THE ROLE OF FOLLISTATIN AND GRANULOCYTE-COLONY STIMULATING FACTOR

IN

HIV-ASSOCIATED PRE-ECLAMPSIA

By

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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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DECLARATION

I, **Siphesihle Mdlalose** declare that:

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

iii. This dissertation does not contain other person's data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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ORAL PRESENTATION

- Mdlalose S, Moodley J and Naicker T (2018). Investigating the angiogenic role of follistatin in HIV-associated pre-eclampsia. Pre-eclampsia Awareness in week KZN (2018).

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- Mdlalose S, Moodley J and Naicker T (2018). The role of follistatin in HIV-associated pre-eclampsia. The 2nd International Meeting on Gynaecology and Obstetrics Pathology (Paris, France), November 2018.

DEDICATIONS

To God- To the alpha and omega. I have stayed strong in faith and prayed throughout the completion of my thesis. I know you have been watching me and helped me through this beautiful journey.

My parents- the emotional and financial support you both have given me is what any child would want. I love you!!

My family and friends- I appreciate the encouragement and the constant question, "when are you submitting?" This question has kept me on the right path and motivated me to work hard.

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LIST OF ABBREVIATIONS

+ve……………………………………. Positive

AIDS…………………………………… Acquired immunodeficiency virus

ART…………………………………. Antiretroviral therapy

CD4…………………………………... Cluster of differentiation

FS………………………………….. Follistatin

FSH…………………………………… Follicle stimulating hormone

FSL3………………………………….... Follistatin-like 3

G-CSF………………………………… Granulocyte-colony stimulating factor

HAART………………………………… Highly active antiretroviral therapy

HIV………………………………….… Human immunodeficiency virus

IFN…………………………………… Interferon

IL…………………………………….. Interleukin

LPS…………………………………. Lipopolysaccharides

MAPK………………………………… Mitogen-activated protein kinase

MFI…………………………………… Median fluorescent intensity

NOX 2……………………………… NADPH Oxidase 2

PE…………………………………….. Pre-eclampsia

PIGF………………………………….... Placental growth factor

ROS………………………………… Reactive oxygen species

SA…………………………………...... South Africa

SA-PE……………………………… Streptavidinphycoerythrin

sEng………………………………….. Soluble endoglin

sFlt1………………………………….. Soluble fms-like tyrosine kinase-1

TGF- β1……………………………… Transforming growth factor- β1

TNF…………………………………… Tumour necrosis factor

-ve………………………………….. Negative

VEGF………………………………… Vascular endothelial growth factor

vs…………………………………… Versus

WHO……………………………….. World health organization

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ABSTRACT

Introduction: KwaZulu-Natal has a high burden of HIV infection and high blood pressure, specifically pre-eclampsia (PE) in pregnancy. Follistatin (FS) and granulocyte-colony stimulating factor (G-CSF) are two glycoproteins involved in PE pathogenesis. FS is an extracellular glycoprotein antagonist of the ligand receptor, activin. G-CSF on the other hand drives the generation of neutrophilic granulocytes from myeloic precursor cells by its inhibition of apoptosis and induction of differentiation. In light of the high maternal mortality and morbidity in South Africa (SA), we investigated the expression of FS and G-CSF in the duality of HIV-associated PE.

Method: Serum samples of normotensive and pre-eclamptic women further stratified by HIV status were collected from a large regional hospital in Durban, and their FS and G-CSF expression was analysed using the Bio-Plex Multiplex Immunoassay.

Results: FS expression was significantly reduced in pre-eclamptic compared to normotensive pregnancies (649.5±116.8 *vs* 2354±353.6; $p<0.001$). However, FS expression did not differ between HIV +ve *vs* HIV –ve groups (1727±291.2 *vs* 1305±306.7; $p=0.13$). Furthermore, we detected significant FS expression across all study groups. The expression of G-CSF was not affected by HIV status *i.e.*, between HIV +ve *vs* HIV -ve groups (14.4±2.3 *vs* 11.6±0.7, $p=0.10$) and there was no significant difference in the expression of G-CSF between normotensive *vs* pre-eclamptic (14.9±2.4 *vs* 11.3±0.5, $p=0.32$). G-CSF expression was notably higher in HIV +ve normotensive when compared to all study groups.

Conclusion: This study demonstrates a downregulation of FS and G-CSF expression in PE, compared to normotensive pregnancies. This finding may be attributed to oxidative stress and its immuno-regulatory role in the hyper-inflammatory milieu of PE. In addition, the downregulation of both analytes may be attributed to the anti-angiogenic micro-environment of PE. HIV status probably had no effect on both analytes albeit, upregulated due to immune reconstitution emanating from HAART. Our novel findings suggest that FS and G-CSF may have a potential predictor test value in early pregnancy, hence work on this is ongoing.

CHAPTER 1

LITERATURE AND BACKGROUND REVIEW

1.1 Maternal mortality

Maternal mortality is the death of a woman during pregnancy, childbirth or within the 42 days after delivery (Hogan *et al.*, 2010). The Safe Motherhood Initiative was introduced in 1987 as a global initiative to raise awareness of the lack of health programmes concerning maternal health in developing countries (Hogan *et al.*, 2010). The World Health Organization (WHO) reports that 830 women die globally due to pregnancy related complications every day, of these deaths 99% of them occur in developing countries (WHO, 2018).

Maternal mortality is high amongst women living in sub-Saharan Africa, where 56 % of people living with HIV are women (UNAIDS, 2018). Notably, pregnancy related complications in South Africa (SA) are a major public health issue (Moodley *et al.*, 2018). Pregnancy places all women at risk for a pregnancy-related event however, some women are at a higher risk of morbidity and mortality (Hirshberg and Srinivas, 2017). The main cause of maternal deaths in SA result from non-pregnancy related infections such as HIV infection, tuberculosis and pneumonia. Reliable data from SA emanating from WHO recommendations for the Safe Motherhood programme underscores HIV/AIDS as the most common cause of maternal deaths (Moodley *et al.*, 2010). The remaining causes of maternal deaths are haemorrhage, high blood pressure, unsafe abortions and complications from delivery (Say *et al.*, 2014). Additionally, hypertensive disorders are the most common medical complication in pregnancy and account for 20.7% of maternal deaths within SA (Saving mothers 2014–2016, 2017).

1.2 HIV and AIDS in South Africa

HIV infection compromises an individual's immune system by causing a progressive loss of CD4+ T cells (Poulton, 2018). Globally more than 2 million HIV infected women become pregnant every year, with the majority residing in sub-Saharan Africa (Moodley *et al.*, 2010). SA has a high HIV infection prevalence with an estimated number of 7.52 million infected in 2018 (Statistics, 2018). Approximately one-fifth of women from SA in their reproductive ages (15-49 years) are HIV +ve, increasing the risk of mother to child HIV transmission (Dellar *et al.*, 2015;Statistics, 2018). This form of HIV transmission occurs at 3 stages:
1) During pregnancy when HIV crosses the placenta.

2) During delivery through the contact of vaginal secretions or blood.

3) During breast-feeding via breastmilk (Poulton, 2018).

1.3 Pre-eclampsia

Hypertension is defined as the presence of a chronic elevation of systemic arterial pressure above a certain threshold (Khaliq *et al.*, 2018). The number individuals affected by hypertension is expected to increase by approximately 560 million people worldwide by the year 2025 (Poulter *et al.*, 2015). Hypertensive disorders are responsible for a large amount of pregnancy complications in developing countries, such as SA (Hutcheon *et al.*, 2011). There are various types of hypertensive disorders however, this study will be looking at pre-eclampsia (PE).

 PE is a systemic syndrome identified by proteinuria, new-onset hypertension and poor placental perfusion in pregnant women (Hutcheon *et al.*, 2011). Maternal mortalities as a result of PE are estimated at 15-20 % in developing countries (Sibai *et al.*, 2005). The risk of a mother dying due to PE are higher in regions such as sub-Saharan Africa in comparison to developed countries (Abalos *et al.*, 2013). In SA, PE accounts for 14.8% of all maternal deaths (Saving mothers 2014–2016, 2017). The diagnostic criteria for PE remains contentious, with angiogenic biomarkers remaining a predictor test for the development of PE (Sibai *et al.*, 2005).

1.3.1   Risk factors of pre-eclampsia

Pre-eclampsia has various risk factors, in fact it is considered "the disease of theories" as its pathogenesis is varied, not concise and unclear (Trogstad *et al.*, 2011). Women who are at high risk of developing PE include those with pre-existing hypertension, chronic kidney disease and women with previous early onset PE (English *et al.*, 2015). Additional risk factors include smoking in pregnancy, multiple gestations and also ethnicity; Black women are at a higher risk when compared to White women (Trogstad *et al.*, 2011). There are familial factors that contribute to PE development, where a family history of PE increases the risk of PE development (Hutcheon *et al.*, 2011). Moreover, numerous evidence shows that paternal genes significantly increase the risk of PE (Agius *et al.*, 2018).

1.3.2   Pathophysiology of pre-eclampsia

For the successful implantation of the placenta, complex interactions occur. Placentation involves trophoblast invasion of maternal spiral arteries by trophoblast cells (Gathiram and Moodley, 2016). The invasion of trophoblasts cells via the decidua results in the transformation of spiral arteries into low-resistance vessels (Trogstad *et al.*, 2011). These trophoblast cells are also responsible for cytokine production, communication of maternal immune cells and angiogenesis (Trogstad *et al.*, 2011). A pregnant woman reacts to the foetal trophoblast through a sterile, low-grade maternal inflammatory response (Ngene and Moodley, 2017).

In pre-eclamptic women, trophoblast invasion is dysregulated and transformation of the myometrial spiral arteries is limited to the decidua during the first trimester of pregnancy (Figure 1.1) (Trogstad *et al.*, 2011;Salman *et al.*, 2018). The consequence of this is reduced blood flow to the placenta leading to poor fetal growth as a result of placental dysfunction (Gathiram and Moodley, 2016). The reduced blood flow results in an ishaemic placenta, which is identified as the central hypothesis governing pre-eclamptic pathogenesis (Chaiworapongsa *et al.*, 2014). The ischaemic placenta releases pro-inflammatory factors into maternal circulation and causes systemic endothelial dysfunction leading to the clinical manifestations of the disease (Chaiworapongsa *et al.*, 2014). The hypoxic micro-environment results in an excess generation of reactive oxygen species (ROS) (Trogstad *et al.*, 2011). The accumulation of ROS causes a self perpetuating inflammatory response which results in chemical destruction and ultimately tissue degradation and cell death (Ngene and Moodley, 2017). The latter contributes to a physiological imbalance causing an exaggerated systemic inflammatory response (Pinheiro *et al.*, 2013).

1.3.3   HIV-associated pre-eclampsia

South Africa has one of the largest public anti-retroviral therapy (ART) programmes in the world (Malaza *et al.*, 2012). The introduction of highly active antiretroviral therapy (HAART) has resulted in an increase in the incidence of PE (Suy *et al.*, 2006). However, HIV is responsible for the immune suppression of an infected women and PE is associated with an elevated immune response; hence a neutralization is expected. However, HAART reconstitutes ones immune response, thus increasing PE development (Phoswa *et al.*, 2018). Nonetheless, the relationship between HIV infection, HAART and PE needs to be further investigated because of the high maternal mortality rate in SA.
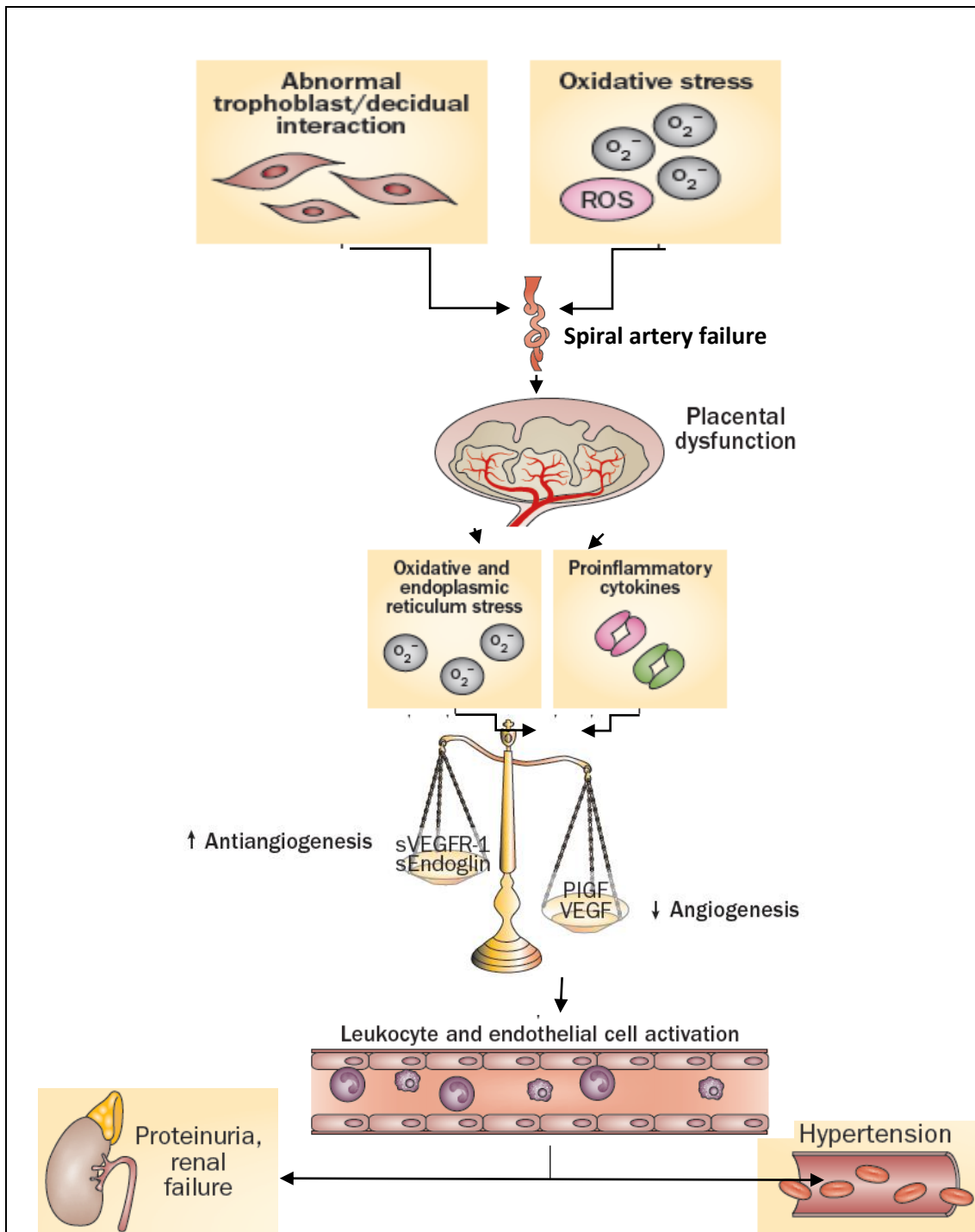
Figure 1.1: A complex model showing the pathophysiology of PE. In pre-eclamptic women there is dysregulation of trophoblast cells invasion, accompanied with oxidative stress. This results in the lack of myometrial spiral artery remodelling causing placental dysfunction. The ischaemic placenta releases ROS. Moreover, an angiogenic imbalance coupled with ROS results in endothelial dysfunction. Leukocyte and endothelial cell activation lead to the maternal clinical manifestations of the disease. Adapted from Chaiworapongsa *et al.* (2014).

1.4 Angiogenesis in pre-eclampsia

Circulating anti-angiogenic proteins such as soluble fms-like tyrosine kinase-1 (sFlt1) and soluble endoglin (sEng) inhibit pro-angiogenic factors [vascular endothelial growth factor (VEGF) and placental growth factor (PlGF)] in PE (Karumanchi, 2016). VEGF plays a role in stabilizing endothelial cells in mature blood vessels and also maintains the endothelium in the kidney, brain and liver; organs severely affected in PE (Wang *et al.*, 2009). PlGF stimulates angiogenesis under conditions of ischaemia, inflammation and wound healing (Wang *et al.*, 2009). Literature shows that VEGF and transforming growth factor (TGF-β1) play an essential role in maintaining the endothelium of the placenta (Karumanchi, 2016). In a normal pregnancy there is vascular homeostasis between angiogenic factors, VEGF and TGF-β1 (Gathiram and Moodley, 2016). However, in PE excess placental excretion of endogenous anti-angiogenic proteins, sFlt1 and sEng inhibit VEGF and TGF-β1 signalling in the vasculature causing endothelial cell dysfunction (Khaliq *et al.*, 2018). The pathogenesis of PE involves the presence of an anti-angiogenic state, whereby the imbalance of angiogenesis is identified as the most important factor (Chaiworapongsa *et al.*, 2014). There have been other proteins with angiogenic activity that have been implicated in PE development however, none have been established to have a predictor test value early in pregnancy (Maynard and Karumanchi, 2011).

1.5 Follistatin

Follistatin (FS) is an extracellular glycoprotein identified as an inhibitor of pituitary follicular stimulating hormone (FSH) secretion. It was first isolated from porcine follicular fluid (Hansen and Plomgaard, 2016). The FS protein functions by preventing the ligand-receptor interaction of activin, which belongs to the TGF-β superfamily (Flanagan *et al.*, 2009). The extracellular FS molecule binds to the activin homodimer with a high affinity, resulting in the restriction of activin bioavailability for the stimulation of FSH secretion (Keutmann *et al.*, 2004). Additionally, FS is an important mediator of cell secretion, development and differentiation in numerous body systems (Sidis *et al.*, 2001). Furthermore, it can be found in the pituitary, reproductive organs and also in the kidney (Blumenfeld and Ritter, 2001).

The distribution of FS occurs among all tissues in which activin is also present (Sidis *et al.*, 2001). TGF-β family members, including activin and myostatin bind to type I and type II receptors and

lead to type I receptor phosphorylation (Zhang *et al.*, 2018). This receptor then phosphorylates the secondary messenger molecule smads, which complexes with a common smad4 (Zhang *et al.*, 2018). This complex then translocates to the nucleus where it activates gene transcription (Zhang *et al.*, 2018). However, in the presence of extracellular FS, the TGF-β superfamily members effects are neutralized as a result of FS binding to them (Figure 1.2) (Zhang *et al.*, 2018).
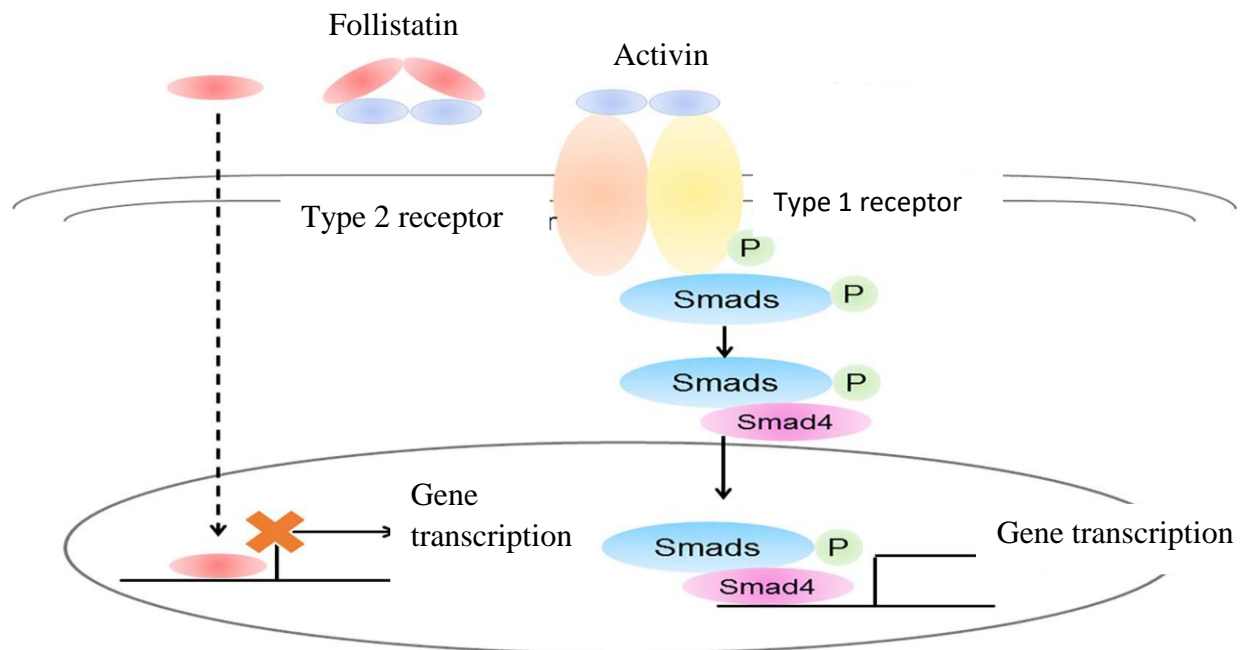
Figure 1.2: Diagram showing the regulation of activin signalling by follistatin. Two FS molecules, which bind activin thereby preventing binding of activin to its receptors. This inhibits gene transcription. Adapted from Zhang *et al.* (2018).

1.5.1   *Follistatin in pre-eclampsia*

Pre-eclamptic symptoms are a result of the ischaemic placenta and studies conducted on FS show that it has a role in regeneration after ischaemic injury (Welt *et al.*, 2002). A rat model with arterial damage showed a reduction in histological defects after the infusion of FS, implicating FS in repair processes (Welt *et al.*, 2002). Moreover, circulating levels of FS are not affected by a woman's reproductive tissues, so FS levels in the serum do not change during a woman's menstrual cycle (Hansen and Plomgaard, 2016). Studies show that the serum concentration of FS in small for gestational age infants is higher than in appropriate for gestational age infants (Sebastiani *et al.*, 2011). This may be attributed to the role of FS in regulating fat, lean mass and muscle growth through its inhibitory effects on activin and myostatin (Anastasilakis *et al.*,

2016).The increase in FS levels could be a compensatory mechanism to regulate the infants' weight. Since we know that PE is associated with a baby's weight we can assume that FS serum levels will be elevated in PE. However, due to oxidative stress occurring in PE there will be a decrease in the levels of FS (Zhang *et al.*, 2018). Previous studies conducted on FS have reported that in PE, FS functions in the inhibition of PE development via its pro-angiogenic role (Zhang *et al.*, 2018).

1.5.2   *Follistatin in HIV-associated pre-eclampsia*

The FS protein is an important regulator of regulatory T cells, natural killer cells and also dendritic cells (Werner and Alzheimer, 2006). An HIV infected woman will have a depletion of CD4+ T cells resulting in an impaired cellular immune response (Okoye and Picker, 2013). Activin acts as a pro-inflammatory agent in diseases such as PE (Refaat *et al.*, 2014). FS on the other hand plays a role in the inflammatory response by direct inhibition of activin (Miyamoto *et al.*, 2011). Additionally, FS plays a role in both innate and humoral immune responses, with evidence implicating the protein in immune response to several pathogens including viruses (Werner and Alzheimer, 2006).

Since PE is associated with inflammation, there will be an upregulation of FS during inflammation (Hedger *et al.*, 2011). This serves to counteract the effect of activin, which acts as a critical amplifier of inflammation (Hedger *et al.*, 2011). Additional inflammatory cytokines such as IL1-β, TNF-α and IFN-γ, also result in the upregulation of FS to mediate an inflammatory response (Hedger *et al.*, 2011). Moreover, activin inhibits endothelial cell proliferation however, FS blocks this inhibitory effect because it is strongly expressed by migrating endothelial cells (Gerritsen, 2008). Literature suggests that FS may be a potential angiogenic biomarker that can counteract the development of PE however, it is unclear how FS expression is affected by HIV status in pre-eclamptic women.

1.6 Granulocyte-colony stimulating factor

Granulocyte-colony stimulating factor (G-CSF) is a 19.6 kDa glycoprotein consisting of 4 antiparallel α helicases. Originally identified in serum as it induced differentiation of the murine myelomonocytic leukemic cell line (Schneider *et al.*, 2006). G-CSF is central in the production of neutrophils in healthy and diseased states in response to infection (Bendall and Bradstock, 2014). The prominent source of G-CSF is monocytes but fibroblasts, mesothelial and endothelial cells have been found to also produce G-CSF (Schneider *et al.*, 2006). Various tissues can produce G-CSF when stimulated by inflammatory mediators such as lipopolysaccharides (LPS), TNF-α, VEGF, macrophages and fibroblasts leading to an immune response (Cai *et al.*, 2017).

In the hematopoietic system, G-CSF drives the generation of neutrophilic granulocytes from myeloic precursor cells by its inhibition of apoptosis and induction of differentiation (Figure 1.3) (Schneider *et al.*, 2006). G-CSF serves as a growth factor that promotes the proliferation and differentiation of neutrophil progenitors (Basu *et al.*, 2002). Additionally, G-CSF released in the blood stream stimulates the mobilization and production of neutrophils within the bone marrow (Bendall and Bradstock, 2014). When an infection occurs there is a typical host response to the infection, such as increased production of neutrophils (Basu *et al.*, 2002). The locally produced G-CSF by tissues, mediates the function of these neutrophils at the infection site (Bendall and Bradstock, 2014).

1.6.1 Granulocyte-colony stimulating factor in pre-eclampsia

Studies have shown that the administration of G-CSF to healthy individuals or experimental animals result in an upregulation of neutrophil levels, 10 to 15 fold greater than normal levels (Basu *et al.*, 2002). Neutrophils are responsible for the production of inflammatory cytokines such as IL-8 (Pinheiro *et al.*, 2013). These neutrophils infiltrate the vasculature of women with PE and provide a chemotactic gradient that attracts more neutrophils (Pinheiro *et al.*, 2013). The neutrophils will subsequently infiltrate into the intimal space where they release ROS consequently resulting in inflammation (Pinheiro *et al.*, 2013). Endothelial cells monocytes and endometrial cells produce G-CSF, which plays an important role in trophoblastic development and placental metabolism (Kamath *et al.*, 2017). There is a paucity of information on G-CSF serum levels in PE. However a study done by Natori *et al.* (2002) showed that G-CSF played a role in promoting angiogenesis in an animal model. There is evidence showing G-CSF has a direct

stimulatory action on mature endothelial cells, thereby promoting angiogenesis. A study conducted by Lee *et al.* (2005) showed G-CSF directly stimulates endothelial cell migration and enhancing angiogenesis-like network formation on Matrix gel culture *in vitro*. This suggests that G-CSF will have a pro-angiogenic effect in PE.

1.6.2   Granulocyte-colony stimulating factor in HIV-associated pre-eclampsia

The G-CSF glycoprotein plays a role in the mediation of neutrophil release in the blood stream (Figure 1.3). Additionally, high circulating levels of G-CSF result in neutrophils altering the body's immune system with an upregulation of inflammation (Cai *et al.*, 2017). Furthermore, G-CSF is also implicated in the increased release of monocytes which counteract the effect of an inflammatory response by releasing anti-inflammatory cytokines (Eyles *et al.*, 2006). This anti-inflammatory role of G-CSF is important in pregnancy because it temporarily suppresses an immune response by its effects on lymphocytes, macrophages and T helper-2 cells (Kamath *et al.*, 2017). In the exaggerated inflammatory milieu of PE, G-CSF would serve to lower the immune response. In HIV infected PE, G-CSF elevates levels of CD4+ T cell subset thereby inhibiting inflammatory infiltration (Peng, 2017). Nonetheless, there is a paucity of data on G-CSF levels in HIV-associated PE. It is plausible to hypothesize that G-CSF may be implicated in an immunoregulatory and angiogenic role; hence be of diagnostic value for the early detection of PE.
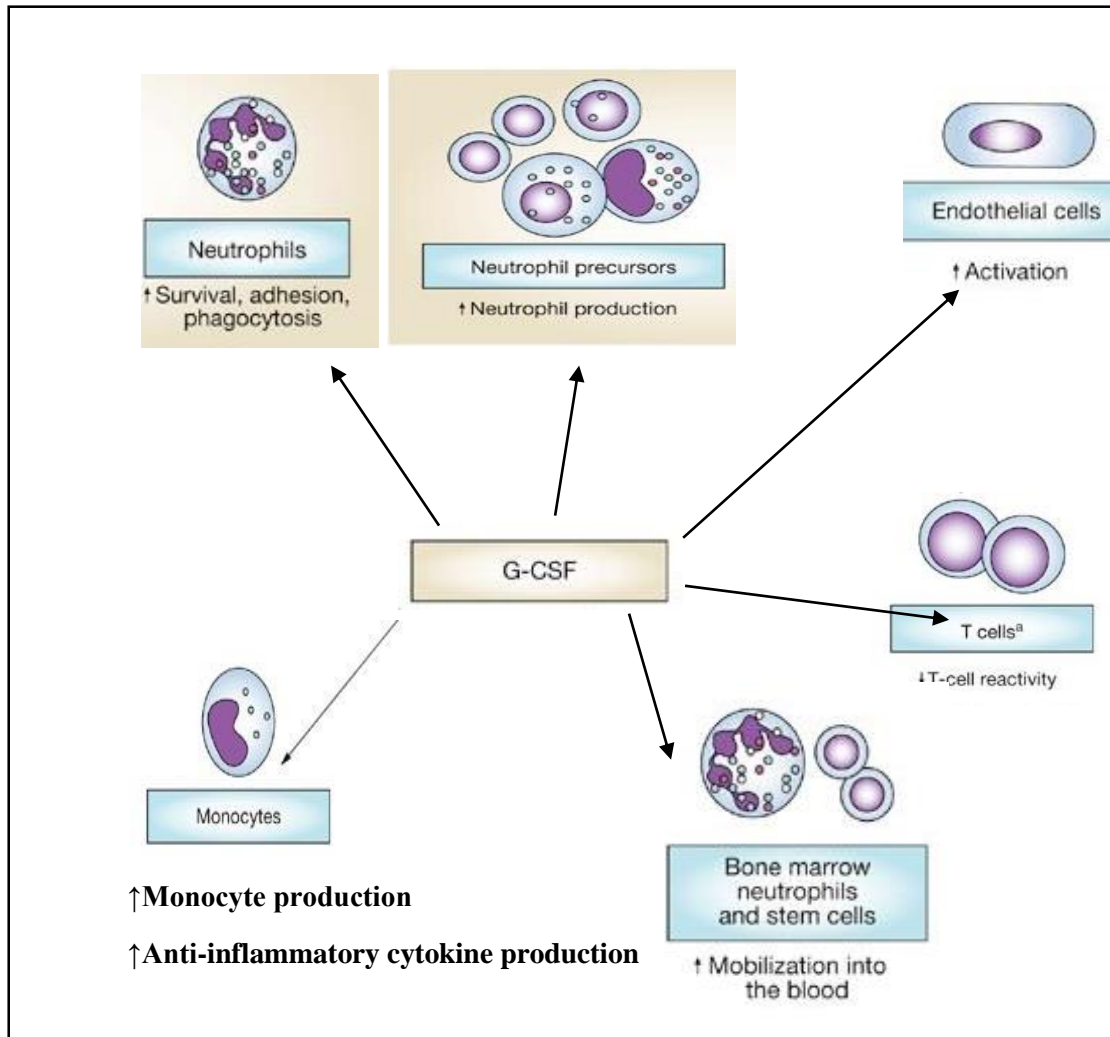
Figure 1.3: Diagram showing basic cellular activities of G-CSF. G-CSF promotes the release of neutrophils from the bone marrow into blood circulation. These neutrophils are involved in phagocytosis and the release of monocytes. G-CSF also acts on neutrophil precursors, increasing neutrophil production. More importantly, G-CSF increases the activation of endothelial cells. Circulating G-CSF plays an important role in an immune response by decreasing T cell reactivity. Adapted from Eyles *et al.* (2006).

1.7 Aim:

To establish the role of FS and G-CSF in HIV-associated normotensive pregnancy and PE using the Bio-Plex Multiplex Immunoassay.

1.8 Objectives:

- To determine the effect of HIV status (HIV +ve *vs* HIV -ve) on FS and G-CSF serum concentrations, irrespective of pregnancy type.
- To determine the effect of pregnancy type (normotensive *vs* PE) on FS and G-CSF serum concentrations, irrespective of HIV status.
- To compare FS and G-CSF serum concentrations across the study population (HIV +ve normotensive pregnant, HIV -ve, normotensive pregnant, HIV +ve PE and HIV -ve PE)
- To correlate FS and G-CSF serum concentration with maternal age, gestational age, parity, blood pressure and maternal weight across the study population.

1.9 Study rationale:

Pre-eclampsia and HIV infection are the major causes of maternal mortality and morbidity cases in SA. In order to introduce treatment methods, it is important to understand the pathogenesis of HIV-associated PE and the role of FS and G-CSF in limiting the effects of both conditions.

CHAPTER 2

HYPERTENSION IN PREGNANCY

The role of follistatin and granulocyte-colony stimulating factor in HIV-associated pre-eclampsia.

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THE ROLE OF FOLLISTATIN AND GRANULOCYTE-COLONY STIMULATING FACTOR IN HIV-ASSOCIATED PRE-ECLAMPSIA

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Abstract

KZN has a high burden of HIV infection and high blood pressure, specifically pre-eclampsia (PE) in pregnancy. Follistatin (FS) and granulocyte-colony stimulating factor (G-CSF) are two glycoproteins involved in PE pathogenesis. In light of the high maternal mortality and morbidity in South Africa (SA), we investigated the expression of FS and G-CSF in the duality of HIV-associated PE. Serum samples of normotensive and pre-eclamptic women were analysed using the Bio-Plex Multiplex Immunoassay. FS expression was significantly reduced in pre-eclamptic compared to normotensive pregnancies (649.5±116.8 *vs* 2354±353.6; *p*<0.001). However, FS expression did not differ between HIV +ve *vs* HIV –ve groups (1727±291.2 *vs* 1305±306.7; *p*=0.13). Furthermore, we detected significant FS expression across all study groups. The expression of G-CSF was not affected by HIV status *i.e.*, between HIV +ve *vs* HIV -ve groups (14.4±2.3 *vs* 11.6±0.7, *p*=0.10) and there was no significant difference between normotensive *vs* pre-eclamptic groups (14.9±2.4 *vs* 11.3±0.5, *p*=0.32). G-CSF expression was notably higher in HIV +ve normotensive when compared to all study groups. This study demonstrated a downregulation of FS and G-CSF expression in PE, compared to normotensive pregnancies. This finding may be attributed to oxidative stress and its immunoregulatory role in the hyperinflammatory milieu of PE. HIV status had no effect on both analytes, albeit upregulated due to immune reconstitution emanating from HAART. Our novel findings suggest that FS and G-CSF may have a potential predictor test value in early pregnancy, hence work on this is ongoing.

Keywords: Pre-eclampsia, HIV, Follistatin, Granulocyte-colony stimulating factor, HAART

Running title: FS and G-CSF in HIV-associated pre-eclampsia.

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Introduction

Globally, an estimated 75% of maternal deaths is attributed to haemorrhage, high blood pressure, abortions and non-pregnancy related infections such as malaria and human immunodeficiency virus (HIV) (1). More than 2 million HIV infected women become pregnant every year, with the majority residing in sub-Saharan Africa (2). In South Africa (SA) more than 21.7% of women are living with HIV and are at child bearing age (3).

Pre-eclampsia (PE) is a hypertensive disorder that affects up to 8 % of all pregnancies annually (4). In SA, PE accounts for 14.8% of all maternal deaths (44). PE is identified by proteinuria, new-onset hypertension and poor placental perfusion in pregnant women (5). Moreover, trophoblast invasion is dysregulated and transformation of the spiral arteries is limited to the decidua during the first trimester of pregnancy (6). The consequence of this deficient invasion is reduced blood flow with subsequent poor foetal growth (7). The reduced blood flow results in an ischaemic placenta, which releases pro-inflammatory factors and generates an excess reactive oxygen species (ROS) into the maternal circulation (8). The resultant endothelial dysfunction contributes to the maternal syndrome, characterised as clinical symptoms of PE (9). Notably, ROS accumulation causes a self-perpetuating inflammatory response which causes chemical destruction and ultimately tissue degrading and cell death (10).

Follistatin (FS) is an extracellular glycoprotein which functions by preventing the ligand-receptor interaction of activin, which belongs to the transforming growth factor-β (TGF-β) superfamily which are essential for regulating cell development and growth (11, 12). FS is also an important mediator of cell secretion, development and differentiation in numerous body systems (13). Moreover, FS plays an important immunoregulatory role in an inflammatory response (14). It is a protagonist in both an innate and humoral immune response, with previous studies implicating the protein in an immune response to viruses (15). Furthermore, FS is involved in angiogenesis (16).

Granulocyte-colony stimulating factor (G-CSF) is a 19.6 kDa glycoprotein consisting of 4 antiparallel α helicases, originally identified in serum as it induced differentiation of the murine myelomonocytic leukemic cell line (17). Various tissues can produce G-CSF when stimulated by inflammatory mediators such as lipopolysaccharides (LPS), tumour necrosis factor-α (TNF-α), vascular endothelial factor (VEGF), macrophages and fibroblasts (18). G-CSF serves as a growth factor that promotes the proliferation and differentiation of neutrophil progenitors (19).

Additionally, G-CSF released in the blood stream stimulates the mobilization and production of neutrophils within the bone marrow (18). Like FS, G-CSF also promotes angiogenesis by inducing the proliferation of endothelial cells (20).

Despite literature demonstrating an upregulation of anti-angiogenic factors in PE, their implementation as a predictor test for PE development is poor in low income countries (21, 22). South Africa is the epicentre of the global HIV pandemic with a high prevalence of PE (12%), hence it is imperative that the association of both conditions is investigated (44). In view of the fact that both FS and G-CSF play a role in the pathogenesis of PE, this study will examine their expression in women based on pregnancy type (normotensive *vs* pre-eclamptic) and HIV status (HIV +ve *vs* HIV -ve).

Materials and methods

Ethical approval (BCA 338/17) and regulatory health authority permission were obtained prior to recruitment of study participants.

Study population: Black pregnant women (n=80) were recruited at the Obstetric unit of a large regional hospital in Durban, SA and divided into 2 study groups viz., normotensive pregnant and pre-eclamptic women. These groups were further stratified by HIV status-into HIV +ve pre-eclamptic (n= 20), HIV –ve pre-eclamptic (n= 20), HIV +ve normotensive (n= 20) and HIV -ve normotensive (n =20).

Inclusion criteria: PE was defined as systolic blood pressure ≥140mmHg and diastolic blood pressure ≥90mmHg and proteinuria occurring after 20 weeks of gestation measured as 300mg of protein in a 24-hour urine sample or measured with a dipstick with a value ≥1+, taken 4-6 hours apart. The HIV status was determined via a routine rapid test at a large regional hospital and CD4+ counts were performed if the woman was HIV +ve. All newly HIV +ve women were initiated on combined anti-retroviral therapy (ART).

Exclusion criteria: Women with chronic diabetes, gestational diabetes, chronic hypertension, connective tissue disorder, chronic renal disease, cardiac disease, sickle cell disease, polycystic ovarian syndrome, abruption placentae, intrauterine death, unknown HIV status, asthma, pre-existing seizure disorders, patients with declined participation, unbooked patients and those who were unable to provide informed consent were excluded from the study.

Sample collection: Following informed consent, venepuncture was used to obtain venous blood samples in K3-EDTA tubes. The samples were centrifuged at 1000 rpm for 10 min at 4°C. The layers were separated i.e. buffy coat, whole blood and serum and stored in cryovials at -80°C until immunoassay.

Bio-plex Multiplex immunoassay: The buffy coat (leucocyte suspension) was used to assess differences in median fluorescent intensity (MFI) of phosphorylated FS (1:4 dilution) using the Bio-Plex® Pro™ Human Cancer Biomarker Panel 1, 16-plex standards (catalogue#171304055M; Bio-Rad Inc., South Africa). G-CSF (CSF-3) (1:1 dilution) was assessed using ProcartaPlex Human Basic Kit (lot#168095000; Thermo Fisher Scientific., Austria). Both FS and G-CSF assays were performed according to the manufacturer's instructions (23) respectively. The assay followed a similar technique to that of a sandwich ELISA, following incubation of antigen samples to the study viz, FS and G-CSF with the capture antibody-coupled magnetic beads. Biotinylated detection antibodies were added to the reaction, along with reporter conjugate, streptavidinphycoerythrin conjugate (SA-PE). After washing, detection of the fluorescence of the SA-PE bound to each bead was carried out using the Bio-Plex®MAGPIX™ Multiplex Reader (Bio-Rad Laboratories Inc., USA).

Data analysis: A protocol was generated on the Bio-Plex Manager™ software version 4.1, to obtain the data from the multiplex system. The measurement of analyte expression for a given bead population in each group was provided as the MFI, which is proportional to the concentration of the analyte bound to the reporter. A positive and negative cell lysate control was used for qualitative verification of the assay. The data was exported into an Excel spreadsheet for statistical analysis.

Statistical analysis: Data was analysed using GraphPad Prism 5.00 for windows (GraphPad Software, San Diego, California USA). Normality test used to check distribution of data (parametric or non-parametric). Data is represented as Mean ± Standard Error of Mean. Statistical analysis across all groups of the same sample population were based on HIV status and pregnancy type, using the One-way ANOVA and the Bonferroni *post-hoc* multiple comparison test. The Mann Whitney's student t-test was performed for comparison between two groups e.g., HIV +ve *vs* HIV -ve or normotensive *vs* pre-eclamptic. The level of significance was considered as a probability level of $p < 0.05$.

Results

Clinical characteristics and patient demographic data

There was no significant difference in maternal age across all study groups (p=0.14). However, there was a significant difference in gestational age between HIV-ve normotensive (39.4 ±0.4 weeks) *vs* HIV +ve pre-eclamptic (31.1±0.9 weeks) (p<0.0001) and HIV +ve normotensive *vs* HIV-ve pre-eclamptic (p<0001). Additionally, there was no significant difference in parity (p=0.25). As expected, a statistical significance was detected for both systolic blood pressure (p<0.0001) and diastolic blood pressure (p<0.0001) between the normotensive and pre-eclamptic group. There was no significant difference in maternal weight across all study groups (p=0.91).

FS expression

HIV status: Regardless of pregnancy type (normotensive *vs* pre-eclampsia), there was no significant difference in FS expression between HIV +ve and HIV-ve groups (1727±291.2 *vs* 1305±306.7, p=0.13; Figure 1A). The MFI of FS was upregulated in HIV infection, albeit non-significantly.

Pregnancy type: FS expression was significantly downregulated in pre-eclamptic compared to normotensive group, irrespective of HIV status (649.5 *vs* 2354±353.6 ±116.8, p<0.001; Figure 1B).

Across all groups: Figure 1C illustrates FS expression across all study groups. There was a significant difference in the expression of FS between the HIV -ve normotensive *vs* HIV +ve pre-eclamptic group (2105±552.8 *vs* 802±213.6, p<0.01), HIV +ve normotensive *vs* HIV +ve pre-eclamptic group (2603±449.1 *vs* 802±213.6, p<0.01) and HIV +ve normotensive *vs* HIV -ve pre-eclamptic group (2603±449.1 *vs* 505.1±99.9, p<0.001). The expression of FS was highest in the HIV +ve normotensive group.

G-CSF expression

HIV status: The expression of G-CSF was not affected by HIV status i.e., between HIV +ve *vs* HIV -ve groups (14.4±2.3 *vs* 11.6±0.7, *p*=0.10; Figure 2A), irrespective of pregnancy type.

Pregnancy type: Regardless of HIV status, there was no significant difference in the MFI of G-CSF between normotensive *vs* pre-eclamptic groups (14.9±2.4 *vs* 11.3±0.5, *p*=0.32; Figure 2B). Additionally, G-CSF was downregulated in the pre-eclamptic group.

Across all groups: Figure 2C illustrates G-CSF expression across all study groups. There was a significant difference between HIV -ve normotensive *vs* HIV +ve normotensive groups (11.6±1.1 *vs* 18.2±4.7, *p*<0.05). The expression of G-CSF was highest in the HIV +ve normotensive group. Nonetheless, the expression of G-CSF was similar between the HIV -ve pre-eclamptic *vs* HIV +ve pre-eclamptic group (11.5±0.8 *vs* 11.1±0.6 *p*>0.05).

Discussion

This study demonstrates a significant downregulation of FS in PE, irrespective of HIV status. These findings are similar to previous studies who attributed this decline to the oxidative stress occurring in PE (24). Notably, the findings of (25) also supports FS downregulation in the hypoxic micro-environment of PE. In cultured human umbilical vein endothelial cells, activin induces ROS production through activating smad2/3 to increase NADPH oxidase 2 (NOX2) activity, which causes endothelial dysfunction (26). The presence of activin promotes ROS production and exacerbates PE development, while FS inhibits this process by neutralizing activin (24). In contrast, FS downregulates NOX2 protein levels to reduce the production of ROS, thus protecting endothelial cells (26). Moreover, FS has an angiogenic role and is strongly expressed in migrating endothelial cells (27). This migration activity of endothelial cells can be inhibited by activin however, FS neutralizes the effect of activin thus promoting angiogenesis (27). Notably, FS administration/infusion in a rat model with arterial damage was shown to reduce tissue damage and promote the repair process, highlighting the regenerative role of FS in post-ischemic injury (28). PE is associated with oxidative stress, high ROS production and anti-angiogenic imbalance.

Hence, the observed downregulation of FS in our pre-eclamptic group compared to the normotensive group.

Based on HIV status, we report no significant difference in FS expression, irrespective of pregnancy type. There was however, an upregulatory trend of FS in HIV infection. Both activin and FS have immunoregulatory roles in an inflammatory response (14). In contrast to activin, FS has an anti-inflammatory response by direct inhibition of activin (29). The imbalance between activin and FS exaggerates the severity of the inflammatory state in diseases such as asthma and carcinogenesis (30). The release of activin precedes the release of other inflammatory cytokines, therefore the modulation of the activin and FS system is a potential therapeutic target in HIV-associated PE which has an inflammatory component (30). The non-significant difference in FS expression in this study may be attributed to the use of highly active antiretroviral therapy (HAART), a standard of care in all HIV infected patients in SA. The disparate immune response of PE and HIV infection would indicate a neutralisation. However, the introduction of HAART has resulted in an increased incidence of PE in HIV infected woman (31). HAART re-establishes immunocompetence thereby, exacerbating the exaggerated immune response of PE (32).

This study also reports a non-significant difference in the expression of G-CSF between the normotensive and pre-eclamptic group, regardless of HIV status; albeit a downregulation in the latter group. Endothelial cells, monocytes and endometrial cells produce G-CSF, which plays an important role in trophoblast invasion and placental metabolism (33). The downregulation of G-CSF may be attributed to the deficient trophoblast invasion in PE, with resulting angiogenic imbalance (9). Nonetheless, there is a paucity of information on G-CSF serum levels in PE. However, G-CSF has a role in promoting angiogenesis in an animal model via a direct stimulatory action on mature endothelial cells (20, 34). A study conducted by (35) showed G-CSF directly stimulates endothelial cell-like network formation on Matrix gel culture in vitro. In light of the latter findings, the downregulation of G-CSF observed in our study is predictable, as PE is an anti-angiogenic state (8).

It is worth mentioning that the administration of G-CSF to healthy individuals or experimental animals results in neutrophilia (36). Neutrophils are responsible for the production of inflammatory cytokines such as interlukin-8 (37). These neutrophils infiltrate the vasculature of women with PE and provide a chemotactic gradient that attracts more neutrophils (38). The neutrophils will subsequently infiltrate into the intimal spaces where they release ROS consequently resulting in inflammation (38). Moreover, (39) showed that a high dosage of G-CSF is associated with an upregulation of inflammation. The unexpected downregulation of G-CSF in PE is attributed to the use of HAART, which re-establishes immunocompetence. In pregnancy,

G-CSF temporarily suppresses the immune response by its effect on lymphocytes, macrophages and T helper-2 cells (33). This immune response suppression is to prevent an exacerbated inflammatory response, such as the one observed in PE.

In our study, there was no significant difference detected in the expression of G-CSF between HIV +ve and HIV -ve groups. However, in the HIV +ve group there was an upregulation of G-CSF expression when compared to the HIV-ve group. The HIV +ve group will have a drop in their CD4+ count as a result of the HIV infection (40). However, the upregulation of G-CSF in the HIV +ve group is to combat the CD4+ drop by increasing the percentage of CD4+ regulatory T cells (41). Moreover, the use of HAART reconstitutes the maternal immune response. This suggests that G-CSF upregulation in the HIV +ve group is to elicit its anti-inflammatory properties (44). Previous studies show that *in vitro*, G-CSF decreases lipopolysaccharide-induced secretion of pro-inflammatory cytokines by increasing the activity of monocytes (36). This consequently results in the increased release of anti-inflammatory cytokines (43). These anti-inflammatory cytokines are to combat the duality of HIV and PE with the adverse effects of HAART.

Limitations

The study was limited by a small sample size and non-inclusion of other ethnicities. The use of HAART may have influenced the expression of our analytes however, further studies are required.

Conclusion

This study demonstrates a downregulation of FS and G-CSF expression in PE, possibly due to oxidative stress and the anti-angiogenic micro-environment of PE. In addition, a limitation of the study was the small sample size. These novel findings suggest that both FS and G-CSF have potential predictor test value for early diagnosis of PE development. HIV status did not influence FS and G-CSF expression, this may be attributed to the effects of the HAART regimen adopted in SA. It is also plausible to assume that the upregulation of FS and G-CSF expression (albeit non-significant) in the HIV +ve group, arises as a result of the immune response in controlling viral infection. Further studies will interrogate FS and G-CSF levels across the trimesters of pregnancy and introduce a larger sample size.

Declaration of interest

There are no conflicts of interest.

Acknowledgments

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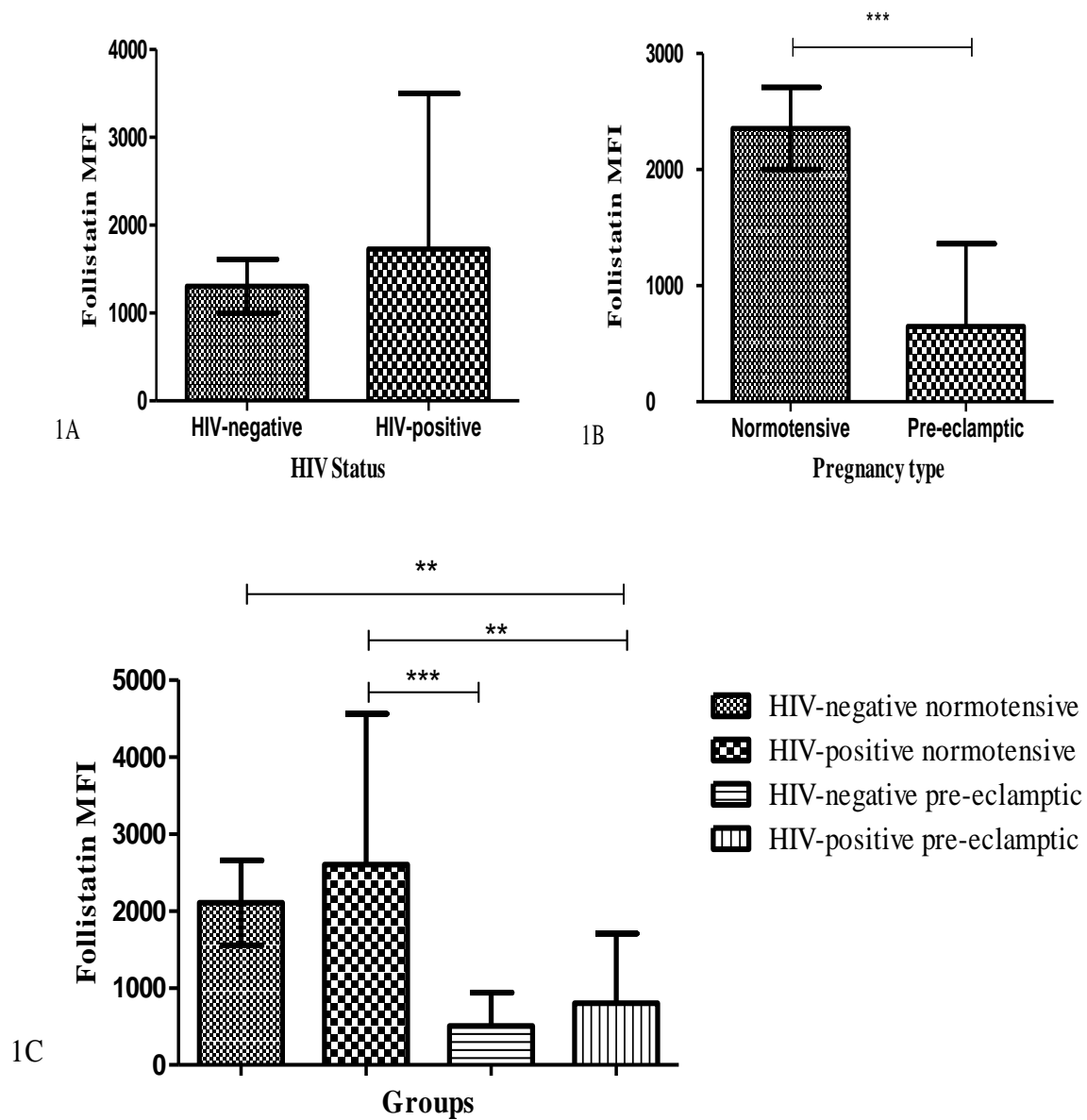
Figure 1: FS expression: (A) based on HIV status; no significance (ns) (B) based on pregnancy type; ***$p<0.001$ and (C) across all study groups;**$p<0.01$ ***$p< 0.001$. Results are represented as mean ± SEM.

Graph 2A error bars adjusted.

2A

2B

2C
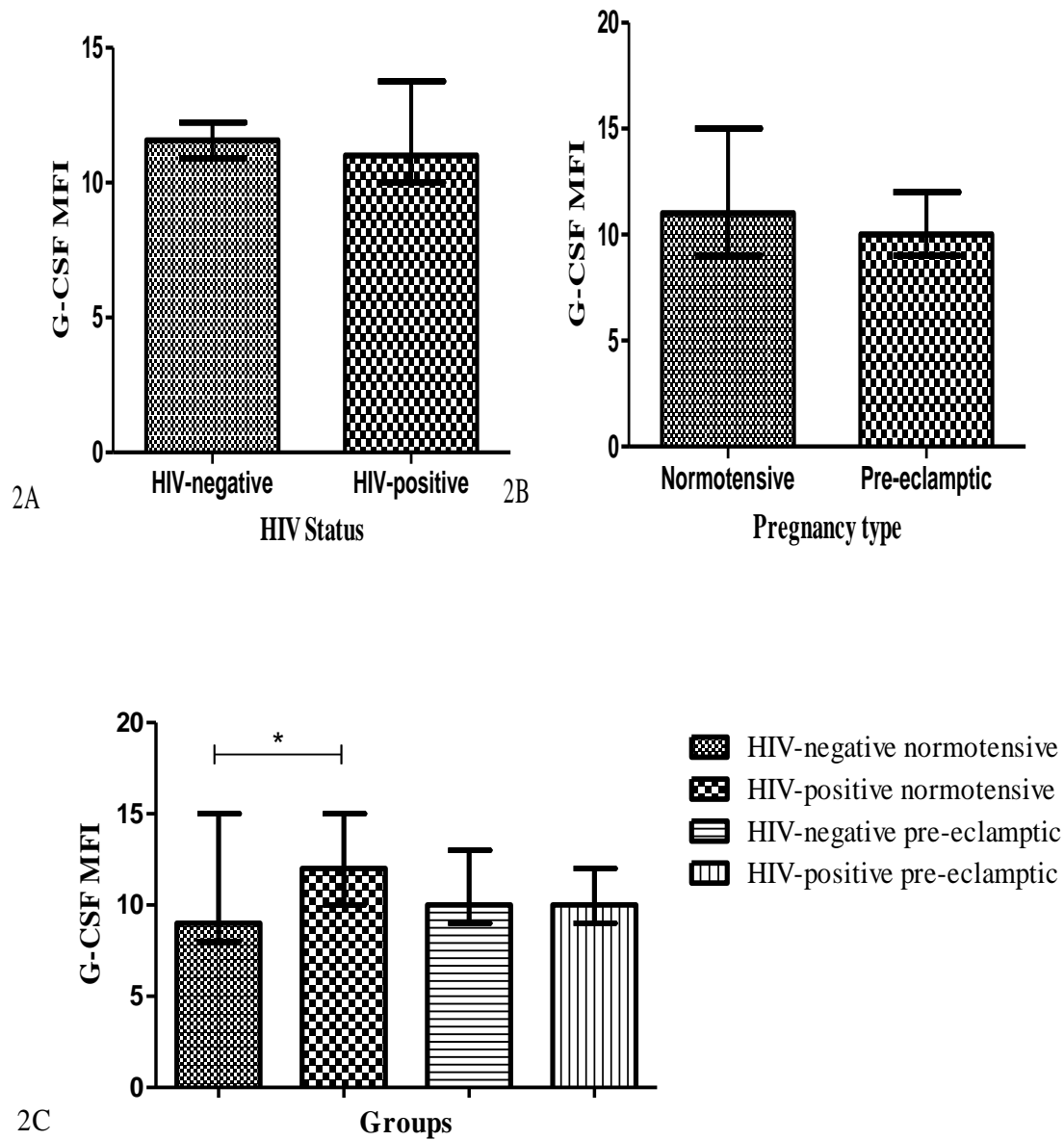
Figure 2: G-CSF expression: (A) based on HIV status; no significance (ns) (B) based on pregnancy type; no significance (ns) and (C) across all study groups; *p<0.05. Results are represented mean ± SEM.

Table 1: Clinical demographic results. Mean ± SEM are represented; One-way ANOVA and the Bonferroni *post hoc* multiple comparison test was used for statistical analysis, n= 80

	HIV –ve normotensive n=20	HIV +ve normotensive n=20	HIV –ve pre-eclamptic n=20	HIV +ve pre-eclamptic n=20	p-value
Maternal Age (years)	25 ± 1	29 ± 1	26 ± 2	29 ± 6	*p*=0.14
Gestational Age (weeks)	39 ± 0.4	38.5 ± 0.5	33 ± 1	31 ± 1	*p*<0.0001
Parity	1 ± 0.3	1.5 ± 0.2	1 ± 0.2	1.3 ± 0.2	*p*=0.25
Systolic BP (mmHg)	120 ± 2	114.6 ± 3	159 ± 4	164 ± 3	*p*<0.0001
Diastolic BP (mmHg)	67 ± 2	67.4 ± 2.0	104.4 ± 2	107 ± 2	*p*<0.0001
Maternal Weight (kg)	77 ± 3	76 ± 4	77 ± 5	77 ± 6	*p*=0.91

CHAPTER 3

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THE ROLE OF FOLLISTATIN IN HIV-ASSOCIATED PRE-ECLAMPSIA

Siphesihle Mdlalose, Thajasvarie Naicker

Optics and Imaging

Introduction
KwaZulu-Natal has a high burden of HIV infection and high blood pressure, specifically pre-eclampsia (PE) in pregnancy. Follistatin (FS) is an extracellular glycoprotein antagonist of the ligand receptor, Activin-A, involved in PE pathogenesis. In light of the high maternal mortality and morbidity in SA, we investigated the expression of FS in the duality of HIV associated PE.

Method
Serum samples of normotensive and pre-eclamptic women stratified by HIV status were collected from a large regional hospital in Durban, and their FS expression was analysed using the Bio-Plex Multiplex Immunoassay.

Results
Irrespective of HIV status, FS expression was significantly reduced in pre-eclamptic compared to normotensive pregnancies (2354 ± 353.6 vs 649.5 ± 116.8; $p < 0.001$). However, FS expression did not differ between HIV +ve vs HIV –ve groups (1727 ± 291.2 vs 1305 ± 306.7; $p = 0.13$)- regardless of pregnancy type. Furthermore, we detected significant FS expression across all study groups ($p > 0.05$).

Conclusion
This study demonstrates a downregulation of FS expression in PE, possibly due to oxidative stress and its immunoregulatory role in the hyperinflammatory milieu of PE. Moreover, the fact that FS did not vary by HIV status may be attributed to the effects of HAART regimen adopted in SA. It is also plausible to assume that the upregulation of FS expression (albeit non-significant) in HIV +ve patients, arises as a result of the immune response in controlling viral infection. Our novel findings suggest that FS may have a potential predicator test value early in pregnancy, hence work on this is ongoing.

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Dear Mr. Mdlalose,

We are happy to inform you your abstract is accepted for a poster presentation during xMAP® Connect 2018 Amsterdam.
This event takes place on November 6th and November 7th 2018 at the following location:
Royal Tropical Institute
Mauritskade 63
1092 AD Amsterdam
The Netherlands

We look forward to your poster presentation during xMAP® Connect Amsterdam.

Kind regards,

Thomas Pracht
Managing Director
Luminex EMEIA

Abstract Acceptance Letter

Date:
October 25, 2018

To
Mr. Siphesihle Mdlalose,
Department of Optics and Imaging
Laboratory Medicine and Medical
Sciences,
University of KwaZulu-Natal
South Africa
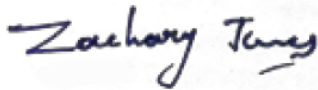
Your Abstract entitled **"The role if follistatin in HIV-associated pre-eclampsia"** has been accepted for **Poster Presentation** at "2nd International Meeting on Gynecology and Obstetrics Pathology 2018 (**Gynecology and Obstetrics Pathology 2018**)" scheduled on November 19-20, 2018 at Paris, France.

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Method: Serum samples of normotensive and pre-eclamptic women stratified by HIV status were collected from a large regional hospital in Durban, and their FS expression was analysed using the Bio-Plex Multiplex Immunoassay.

Results: Irrespective of HIV status, FS expression was significantly reduced in pre-eclamptic compared to normotensive pregnancies (649.5 ± 116.8 *vs* 2354 ± 353.6; $p<0.001$). However, FS expression did not differ between HIV +ve *vs* HIV −ve groups (1727 ± 291.2 *vs* 1305 ± 306.7; $p=0.13$) - regardless of pregnancy type. Furthermore, we detected significant FS expression across all study groups ($p>0.05$).

Conclusion: This study demonstrates a downregulation of FS expression in PE, possibly due to oxidative stress and its immunoregulatory role in the hyperinflammatory milieu of PE. Moreover, the fact that FS did not vary by HIV status may be attributed to the effects of HAART regimen adopted in SA. It is also plausible to assume that the upregulation of FS expression (albeit non-significant) in HIV +ve patients, arises as a result of the immune response in controlling viral infection. Our novel findings suggest that FS may have a potential predicator test value early in pregnancy, hence work on this is ongoing.

CHAPTER 4

SYNTHESIS

Maternal mortality within the African continent is high, with sub-Saharan Africa accounting for 85 % of the maternal mortality rate (Amzat, 2015). Whilst pregnancy places most women at a higher risk for a pregnancy-related event, some women are at higher risk of mortality than others (Hirshberg and Srinivas, 2017). More than 75 % of pregnancy related deaths are caused by hypertension and HIV, so clarification of the interaction in the duality of these conditions is imperative (Say *et al.*, 2014). There are more than 2 million pregnancies in HIV infected women worldwide, with the majority occurring within sub-Saharan Africa (Moodley *et al.*, 2010). In SA, one-fifth of women in their reproductive ages are HIV +ve (Statistics, 2018). Furthermore, hypertensive disorders such as PE contribute directly to maternal deaths occurring in SA (Moodley, 2010). Notably, PE accounts for 14.8% of all maternal deaths occurring within SA (Saving mothers 2014–2016, 2017).

Globally, PE affects 5-7% of pregnancies, with immunological and genetic factors implicated in its pathogenesis (Salman *et al.*, 2018). A defective trophoblast and the non-physiological conversion of myometrial spiral arteries contributes to placental ischaemia and the ensuing hypoxia which characterizes PE (Chaiworapongsa *et al.*, 2014). Moreover, an angiogenic imbalance in favour of elevated anti-angiogenic factors such as sFlt1 and sEng exist in PE together with a concomitant downregulation of pro-angiogenic factors [PIGF and VEGF] (Maynard and Karumanchi, 2011;Ngene and Moodley, 2017).

This study demonstrates a downregulation of FS in PE compared to the normotensive pregnant groups, irrespective of HIV status. These findings are corroborated by Zhang *et al.* (2018) who attributed the decline of FS to the high oxidative stressed micro-environment of PE. Similarly a longitudinal analysis also showed that serum FS levels are lower in women with PE compared to normotensive pregnant women (Garces *et al.*, 2015). FS is a glycosylated single-chain protein observed in the inhibition of FSH secretion (Kelaini *et al.*, 2018). The local role of FS is to neutralize the effect of activin by binding to its receptor thereby restricting activin bioavailability for stimulation of FSH (Keutmann *et al.*, 2004). In PE, serum activin is significantly higher compared to normotensive pregnant women (Yu *et al.*, 2011). Moreover, in PE- activin induces oxidative stress via the activation of smad2/3 promoting the release of NADPH oxidase 2 (NOX2), causing a hypoxic environment that results in FS downregulation (Zhang *et al.*, 2018). Furthermore, FS plays a role in tissue restoration and is involved in endothelial cell differentiation, including angiogenesis (Kelaini *et al.*, 2018). It is well established that an

angiogenic disproportion in favour of anti-*vs* pro-angiogenic state prevails in PE (Gathiram and Moodley, 2016). Since FS is a pro-angiogenic molecule, the downregulation of FS in PE is plausible , in view of the decreased angiogenesis (Turcotte *et al.*, 2017). Additionally, our findings are supported by Charkiewicz *et al.* (2018) who reported that pro-angiogenic factors (VEGF and PIGF) are downregulated, with a concurrent significant decline in FS expression in women with PE.

In contrast, a study conducted by Founds *et al.* (2015) described an upregulation of follistatin-like 3 (FSL3) over the course of pregnancy in women who subsequently developed PE. This was attributed to the higher insulin resistance in PE and/or to the effects of hypoxia. Results are contradictory in that enhanced serum FS levels have been reported in PE compared to normotensive pregnant women (Keelan *et al.*, 2002). However, D'Antona *et al.* (2000) reported the inverse.

This current study reports a non-significant difference albeit, an upwards trend in FS between the HIV +ve compared to the HIV –ve group, irrespective of pregnancy type. In HIV infection there is a loss of CD4+ cells and the suppression of the immune system (Poulton, 2018). Klein *et al.* (1996) showed that FS levels increased in sheep after the administration of LPS , illustrating the role of FS in the acute phase of an inflammatory response .Moreover, FS administration to mice reduces the levels of LPS, suggesting that FS has an anti-inflammatory effect (Hansen and Plomgaard, 2016). Notably, the anti-inflammatory effect of FS has been shown in patients with sepsis (Phillips *et al.*, 2001).

Moreover, inflammation related to hypoxia induces activin, which increases FSL3 transcription via the smad proteins (Founds *et al.*, 2015). Activin is expressed within inflammatory cells, promoting an inflammatory response (Monsuur *et al.*, 2018). The latter studies suggest that in an HIV infection, FS expression would be increased to neutralize the effects of activin. Confounding our results, is the fact that a standard of care practice in SA is HAART. This standard of care induces immune reconstitution in HIV infected pre-eclamptic women (Phoswa *et al.*, 2018). This implies that serum levels of activin would rise to elicit a systemic inflammatory response that is exacerbated in PE. The interaction of activin with smad proteins and the stimulation of nitric oxide metabolites is inhibited by the upregulation of FS (Phillips *et al.*, 2001). Therefore, we hypothesize that the FS upregulation in HIV +ve pre-eclamptic woman is a compensatory mechanism to neutralize the effects of activin, a release stimulated by the immune reconstitution effect of HAART.

In our study, there was no significant difference in the expression of G-CSF between the pre-eclamptic *vs* normotensive group, irrespective of HIV status. However, there was an observed down regulatory trend of G-CSF in PE. G-CSF is a glycoprotein that induces the differentiation of myelomocytic leukemic cell line (Schneider *et al.*, 2006). The G-CSF receptor is a single homodimer receptor which mediates the effects of G-CSF (Bendall and Bradstock, 2014). This receptor signals via STAT3, STAT5 and the MAPK cell signalling pathways (Hamilton *et al.*, 2016). It is plausible that STAT3 is downregulated in HIV infection negatively influencing dendritic cell function, thereby interfering with the mechanism that links innate and adaptive immunity.

Literature suggests that G-CSF promotes angiogenesis inducing the proliferation of endothelial cells (Kohlstedt *et al.*, 2018). Lee *et al.* (2005), showed that recombinant G-CSF when administrated in low doses effectively augments ischaemia, inducing angiogenesis *in vivo*. G-CSF and VEGF mobilize endothelial cells from the bone marrow into peripheral circulation and promote angiogenesis (Okazaki *et al.*, 2006). In PE, the angiogenic imbalance in favour of anti-angiogenic factors *vs* pro-angiogenic factors (G-CSF) results in endothelial dysfunction (Kalumba *et al.*, 2013).

Notably, endothelial cells produce G-CSF, since PE is characterized by endothelial dysfunction there will be a downregulation in G-CSF production. This was observed in our study as G-CSF was downregulated in the pre-eclamptic group. The production of G-CSF can be induced *in vitro* by TNF-α , IL-1 and bacterial LPS (Daniels and Lewin, 2008). These cytokines are present in pre-eclamptic women as a result of oxidative stress eventuating in an increase in G-CSF. The G-CSF downregulation noted in our study could be attributed to HAART as it reconstitutes the maternal immune response.

Furthermore, G-CSF expression in our study were similar between HIV +ve *vs* HIV –ve groups, regardless of pregnancy type. G-CSF plays an important role in the mediation of neutrophil release in the blood stream, with neutropenia being associated with an upregulation of inflammation (Cai *et al.*, 2017). The presence of G-CSF in the blood stream alters the body's immune system, eliciting an immune response to an infection (Basu *et al.*, 2002). Studies have highlighted the beneficial effects of G-CSF after it has been administrated in different inflammatory conditions (Jeannin *et al.*, 2018). A study conducted by Martins *et al.* (2010) showed that G-CSF has anti-inflammatory properties *in vitro* and *in vivo*. In addition, Boneberg *et al.* (2000) showed that *in vitro*, G-CSF decreases LPS-induced secretion of pro-inflammatory

cytokines by increasing mobilization of human monocytes. This suggests that G-CSF has an anti-inflammatory role by increasing the activation of monocytes, which release anti-inflammatory cytokines (Eyles *et al.*, 2006). Notably, in mice G-CSF mobilizes CD34+ progenitors which favour T-regulatory cell expansion, protecting the mice from developing type 1 diabetes (Kared *et al.*, 2005).

Our study demonstrated that G-CSF has an upregulatory trend in the HIV +ve group compared to the HIV –ve group. Since HIV infection is characterized by the drop in an HIV infected individuals CD4+ count, mice who were administrated with G-CSF showed an increased percentage of CD4+ regulatory T cells (Peng, 2017;Poulton, 2018). Hence in HIV infection, the compensatory action of G-CSF would be to promote an immune response. Nonetheless, HAART establishes an individual's immune response thus, the upregulation of G-CSF in HIV infection is to mobilize CD4+ cells.

In conclusion, this study reports the downregulation of FS and G-CSF in PE compared to normotensive pregnancies reflecting the anti-angiogenic state of PE. Both FS and G-CSF were upregulated albeit non-significantly in HIV infection, due to their immunoregulatory role in both innate and adaptive response. However, HAART may have affected analyte expression due to immune reconstitution in HIV infection further exacerbating the immune response of PE.

Future studies will include a larger sample size, stratification of the PE group by gestational age and the consideration of the duration of HAART.

CHAPTER 5

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APPENDICES

APPENDICE 1: BREC APPROVAL

UNIVERSITY OF
KWAZULU-NATAL

INYUVESI
YAKWAZULU-NATALI

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Biomedical Research Ethics Administration
Westville Campus, Govan Mbeki Building
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17 April 2018

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 you that your response received on 03 April 2018 to BREC letter dated 16 March 2018 has
been noted by a sub-committee of the Biomedical Research Ethics Committee.

Your request received on 07 March 2018 to append the studies below to the above study has now been
approved by a sub-committee of the Biomedical Research Ethics Committee

Name	Student number	Title
Deneshree Varaden	211510564	Morphometric image analysis of placental clec2d and HLA-G immunolocalization in HIV-associated pre-eclampsia
Merantha Moodley	214514757	The role of histone 2A in NETosis of HIV-associated pre-eclampsia
Kyle Kupsamy	214504430	EGF and HGF in HIV-associated pre-eclampsia
Mikaila Moodley	214558958	Immunoglobulin isotypes (IgG1, IgG2, IgG3, IgG4, IgM, IgA) in HIV-associated pre-eclampsia
Charlene Mukasa Sangany	211559876	The role of acidic and basic fibroblast growth factor in HIV-associated pre-eclampsia
Mduduzi Mazibuko	214504614	The role of sTIE2 and sHER2 in HIV-associated pre-eclampsia
Siphesihle Mdlalose	217011521	The role of follistatin and G-CSF in HIV-associated pre-eclampsia

This approval will be ratified at the next BREC meeting to be held on 08 May 2018.

Yours sincerely

Mrs A Marimuthu
Senior Administrator: Biomedical Research Ethics

APPENDICE 2: Follistatin standard curve
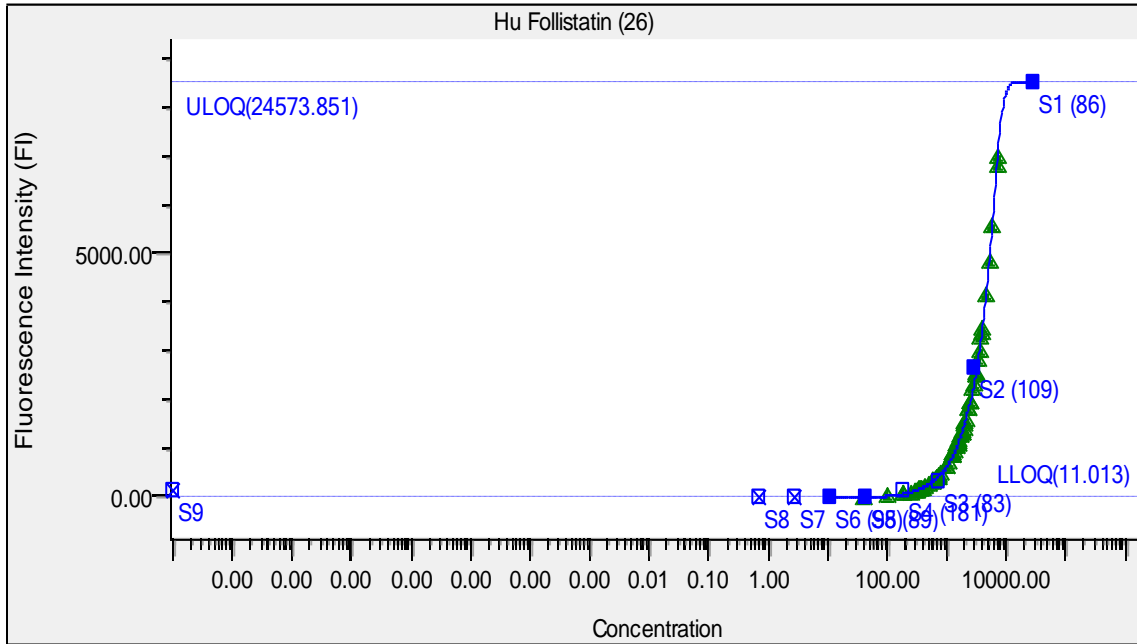
APPENDICE 3: G-CSF standard curve