibitory activity of TSP.

It is important to note that the HIV positive group in this study received dual antiretroviral therapy (HAART + Nevirapine). It has been previously demonstrated that HAART may influence the HIV-1 matrix protein p17 to induce the secretion of TSP-1 (39).

Our results demonstrate significant upregulation of TSP-2 in HIV-associated preeclampsia. The effect of TSP-2 in the hypoxic, inflammatory microenvironment of preeclampsia combined with the anti-angiogenic effect of TSP-2 reduces its bioavailability for VEGF binding.

CONCLUSION

In conclusion, this study demonstrates significant upregulation of TSP-2 in preeclamptic vs normotensive pregnancies as well as when stratified by HIV status. Additionally, a significant elevation of TSP-2 in HIV-associated preeclampsia was also noted. TSP-2 has predictor test value in the early diagnosis of preeclampsia due to its role in the remodelling of vasculature, angiogenesis regulation, apoptosis and inflammation. Finally, further large scale studies are required to confirm its biomarker value for preeclampsia development.

CONFLICT OF INTEREST

There are no conflicts of interest.

ACKNOWLEDGEMENTS

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CHAPTER 3

Original Article: The role of soluble E-selectin (sE-selectin) in HIV-associated preeclampsia

Chapter 3 is an original article submitted to Archives of Gynecology and Obstetrics, which is a DOHET accredited international journal. It investigates the role of soluble E-selectin (sE-selectin) in HIV-associated preeclampsia. This study revealed a significant elevation of sE-selectin in HIV-negative preeclamptic pregnancies compared to HIV-negative normotensive groups.

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The Role of soluble E-selectin (sE-selectin) in HIV Associated Preeclampsia
--Manuscript Draft--

Abstract:

ABSTRACT
Purpose: To determine the serum concentration of soluble E-selectin (sE-selectin) in HIV associated preeclampsia.
Methods: The study population (n = 72) consisted of normotensive pregnant (n = 36) and preeclamptic (n = 36) women stratified by HIV status (negative vs. positive). Serum concentrations of sE-selectin were quantified using the Bio-Plex immunoassay.
Results: Based on pregnancy type and HIV status, serum sE-selectin levels were elevated in the preeclamptic HIV-negative group compared to the normotensive HIV-negative group (p = 0.0070). Gestational age, systolic blood pressure, diastolic blood pressure and baby weight were statistically different across the study groups (p < 0.0001).
Conclusion: This study demonstrates an elevation of sE-selectin in preeclamptic HIV-negative compared to the normotensive HIV-negative group. However, based on HIV status, there was no significant difference observed in preeclamptic HIV-positive and normotensive HIV-positive groups. Therefore, sE-selectin may be used as a biomarker or an early identifier of preeclampsia.

Other Authors:	Olive P Khaliq
	Jagidesa Moodley
	Thajasvarie Naicker
Order of Authors (with Contributor Roles):	Girija Naidoo
	Olive P Khaliq
	Jagidesa Moodley

| | Thajasvarie Naicker |

The Role of soluble E-selectin (sE-selectin) in HIV Associated Preeclampsia

Girija Naidoo[a], Olive P Khaliq[a] Jagidesa Moodley[b] and Thajasvarie Naicker[a]

[a]Optics and Imaging Centre, Doris Duke Medical Research Institute, College of Health Sciences, University of KwaZulu-Natal, Durban, KwaZulu-Natal, South Africa

[b]Department of Obstetrics and Gynecology and Women's Health and HIV Research Group, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, South Africa.

Corresponding author: Girija Naidoo

Address: Optics and Imaging Centre,

Doris Duke Medical Research Institute,

College of Health Sciences,

University of KwaZulu-Natal,

719 Umbilo Road,Durban,

KwaZulu-Natal, South Africa

Email address: 215028434@stu.ukzn.ac.za; naickera@ukzn.ac.za

Telephone number: (+27) 31 260 4435

ABSTRACT

Purpose: To determine the serum concentration of soluble E-selectin (sE-selectin) in HIV associated preeclampsia.

Methods: The study population ($n = 72$) consisted of normotensive pregnant ($n = 36$) and preeclamptic ($n = 36$) women stratified by HIV status (negative *vs*. positive). Serum concentrations of sE-selectin were quantified using the Bio-Plex immunoassay.

Results: Based on pregnancy type and HIV status, serum sE-selectin levels were elevated in the preeclamptic HIV-negative group compared to the normotensive HIV-negative group ($p = 0.0070$). Gestational age, systolic blood pressure, diastolic blood pressure and baby weight were statistically different across the study groups ($p < 0.0001$).

Conclusion: This study demonstrates an elevation of sE-selectin in preeclamptic HIV-negative compared to the normotensive HIV-negative group. However, based on HIV status, there was no significant difference observed in preeclamptic HIV-positive and normotensive HIV-positive groups. Therefore, sE-selectin may be used as a biomarker or an early identifier of preeclampsia.

KEYWORDS

HIV; preeclampsia; sE-selectin

Introduction

The Human Immunodeficiency Virus (HIV) infection is a global health concern, with sub-Saharan Africa being the region most affected by the epidemic [1]. In South Africa (SA), the prevalence of HIV infection is exceedingly high, with 7.5 million people being infected [2]. The epicentre of this epidemic is the province of KwaZulu-Natal where the prevalence of HIV infection during pregnancy is approximately 41.1% [3]. In 2018, one-fifth of women in SA, in the reproductive ages between 15 to 49 years were HIV positive; hence, this presents a sombre quandary for health care professionals [2]. Furthermore, both HIV infection and preeclampsia are common causes of maternal mortality [4].

Preeclampsia (PE) is a major obstetric concern affecting approximately 2-8% of pregnancies globally, while in SA maternal deaths caused by hypertensive disorders of pregnancy is 14.8% [5, 6]. In order for PE to be effectively treated, both the fetus and placenta require urgent delivery [7]. The placenta is crucial for the growth and development of the fetus as it continually develops and adapts to meet increasing fetal metabolic requirements [8, 9]. Maternal structural modifications also occur during healthy pregnancies where cytotrophoblasts invade the decidua and myometrium of the uterus and remodel the spiral arteries [10]. The above is necessary for efficient supply of blood (containing oxygen and nutrients) to the fetus [11]. In PE, however, cytotrophoblast invasion is limited resulting in the incomplete physiological conversion of the myometrial spiral arteries [11, 12]. The maladaptation of the uterine spiral arterioles leads to placental hypoxia/stress from reduced blood flow and physiological assessment indicates endothelial dysfunction, vasospasm, a systemic inflammatory response and widespread multi-organ involvement [13].

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The circulating adhesion molecule soluble E-selectin (sE-selectin), expressed on endothelial cells, is activated by inflammation or stress [14]. This adhesion molecule plays a vital role in transporting immune cells to areas of inflammation and facilitates interaction between circulating leukocytes and the endothelium. Ligands found on the surface of endothelial cells bind to sE-selectin resulting in leukocytes being recruited to the endothelium [14-16]. Since endotheliosis is a pathognomonic lesion of PE, levels of sE-selectin are altered in this disorder [16]. The activation and damage of endothelial cells contribute to the increasing sE-selectin levels [16]. The release of cytokines like tumour necrosis factor-alpha (TNF-α) upregulates the expression of sE-selectin [15].

Cytokines can also be categorised as T helper 1 (Th1) cytokines and T helper 2 (Th2) cytokines, where Th1 cytokines have the ability to induce inflammatory and cytotoxic reactions while Th2 cytokines are associated with humoral immunity [17]. These cytokines are involved in HIV infection pathogenesis where HIV positive individuals display a shift from Th1 immune response to Th2 immune response [18].

Activation of the immune system and release of inflammatory cytokines are associated with HIV infection [19]. The regulatory HIV protein *Tat* activates pro-inflammatory cytokine production and induces an elevation in mediators of inflammation such as sE-selectin [20]. HIV infection influences soluble adhesion molecule expression and in serum, leads to abnormal adhesion molecule levels [21].

In view of the altered sE-selectin expression in PE, there is a possibility that this protein may have value as a predictive biomarker of PE development. The identification of predictive biomarkers is a challenge as the development of PE commences in the first trimester of pregnancy, prior to the appearance of clinical symptoms [12]. Hence, further research is required in order to identify potential biomarkers that may be utilised as early indicators of PE development. Therefore, the aim of this study was to compare, for the first time, the role of sE-selectin in preeclamptic and normotensive pregnancies compromised by HIV infection.

Methods and materials

Study population

Institutional ethical approval (BCA338/17) was obtained. The study utilised serum samples retrospectively collected from women who attended a large regional hospital in Durban, South Africa. The study population ($n = 72$) consisted of normotensive pregnant ($n = 36$) and preeclamptic ($n = 36$) women. Both these groups were further stratified by HIV status. Patients unable to provide informed consent were excluded. Women with a history of other co-existing medical disorders and infections other than PE and HIV were excluded from the study. Normotensive primigravid and multigravid women constituted the control group. PE is a pregnancy-specific condition characterised by new-onset high blood pressure (systolic blood pressure ≥140mmHg or diastolic blood pressure ≥90mmHg) together with one or more of the following conditions: without proteinuria or with proteinuria (urinary protein ≥300 mg per 24 hours), maternal organ dysfunction, liver and renal injury or intra-uterine growth restriction, with such characteristics developing at or after 20 weeks of gestation [22].

MilliPlex multiplex method

The MilliPlex Human Angiogenesis Magnetic Bead Panel 2 kit was used to quantify the concentration of sE-selectin according to the manufacturer's instructions (MILLIPLEX® MAP Human Angiogenesis Panel 2, catalogue no: HANG2MAG-12K). The bead-based flow cytometric mechanism of this immunoassay enabled multiplex analyses. The assay included the incubation of the antigen sample, *i.e*., sE-selectin, with the capture of antibody-coupled beads. Subsequent to washing, which ensured the removal of unbound substances, an additional incubation with biotinylated detection antibodies was carried out. The plate was thereafter washed to remove any unbound biotinylated detection antibodies and the beads were incubated with streptavidin-phycoerythrin (SA-PE) which is a reporter conjugate. Following a third and final wash to clear the plate of excess SA-PE, the beads flowed through an array reader which determined the fluorescence emitted from bound SA-PE. The Bio-Plex® MAGPIX™ Multiplex Reader (Bio-Rad Laboratories Inc., USA) was used to read the samples and the data obtained was analysed using Bio-Plex Manager™ analysis software (version 4.1).

Statistical analysis

GraphPad Prism version 5.00 (GraphPad Software, San Diego, California, USA) was used for all statistical analysis. The data was non-parametrically distributed. Descriptive statistics were used to summarise the data and non-parametric data was presented as median and interquartile range. A Mann-Whitney U test was used to determine statistical significance based on pregnancy type (normotensive *vs*. preeclamptic) and HIV status (negative *vs*. positive). One-way ANOVA, *i.e*., a Kruskal-Wallis test as well as the Dunn's *post hoc* test (for multiple comparisons) were performed in order to determine statistical significance among all study groups. A value of $p < 0.05$ was considered to be of statistical significance.

Results

Clinical characteristics

Table 1 displays patient demographics across all study groups. Gestational age, systolic and diastolic blood pressure, as well as baby weight, were statistically significant across the study groups ($p < 0.0001$). A lower gestational age was observed in the PE group compared to the normotensive group ($p = < 0.0001$). A lower baby weight was also observed in the preeclamptic group compared to the normotensive group ($p = < 0.0001$). There was no statistical significance observed in maternal age, maternal weight, parity and gravidity (table 1).

Serum concentrations of sE-selectin

Pregnancy type

Based on pregnancy type, normotensive (mean = 110.6 ng/ml; 95% CI: 125.5-95.69) *vs*. preeclamptic (mean = 123.6 ng/ml; 95% CI: 140.4-106.7), there were no significant difference in the levels of sE-selectin ($p = 0.0708$) irrespective of HIV status as shown in Table 2 and Fig. 1a. However, an upward trend in sE-selectin level, albeit non-significant, was noted between the preeclamptic compared to the normotensive group.

HIV status

As shown in Table 2 and Fig. 1b, based on HIV status, negative (mean = 114.7 ng/ml; 95% CI: 129.8-99.65) *vs*. positive (mean = 119.4 ng/ml; 95% CI: 136.3-102.5), there was no significant difference in sE-selectin levels ($p = 0.7227$) regardless of pregnancy type. However, an increase in the concentration of sE-selectin was noted in the HIV-positive group compared to the HIV-negative group.

Pregnancy type and HIV status

Based on pregnancy type and HIV status, the concentration of sE-selectin was statistically significant between the normotensive HIV-negative and preeclamptic HIV-negative group ($p = 0.0070^{**}$) (Table 2 and Fig. 2a). Furthermore, as displayed in Table 2 and Fig. 2b-e, no significant difference was observed in normotensive HIV-positive group and the preeclamptic HIV-positive group ($p = 0.5495$), the normotensive HIV-negative and normotensive HIV-positive group ($p = 0.0557$), the preeclamptic HIV-negative and preeclamptic HIV-positive group ($p = 0.1835$) as well as normotensive HIV-negative group and the preeclamptic HIV-positive ($p = 0.0888$).

Table 1 Patient demographics in normotensive HIV negative; normotensive HIV positive; preeclamptic HIV negative and preeclamptic HIV positive pregnant women (*n* = 72)

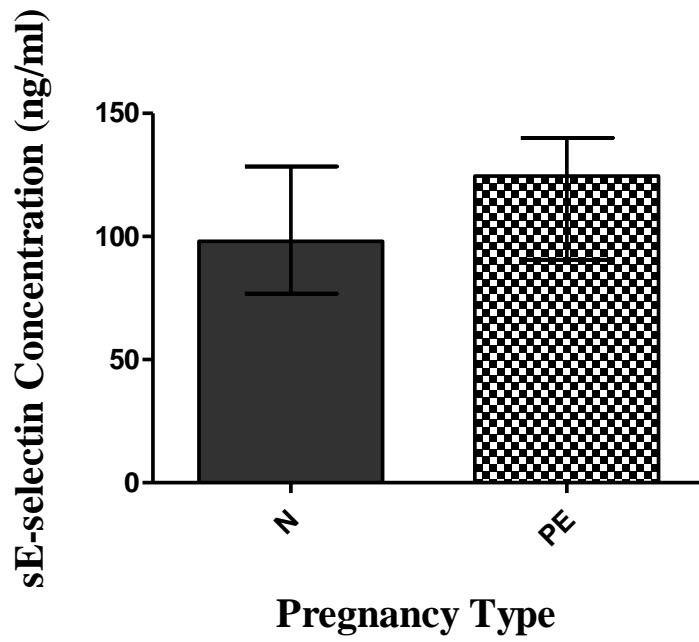|	Normotensive HIV negative (n=14)	Normotensive HIV positive (n=22)	Preeclamptic HIV negative (n=18)	Preeclamptic HIV positive (n=18)	*p*-Value
Maternal age (years)	24.50 (29.25-21)	24.50 (29-22)	27.50 (36.50-21.00)	27 (38.50-22)	0.5869
Maternal weight (kg)	70.10 (76-54.83)	70.35 (79.45-60.75)	87 (95-65)	74 (86.70-66.50)	0.0784
Gestational age (weeks)	38 (40.25-37)	37.50 (38.25-37)	30 (33.50-27.25)	30.50(32.25-28)	< 0.0001***
Parity	1 (1.250-0)	1 (2-0)	1 (2.250-1)	1 (2-0)	0.4408
Gravidity	2 (2.250-1)	2 (3-1)	2 (3.250-2)	2 (3-1)	0.4317
Systolic blood pressure (mmHg)	105.5 (115.5-102)	108.5 (115-100.8)	154 (165-148.5)	157.5 (169.3-146.5)	< 0.0001***
Diastolic blood pressure (mmHg)	68.50 (74.75-62.50)	71.50 (75-64.75)	102 (109-95.25)	99.50 (106.5-97)	< 0.0001***
Baby weight (kg)	3.340 (3.870-3.000)	3.00 (3.730-3.038)	1.880 (2.908-1.478)	2.175 (2.693-1.335)	< 0.0001***

Data represented as median (interquartile range); *** *p* < 0.001

Table 2 Serum concentrations (ng/ml) of sE-selectin Across All Groups

	sE-selectin		*p*-Value
N vs PE	98.06 (128.4-76.78)	124.6 (140.1-90.56)	0.0708
HIV- vs HIV+	99.24 (131.8-77.36)	116.8 (136.7-85.61)	0.7227
PE- vs N-	131.8 (142.4-91.59)	94.62 (106.4-61.34)	0.0070**
PE+ vs N+	107.1 (138.2-80.37)	123.5 (139.0-85.46)	0.5495
N- vs N+	94.62 (106.4-61.34)	123.5 (139.0-85.46)	0.0557
PE- vs PE+	131.8 (142.4-91.59)	107.1 (138.2-80.37)	0.1835
PE+ vs N-	107.1 (138.2-80.37)	94.62 (106.4-61.34)	0.0888

N = Normotensive, PE = Preeclampsia, HIV = Human Immunodeficiency Virus, - = HIV negative, + = HIV positive

Data represented as median (interquartile range); ** $p < 0.05$
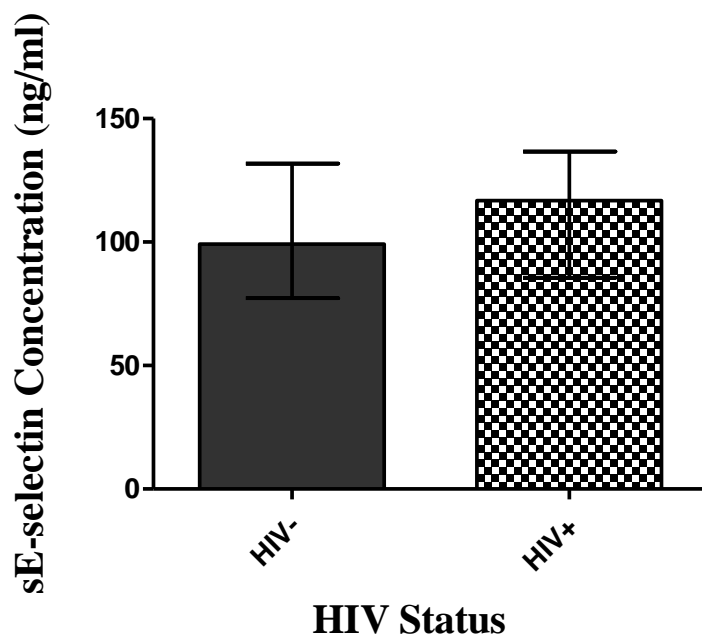
Fig. 1 (a) Serum concentrations of sE-selectin (ng/ml): Normotensive (N) and Preeclampsia (PE) groups.

(b) Serum concentrations of sE-selectin (ng/ml): HIV-negative (HIV-) and HIV-positive (HIV+) groups.

a
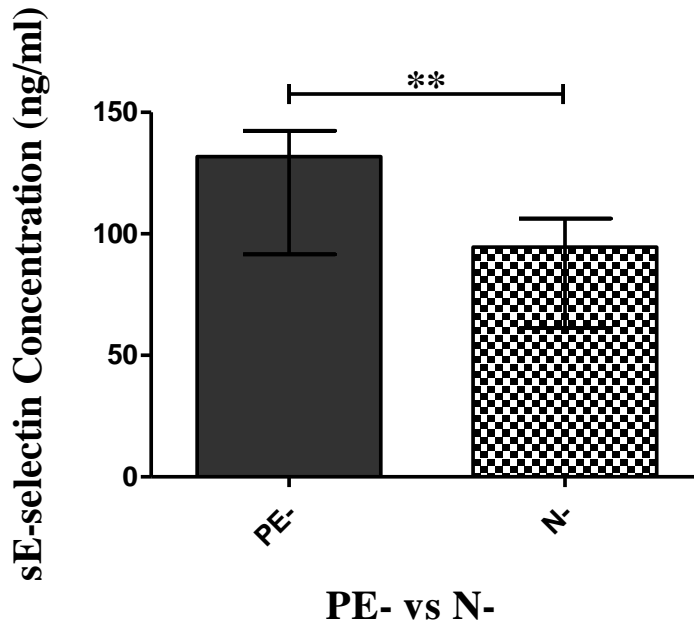
b

Fig. 2 **(a)** Serum concentrations of sE-selectin (ng/ml): Preeclamptic HIV-negative (PE-) and Normotensive HIV-negative (N-) groups. Serum levels of sE-selectin were significantly elevated in PE- groups compared to N- groups (p=0.0070[**]). **(b)** Serum concentrations of sE-selectin (ng/ml): Preeclamptic HIV-positive (PE+) and Normotensive HIV-positive (N+) groups.

Fig. 2 **(c)** Serum concentrations of sE-selectin (ng/ml): Normotensive HIV-negative (N-) and Normotensive HIV-positive (N+) groups. **(d)** Serum concentrations of sE-selectin (ng/ml): Preeclamptic HIV-negative (PE-) and Preeclamptic HIV-positive (PE+) groups.
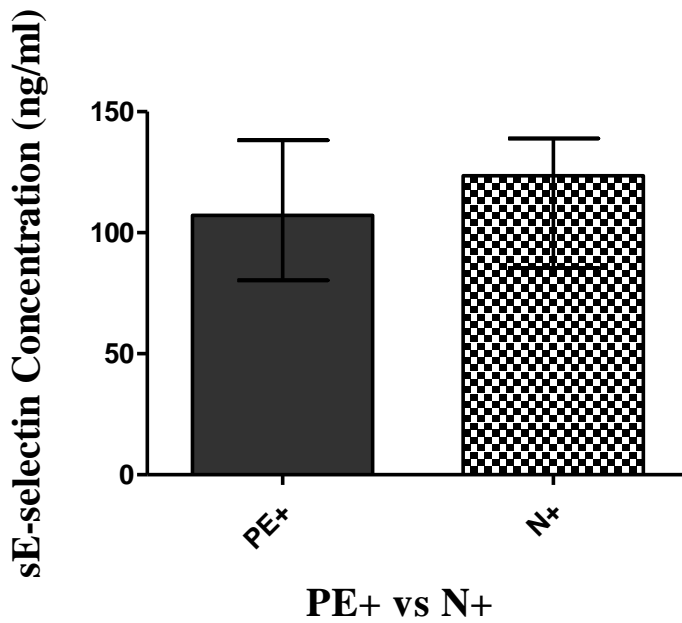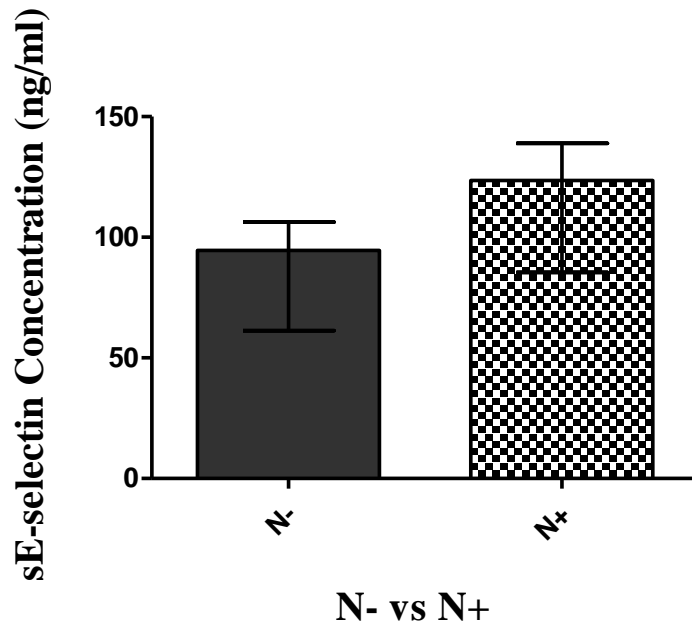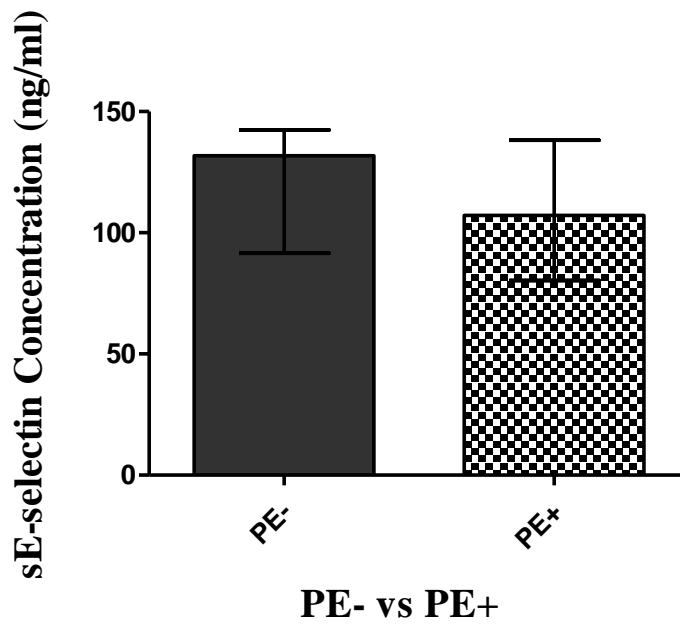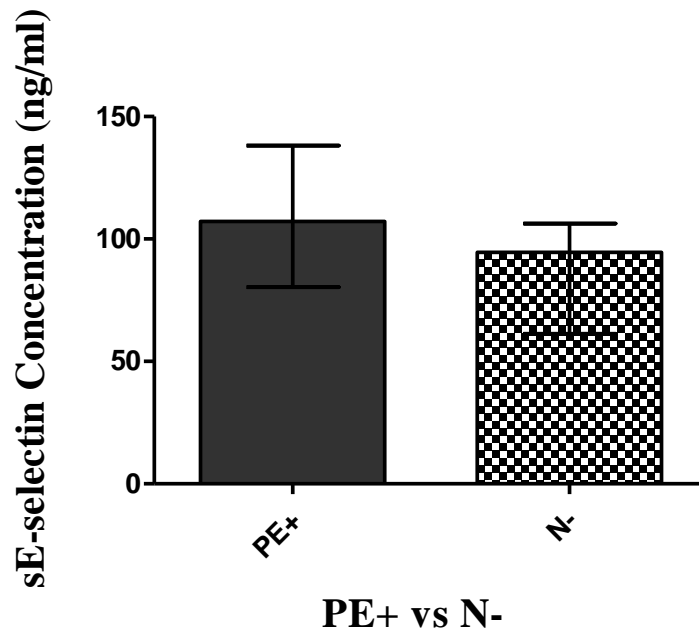
Fig. 2 (e) Serum concentrations of sE-selectin (ng/ml): Preeclamptic HIV-positive (PE+) and Normotensive HIV-negative (N-) groups.

Discussion

Based purely on pregnancy type, this study found no significant differences between the normotensive group (HIV positive and HIV negatives combined) and preeclamptic (HIV positive and negative combined) group. These findings are similar to others who reported no significant difference of sE-selectin in normotensive compared to PE pregnancies [23-25]. No significant difference was noted in normotensive positive pregnancies compared to PE positives; also, no statistical difference was found between normotensive negatives and PE positives.

The non-significance noted in the current study could be due to antiretroviral therapy given to HIV positive pregnant women. Kristofferson *et al*., (2009), investigated the effect of antiretroviral therapy in non-pregnant HIV positive patients and reported a decrease in levels of endothelial dysfunction markers in HIV positive patients [26]. Another interesting factor that comes into play is that sE-selectin has been reported to entrap itself on ligands of circulating leukocytes in order to prevent them from relocating to areas of inflammation, in this way, exaggerated inflammation is prevented [27]. This may also be the reason why this study found no significance in normotensive positive vs PE positive and normotensive negative vs PE positive groups.

Interestingly, in this study, a significant increase in levels of sE-selectin was found in preeclamptic HIV negative compared to normotensive HIV negative groups (p=0.0070[**]). Carty *et al*., (2012) studied levels of sE-selectin in pregnancies complicated with PE alone and reported elevated levels of sE-selectin in early pregnancies that later developed into PE. The pathophysiology of PE involves poor placentation, endothelial dysfunction, inflammation and oxidative stress [16]. Soluble E-selectin is a cell adhesion molecule found on endothelial cells [16]. The main function of sE-selectin is to control leukocyte cohesion on endothelial cells and the migration of white blood cells to perivascular tissue during inflammation [14]. Therefore, upregulation of sE-selectin is an indication of endothelial dysfunction, which is one of the clinical features of PE.

Regardless of pregnancy type, but based purely on HIV status, an upward trend was noted in normotensive positive compared to normotensive negative pregnancies albeit non-significantly. sE-selectin levels between PE negatives and PE positives showed no significant difference; however the PE positive group showed an upward trend of sE-selectin compared to PE negatives. Levels of sE-selectin showed no significant difference between the HIV positive group and the HIV negative group, with an upward trend observed in the HIV positive group.

Our results agree with findings of Hoffman *et al*., (2018) who reported no significant difference in sE-selectin levels in HIV positive compared to HIV negative non-pregnant patients [14]. Hoffman *et al*., (2018) observed levels of sE-selectin in chronic HIV positive patients who were on treatment and discovered no correlation of the adhesion molecule with inflammation [14]. In addition, Rönsholt *et al*. (2013) reported a decrease in sE-selectin in patients on HIV treatment compared to untreated patients [28]. More interestingly, Graham *et al*., (2013) described levels of sE-selectin to be elevated in HIV positive acute patients compared to HIV positive chronic patients which may indicate that the admission of HAART may decrease levels of inflammation in HIV positive patients who have been on chronic HIV treatment [29]. In the current study, HIV positive patients showed an upward trend of sE-selectin, probably due to immune activation during infection. According to Rönsholt *et al*., (2013), levels of sE selectin are altered due to HIV treatment but are not regulated to normal levels noted in HIV negative patients [28].

As far as we are aware, this is the first study to report sE-selectin levels in preeclamptic patients with HIV infection. Furthermore, it is standard practice for pregnant individuals to be tested for HIV and to be treated accordingly. Our results showed no significant difference in levels of sE-selectin in preeclamptic HIV positive patients compared to preeclamptic HIV negative patients. However, an upward trend was noted in preeclamptic HIV positive patients. This may be due to inflammation triggered by cytokines such as IL-1 and the tumour necrosis factor-alpha (TNF-α). Tumour necrosis factor-alpha activates sE-selectin [15]. Furthermore, Calza *et al*., (2009) reported a correlation between sE-selectin, viral load and $CD4^+$ in HIV non-pregnant positive patients [30]. In contrast, Rönsholt *et al*., (2013) found no correlation between sE-selectin, viral load and $CD4^+$ in non-pregnant HIV positive patients.

The HIV protein *Tat*, is a protein that elevates the release of pro-inflammatory cytokines and upregulates the expression of sE-selectin by functioning as a cytokine during endothelial cell activation [31]. The *Tat* protein also stimulates the upregulation of the adhesion molecule ICAM-1 on endothelial cells, while also upregulating mediators of inflammation such as VCAM-1 to inhibit cell migration [31, 32], hence the upward trend noted in preeclamptic HIV positive patients in this study.

Strengths and Limitations

This study is the first to report on levels of sE-selectin in individuals with both HIV and PE. However, larger sample size is required to confirm our findings. Also important to note, the HIV patients were not grouped according to their duration and type of treatment.

Conclusion

This study demonstrates upregulation of sE-selectin in HIV negative preeclamptic compared to normotensive HIV negative pregnancies. Soluble E-selectin could possibly be used diagnostically, as an early indicator of PE development due to its role in endothelial dysfunction; however; further large scale studies are required.

Author's contribution

G Naidoo: Project development, data analysis and manuscript writing.

OP Khaliq: Manuscript writing.

J Moodley: Manuscript writing and editing.

T Naicker: Project development and manuscript editing (Supervisor).

Compliance with ethical standards

Conflict of interest

There are no conflicts of interest.

Ethical approval

Ethical approval was obtained from the Biomedical Research Ethics Committee, University of KwaZulu-Natal (reference number: BCA338/17).

Statement of human rights

This is a retrospective study; hence patient demographics were recorded from patient data forms available at the Optics and Imaging Centre, Doris Duke Medical Research Institute, College of Health Sciences, University of KwaZulu-Natal.

Informed consent

Informed consent was obtained from patients; patients unable to provide informed consent were excluded from this study.

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CHAPTER 4

4.1 SYNTHESIS

HIV infection and preeclampsia are common causes of maternal mortality in SA (Saving-Mothers-Report, 2017). Preeclampsia (PE) only occurs during pregnancy with characteristic features that include impaired placentation, systemic inflammation, as well as endothelial dysfunction (Rana *et al.*, 2019). The circulating adhesion molecule sE-selectin is a marker of endothelial dysfunction (Hoffman *et al.*, 2018). The activation and damage of endothelial cells lead to increased levels of this adhesion molecule (Carty *et al.*, 2012). In PE, endothelial cell activation and dysfunction results in endothelial cells secreting the glycoprotein TSP-2; this indicates a possible association between TSP-2 and PE (Stenczer *et al.*, 2011). The development of PE begins during the early stages of pregnancy and is followed by the

manifestation of clinical symptoms (Gathiram and Moodley, 2016). This makes it difficult to identify predictor tools that can be utilised in the early diagnosis of PE. Taking into consideration the altered levels of sE-selectin and TSP-2 in PE, these analytes may have value as early indicators of PE. Therefore, the aim of this study was to investigate the role of sE-selectin and TSP-2 in HIV-associated PE.

Based solely on pregnancy type, this study reports no significant difference of sE-selectin between normotensive (HIV positive and HIV negative combined) and preeclamptic (HIV positive and HIV negative combined) pregnancies. sE-selectin is a marker of endothelial dysfunction that is elevated in conditions associated with inflammation (Raffray *et al.*, 2017; Hoffman *et al.*, 2018).

This study demonstrates no significant difference of sE-selectin expression between normotensive HIV-positive compared to the preeclamptic HIV-positive groups. In addition, the normotensive HIV-negative and preeclamptic HIV-positive groups also did not differ significantly. The administration of antiretroviral therapy to pregnant HIV-positive women may account for the non-significance reported in this study. While the HIV regulatory protein *Tat* stimulates endothelial dysfunction by binding to integrin receptors, HIV-positive patients receiving antiretroviral therapy have been reported to exhibit decreased levels of endothelial dysfunction markers (Kristoffersen *et al.*, 2009; Anand *et al.*, 2018). Interestingly, sE-selectin can remain attached to ligands present on the surface of circulating leukocytes thereby preventing these cells from migrating to inflammatory sites. This ability of sE-selectin prevents destructive inflammation during exaggerated leukocyte release and could explain the non-significance found in this study between the normotensive HIV-positive and preeclamptic HIV-positive groups as well as between the normotensive HIV-negative and preeclamptic HIV-positive groups (Raffray *et al.*, 2017).

A significant elevation of sE-selectin was also observed between the preeclamptic HIV-negative group and the normotensive HIV-negative group ($p=0.0070^{**}$). The concentration of circulating adhesion molecules are elevated in PE (Lylla *et al.*, 1999). Increased levels of sE-selectin have been reported in early pregnancies which were later complicated with PE (Carty *et al.*, 2012). Reduced placentation, placental ischaemia with resultant increase in oxidative stress, dysfunction of the endothelium and inflammation contribute to the pathophysiology of PE (Geldenhuys *et al.*, 2018). Soluble E-selectin is synthesised and transported to the endothelial cell surface where it facilitates the adhesion of leukocytes to endothelial cells (Kim *et al.*, 2004; Feng, 2017). During inflammation, sE-selectin regulates the movement of white blood cells into perivascular tissue (Hoffman *et al.*, 2018). Hence, an elevation of sE-

selectin indicates dysfunction of the endothelium, which is involved in the pathophysiology of PE.

Irrespective of pregnancy type and based solely on HIV status, this study reports no significant difference between normotensive HIV-positive and normotensive HIV-negative groups, however the normotensive HIV-positive group exhibited an upwards trend of sE-selectin compared to the normotensive HIV-negative group. Although levels of sE-selectin were non-significant between the preeclamptic HIV-negative and preeclamptic HIV-positive groups, an upwards trend of sE-selectin was observed in the preeclamptic HIV-positive compared to the preeclamptic HIV-negative group. No significant difference was observed in sE-selectin levels between the HIV-positive and HIV-negative groups, however the HIV-positive group showed an upwards trend of sE-selectin compared to the HIV-negative group.

Hoffman *et al*., (2018) reported that levels of sE-selectin did not differ significantly in HIV positive compared to HIV negative non-pregnant patients which is in agreement with the results reported in this study (Hoffman *et al.*, 2018). Furthermore, decreased sE-selectin levels have been reported in HIV positive patients receiving treatment compared to patients not receiving HIV treatment (Rönsholt *et al.*, 2013). It is also interesting to note that sE-selectin levels are reported to be elevated in patients with acute HIV compared to patients with chronic HIV (Graham *et al.*, 2013). This could be indicative of HAART admission possibly lowering inflammation levels of patients that are on chronic HIV treatment. The immune activation that occurs during HIV infection may account for the upwards trend of sE-selectin noted in the HIV-positive group of this study. In addition, HIV treatment reportedly alters sE-selectin levels but does not regulate it to the normal levels observed in patients that are HIV-negative (Rönsholt *et al.*, 2013).

To the best of our knowledge, this is the first study to report dysregulated levels of sE-selectin in preeclamptic patients compromised by HIV infection. Notably, during pregnancy, testing for HIV and receiving appropriate treatment regimens is standard practice. In this study, levels of sE-selectin showed no significant difference between the preeclamptic HIV-positive group and the preeclamptic HIV-negative group, with an upwards trend observed in the preeclamptic HIV-positive group. This could be explained by cytokines such as IL-1 and TNF-α eliciting an inflammatory response. Tumour necrosis factor-alpha is also responsible for the activation of sE-selectin.

Furthermore, the upwards trend of sE-selectin observed in the preeclamptic HIV-positive group in this study may be due to the HIV protein *Tat*. The *Tat* protein functions as a cytokine

during the activation of endothelial cells thereby elevating the release of pro-inflammatory cytokines and upregulating sE-selectin expression (Jiang *et al.*, 2018). This HIV protein induces elevation of the ICAM-1 adhesion molecule on endothelial cells and also upregulates VCAM-1 which is a mediator of inflammation that inhibits cell migration (Jiang *et al.*, 2018;Padayachee *et al.*, 2019).

As far as we know, this is the first study to report that TSP-2 is upregulated in preeclamptic patients with HIV infection. Regardless of HIV status and based on pregnancy type, this study reports a significant difference in the serum concentration of TSP-2. TSP-2 is an extracellular glycoprotein that affects the proliferation as well as invasion of cells by aiding in communication between cells and the extracellular matrix. TSP-2 is also involved in the remodelling of vasculature and could possibly explain the defective trophoblast cell invasion of PE (Lawler, 2000;Mirochnik *et al.*, 2008;Stenczer *et al.*, 2011). Since the bioavailability of proteases is regulated by TSP-2, cell migration together with physiological conversion of spiral arteries would also be regulated by TSP-2 (Pellerin *et al.*, 1994;Bornstein *et al.*, 2000).

Additionally, microRNAs are involved in the regulation of trophoblast migration and invasion. The growth of trophoblasts, migration as well as invasion is reported to be stimulated by overexpression of miR-221-3p, while the knockout of this microRNA is shown to have the opposite effect (Yang *et al.*, 2019). Moreover, Yang *et al*., (2019) discovered an elevation of TSP-2 microRNA expression in PE which was also negatively correlated with miR-221-3p (Yang *et al.*, 2019). The findings of Yang *et al*., (2019) corroborate the upregulation of TSP-2 in PE observed in this study.

TSP-2 expression is regulated by hypoxia (Stenczer *et al.*, 2011). The expression of HIF-1α is reported to be elevated in preeclamptic pregnancies (Verma *et al.*, 2018). Hence, the TSP-2 upregulation in preeclamptic pregnancies noted in this study may be due to the ischaemic microenvironment associated with PE.

Dysfunction of the endothelium and endotheliosis are characteristic features of PE (Sani *et al.*, 2019). In PE, there is an elevation of anti-angiogenic proteins such as sFlt1 and sEng which is accompanied by a concomitant downregulation of VEGF and PlGF (Govender *et al.*, 2015;Ngene *et al.*, 2019). Levels of sFlt1 are reported to be higher in HIV-negative (preeclamptic and normotensive) compared to HIV-positive pregnancies, therefore suggesting that the exaggerated immune response in PE is neutralised (Govender *et al.*, 2013). Soluble Fms-like Tyrosine Kinase 1 (sFlt1) dysregulates angiogenesis by blocking the function of pro-angiogenic proteins such as PlGF and VEGF (Nejad *et al.*, 2019).

The systemic endothelial damage which arises from the angiogenic inhibitory action of TSP-2 may account for the upregulation of TSP-2 reported in this study. The angiogenic nature of TSP-2 results in systemic endothelial damage. Interaction between TSP-2 and the CD36 receptor is responsible for the anti-angiogenic properties displayed by TSP-2 (Silverstein and Febbraio, 2009). The binding of TSP-2 to CD36 induces endothelial cell apoptosis and thus inhibits angiogenesis (Koch *et al.*, 2011;Fei *et al.*, 2017).

TSP-2 plays a role in assembling constituents of connective tissue and is upregulated during the remodelling of tissue (Bornstein *et al.*, 2000;Bornstein *et al.*, 2004). TSP-2 is reported to regulate tissue inflammation in rheumatoid arthritis and also has the ability to activate regulatory anti-inflammatory T-cells thereby acting as a suppressor of inflammation (Park *et al.*, 2004;Papageorgiou *et al.*, 2012). The elevation of serum TSP-2 noted in this study is indicative of the exaggerated inflammatory state seen in PE.

Based on HIV status, this study reports a significant upregulation of TSP-2 in HIV-positive women. TSP-2 binds to the HIV protein *Tat* (Rusnati et al., 2000). This protein is similar to VEGF in sequence which makes it a powerful angiogenic factor (Zhou *et al.*, 2013). Hence, in this study it is possible that an antagonistic angiogenic activity is stimulated in HIV infection when TSP-2 binds to the *Tat* protein.

In addition, the inclination of TSP-2 to bind to the HIV protein gp120 via CD36 could explain the elevated levels of TSP-2 noted in the HIV-positive group of this study (Crombie, 2000). Notably, in this study, all HIV-positive women received dual antiretroviral therapy (HAART + Nevirapine). HAART also has been reported to possibly stimulate TSP-1 secretion by influencing p17 which is a matrix protein of HIV (Caccuri *et al.*, 2014).

This study also reports a significant elevation of TSP-2 in HIV-associated PE. The bioavailability of TSP-2 for VEGF binding is reduced due to the anti-angiogenic properties displayed by TSP-2 together with its role in the hyper inflammatory, hypoxic microenvironment of PE.

4.2 CONCLUSION

To the best of our knowledge, this is the first study to report on the serum concentrations of sE-selectin and TSP-2 in HIV-associated preeclamptic and normotensive pregnancies. A

significant upregulation of sE-selectin was observed in preeclamptic HIV-negative compared to normotensive HIV-negative pregnancies. However, based on HIV status, there was no significant difference of sE-selectin expression observed between the preeclamptic HIV-positive and normotensive HIV-positive groups. Furthermore, a significant elevation of TSP-2 was demonstrated in preeclamptic compared to normotensive pregnancies, irrespective of HIV status. Based on HIV status, a significant upregulation of TSP-2 was observed in HIV-positive women and in HIV-associated PE. This study therefore reflects the biomarker value of sE-selectin and TSP-2, as early indicators of PE development.

4.3 FUTURE RESEARCH

In light of the role that sE-selectin plays during dysfunction of the endothelium, this adhesion molecule could possibly be used as an early indicator of PE. The role of TSP-2 in vasculature remodelling, inflammation and as a regulator of angiogenesis makes it suitable to be used diagnostically for the early detection of PE. Lastly, further large scale studies to confirm the biomarker value of sE-selectin and TSP-2 in PE is crucial as this will aid in developing therapeutic interventions and specialised care for affected women.

CHAPTER 5

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APPENDICES

APPENDIX 1

24 May 2019

Prof T Naicker
Discipline of Optics and Imaging
School of Laboratory Medicine and Medical Sciences
naickera@ukzn.ac.za

Dear Prof Naicker

Title of Project: Exploring the pathogenesis HIV associate pre-eclampsia syndrome in a homogenous South African population group.
BREC Ref No.: BCA338/17

We wish to advise that your request dated 30 April 2019 to add the studies below to the above study has been noted and approved by a sub-committee of the Biomedical Research Ethics Committee.

Studies added to the above study:

| STUDENT	TITLE	DEGREE
Seke Nzau MAfuika	The role of Tenascin C in HIV associated preeclampsia	MMedSci
Samukelisiwe Sibiya	The role of human complement proteins C3b/IC3b and C4 in HIV associated preeclampsia	MMedSci
Phumelelle Kikine	The role of human complement proteins Factor B and Factor P/Properdin in HIV associated preeclampsia	MMedSci
Zinhle Pretty Mlambo	The role of Apolipoprotein A1 and A2 in HIV associated preeclampsia	MMedSci
Saieshni Pillay	The role of VEGFR-3 in the placenta and placental bed in HIV associated preeclampsia	MMedSci
Girija Naidoo	The role of soluble E-selectin and Thrombospondin-2 in HIV associated preeclampsia	MMedSci
Mbuso Herald Mthembu	The role of Endothelin-1 in HIV associated preeclampsia	MMedSci

The committee will be notified of the above approval at its next meeting to be held on 11 June 2019.
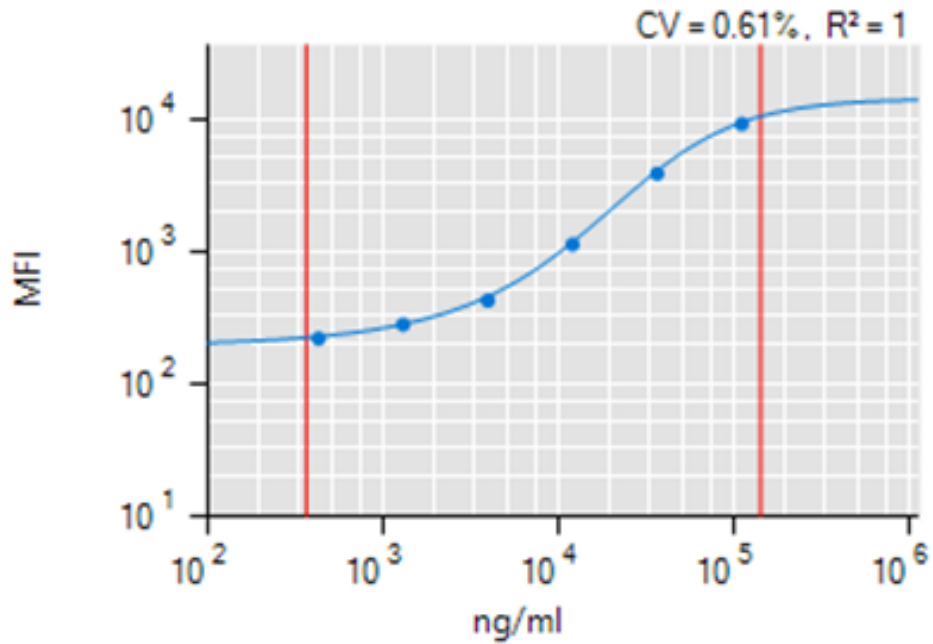
Yours sincerely

Prof V Rambiritch
Chair: Biomedical Research Ethics Committee

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APPENDIX 2

E-Selectin (30)

CV = 1.45%, R² = 1
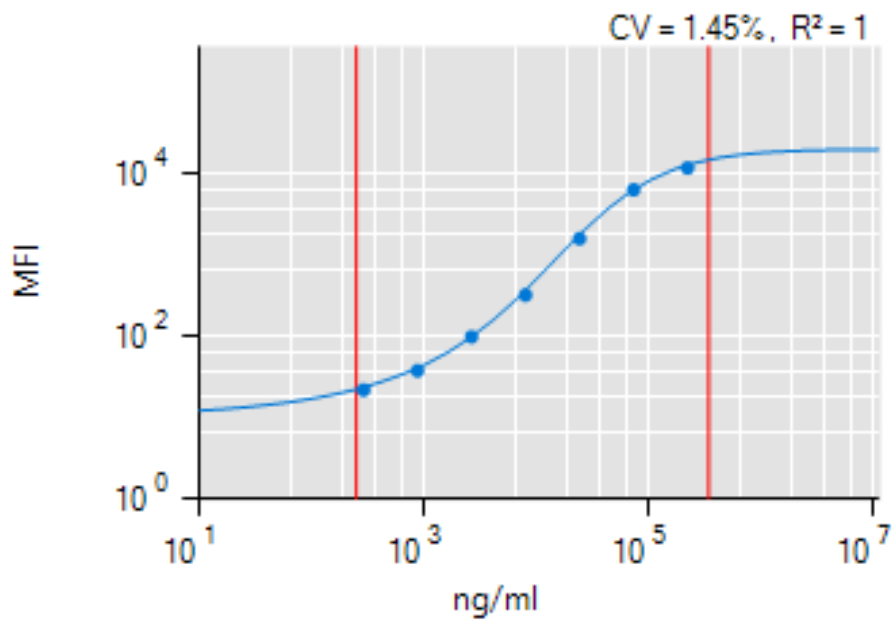
Thrombospondin-2 (42)

CV = 0.61%, R² = 1

Standard curves of sE-selectin and TSP-2