TRANSFORMING GROWTH FACTOR BETA 1-3 IN HIV ASSOCIATED PRE-ECLAMPSIA

By

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PREFACE

This study contains original work done by the author, it has not been submitted in any form to any other University. Work done by other authors has been used and has been duly acknowledged in the text.

The research within this dissertation was conducted in the Optics and Imaging centre, Doris Duke Medical Research Institute, College of Health Science, University of Kwa-Zulu Natal, Durban, South Africa under the supervision of Professor Thajasvarie Naicker.

DECLARATION

I, **Zamahlabangane Sisiziwe Mtshali** declare that:

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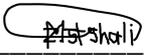
Signed: _____Date: 30/01/17

DEDICATION

To mother – Thank you for your endless love and support that you have shown me throughout my life, I am truly blessed to have you as my parent. Without you I wouldn't have been granted this beautiful opportunity therefore I dedicate this Dissertation to you as my inspiration.

To God- Thank you for the gift of life and the many blessing you have showered upon me, I truly thankful. My life is prosperous day by day all thanks to your everlasting love. Thank you almighty.

To Lindokuhle and Ayabonga thank you for being in my life and motivating me excel in everything I do, you are truly blessings

"But those who hope in the Lord will renew their strength; they will fly up on wings like eagles; they will run and not be tired; they will walk and not be weary."

Isaiah 40:31

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LIST OF ABBREVIATIONS

WORD/S	ABBREVIATION
Acquired Immunodeficiency Syndrome	AIDS
Blood Pressure	BP
endoglin	Eng
Early onset pre-eclampsia	EOPE
Highly Active Antiretroviral Therapy	HAART
Human Immunodeficiency Virus	HIV
Intraurine growth restriction	IUGR
Late onset pre-eclampsia	LOPE
millennium development goals	MDG
milligram	Mg
Magnesium sulfate	$MgSO_4$-
millimetres mercury	mmHg
Maternal Mortality Ratio	MMR
Normotensive HIV Negative	N-
Normotensive HIV Positive	N+
Pre-Eclampsia	PE
Pre-Eclamptic HIV Negative	PE-
Pre-Eclamptic HIV Positive	PE+
picograms per milliliter	pg/ml
Placental Growth Factor	PlGF
soluble endoglin	sEng
soluble fms -like tyrosine kinase 1	sFlt-l
Transforming Growth Factor beta	TGF-β

Transforming Growth Factor beta 1   TGF-β1

Transforming Growth Factor beta 2   TGF-β2

Transforming Growth Factor beta 3   TGF-β3

ABSTRACT

Introduction: Pre-eclampsia is a clinical syndrome that complicates approximately 3-8% of all pregnancies and is the second leading cause of maternal mortality. The maternal imbalance of pro-angiogenic and anti-angiogenic factors evident in pre-eclampsia has influenced investigation of circulating levels of TGF-β in normotensive and pre-eclamptic women to determine if this protein can be used as a biomarker for pre-eclampsia development. The aim of this study was to determine the level of transforming growth factor β1, β2 and β3 in serum of HIV associated pre-eclampsia

Methods: The study consisted of 76 participants including normotensive HIV negative (19), normotensive HIV positive (19), pre-eclamptic HIV negative (19) and pre-eclamptic HIV positive (19). The TGF-beta levels were measured using the Bio-plex pro assay kit and the results were read using the Bio-Plex® MAGPIX ™ Multiplex Reader**.**

Results: There was no significant difference in TGF-β1 and TGF- β2 levels between pre-eclamptic and normotensive groups (p=0.3921 and 0.9265) respectively. Also based on HIV status there was no significant difference in beta-1 and beta-2 levels amongst HIV positive and HIV negative groups (P=0.6312 and 0.5655) respectively. Moreover, TGF-β3 levels showed no significant difference between normotensive and pre-eclamptic groups; however they differed significantly between the HIV negative and HIV positive groups; p=0.0461.

Conclusion: No significant difference in TGF-β1 and β2 serum levels between the pre-eclamptic and normotensive pregnancy women was observed, however TGF-β3 levels differed significantly between HIV positive and HIV negative women. These findings support the predictor test value of TGF-β3 as a biomarker for HIV associated pre-eclampsia

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 MATERNAL MORTALITY

Pre-eclampsia is among the top three leading causes of maternal mortality and morbidity among pregnant women (Abalos et al., 2013). Globally, more than 500 000 women die annually from pregnancy related causes such as preeclampsia/ eclampsia. Approximately 99% of deaths are occurring in developing countries (Abalos et al., 2013). The lifetime risk of a women dying from maternal related causes is 1 in 39 in sub-Saharan Africa compared to 1 in 3800 in developed countries (Abalos et al., 2013).

In 2013, approximately 2 890 000 maternal deaths were reported, of which Sub-Saharan Africa accounted for 67% and Asia 24% (Singla et al., 2017). The maternal mortality ratio (MMR) is not consistently distributed throughout the world, in developing countries the MMR is15 times higher when compared to developed countries (Islam, 2007). Various socio-economic factors including poor access to quality health care are major contributors to maternal mortality (Keskinkılıç et al., 2017).

To reduce MMRs, the WHO has adopted and implemented initiatives to promote mother-care survival as well as safe pregnancy. However, countries such as sub-Saharan Africa are far from attainment of the new sustainable development goal targets of reducing maternal mortality by 2030 (Srofenyoh et al., 2016, Nachbar et al., 1998, Organization, 1996)

1.2 PRE-ECLAMPSIA

1.2.1 Pre-eclampsia definition and diagnosis

Pre-eclampsia is defined as new onset gestational hypertension with a systolic blood pressure (BP) ≥140 mmHg, diastolic BP ≥90 mmHg and proteinuria ≥ 0.3g per 24 hours

(Mammaro et al., 2009). The disorder is specifically unique to human pregnancy (Brennan et al., 2014). Globally, pre-eclampsia (PE) complicates approximately 3-8% of all pregnancies, making it one of the leading causes of maternal and perinatal morbidity (Bergström et al., 1991, Faupel-Badger et al., 2011). The syndrome is associated with peripheral vasoconstriction along with reduction in arterial compliance whilst proteinuria is associated with glomerular endotheliosis (Pepper, 1997). The latter is associated with glomerular endothelial cells swelling and endothelial fenestrations being compromised (Lafayette et al., 1998). For these reasons pre-eclampsia has been reported to target the endothelium (Bosio et al., 1999).

1.2.2 Pre-eclampsia classification

Pre-eclampsia may be classified into two categories based on the gestational period and onset of symptoms (Tranquilli *et al*., 2014)

- Early onset pre-eclampsia (EOPE): presentation of signs and symptoms < 33 weeks + 6 days

- Late onset pre-eclampsia (LOPE): presentation of signs and symptoms >34 weeks. This second category has since been divided into 2 subcategories;

    - Preterm pre-eclampsia (starting from 34 weeks + 1 day to 37 weeks + 0 day) and

    - Term pre-eclampsia (occurring after 37 weeks +1 day).

Importantly, EOPE and LOPE are considered as 2 different entities sharing common presentations (Valensise et al., 2013, Redman and Sargent, 2005). Maternal and perinatal outcomes are generally poor in EOPE compared to those observed in LOPE (Mitsui et al., 2015, Madazli et al., 2003)

1.2.3 Pre-eclampsia pathogenesis

The exact mechanism of pre-eclampsia pathogenesis remains unclear although it is evident that the key organ of dysfunction is the placenta (George and Granger, 2010). Risk factors associated with the syndrome include obesity, pre-existing hypertension, multiple pregnancies and age (>40 years) (Thäle and Schlitt, 2008). PE development is proposed to be a two stage process including the pre-clinical (asymptomatic) and the clinical (symptomatic) phase (Govender et al., 2013). The pre-clinical stage is characterised by placental hypoxia, immune dysregulation and oxidative stress which is a result of abnormal placentation (Govender et al., 2013). The clinical stage however includes the release of soluble factors sFlt1 and sEng into the circulation resulting in clinical symptoms such as hypertension, proteinuria and intrauterine growth restriction (Govender et al., 2013)

1.2.3.1 Normal placentation

The pregnancy process involves the formation of new blood vessels from pre-existing vessels through angiogenesis and vascular remodelling therefore permitting placental development and functioning (Faupel-Badger et al., 2011). Placentation is regulated by numerous pro and anti-angiogenic factors which work together to maintain an angiogenic balance (Faupel-Badger et al., 2011)

The placenta is an organ that supports the early life development of the foetus, it has unique endocrine and immunomodulatory properties which facilitate implantation, trophoblast invasion, proliferation and differentiation, vasculogenesis and angiogenesis (Gude et al., 2004). The placenta allows for gestational adaptation of the mother, it additionally aids

nutrient transfer, waste removal, metabolism as well as growth and development of the foetus (Gude et al., 2004). The placenta is the main organ involved in pre-eclampsia pathogenesis therefore its delivery is the only known treatment for the condition (BROWN, 1995).

Normal placentation includes trophoblast invasion into uterine spiral arteries, this results in the decrease in vascular resistance, loss of smooth arteriolar muscle as well as terminal vessel dilation which results in the increase in the spiral artery diameter hence enabling an adequate blood supply to the growing foetus (Wang et al., 2009).

1.2.3.2 Abnormal placentation

In PE cytotrophoblast migration is reduced whilst endovascular invasion is limited to the decidua (Meekins et al., 1994). Moreover, the physiological transformation of the spiral artery is limited to the decidua hence the diameter of the spiral arteries remain small and of high resistance, this eventuates in inadequate blood supply to the foetus (Faupel-Badger et al., 2011). Narrowing of the artery also contributes to the increase in blood pressure which consequently leads to PE development (Levy and Murphy, 2002, Meekins et al., 1994). A lack of physiological spiral artery remodelling may emanate from the influence of cytokines on migratory trophoblast cells within the decidua and myometrium (Naicker et al., 2003).
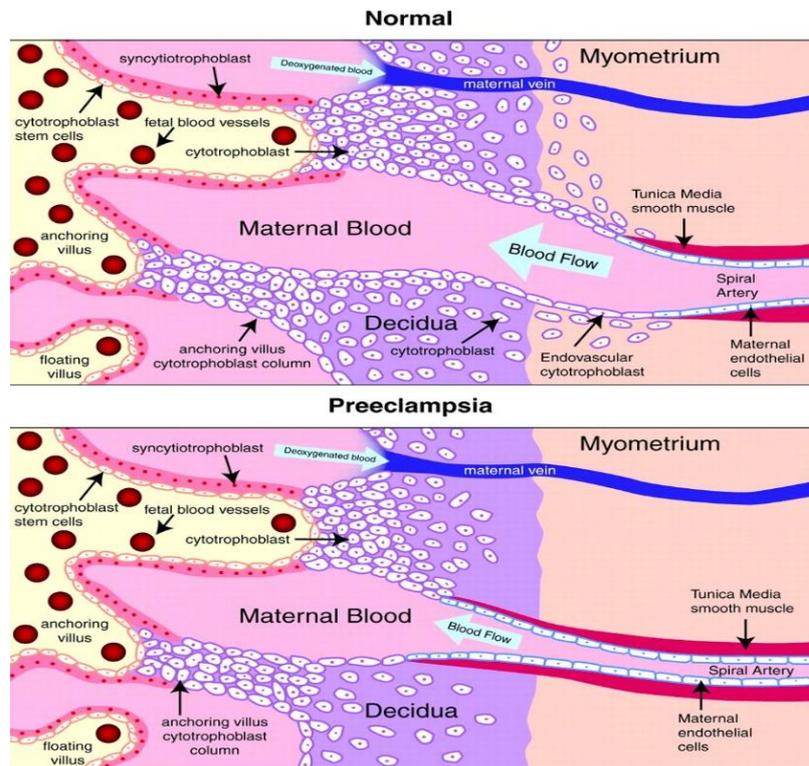
Figure 1: Normal versus abnormal placentation (adopted from (Powe et al., 2011))

1.2.4 PE management and treatment

The only curative treatment for pre-eclampsia is delivery although it can be managed using therapeutics (Ayoubi, 2011). Management of PE requires multiple disciplines including a paediatrician, an obstetrician and an anaesthetist, induced preterm delivery may be conducted considering minimal maternal and foetal risk(Ayoubi, 2011). Two interrelated factors determine the criteria for delivery namely the gestational age at diagnosis *ie*. foetal weight and the severity of the PE condition (Ayoubi, 2011). The WHO recommends the use of low dose aspirin and calcium for prevention of PE (LeFevre, 2014). Depending on the severity of the hypertension different antihypertensive drugs can be used, in France nicardipine, clonidine, labetalol and di-hydralazine are the authorized drugs for treatment (Ayoubi, 2011, Duley et al., 2006).

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1.3. HUMAN IMMUNODEFICIENCY VIRUS

Human Immunodeficiency Virus (HIV) infection is a major public health concern that has claimed over 35 million lives globally (Organization, 2010). In South Africa approximately 30% of antenatal attendees are living with the infection (HIV/AIDS, 2016, Sebitloane et al., 2009).

HIV weakens the immune system by targeting defence cells specifically the CD4 T lymphocytes. Consequently, an individual who becomes HIV infected becomes susceptible to numerous infections and diseases. Antiretroviral drugs suppress the viral load thereby delaying disease progression (Organization, 2010) (Group, 2015a, Group, 2015b).

1.4 HIV ASSOCIATED PRE-ECLAMPSIA

The routine use of highly active antiretroviral therapy (HAART) has contributed to decrease in mortality rates among HIV infected individuals, although adverse long-term complications such as lipodystrophy and cardiovascular disease are associated with the infection (Group, 2003).

Normal human pregnancy exists as a pro-inflammatory state while pre-eclampsia is an over-expressed immune state (Lorquet et al., 2010). HIV infection is associated with a reduced immune state due to CD4 lymphocyte depletion therefore HIV associated pre-eclampsia is a combination of opposing immunities, resulting in a neutral state which is proposed to be the reason of reduced incidence in untreated patients (Wimalasundera et al., 2002).

Prior to the pre-HAART era, HIV infected women were unlikely to develop pre-eclampsia when compared to the general population, however routine HAART usage has increased

the incidence of PE development (Stratton et al., 1999, Wimalasundera et al., 2002). It is debatable whether HAART usage predisposes women to hypertensive disorders such as pre-eclampsia when compared to the untreated individuals (Suy et al., 2006).

1.5 TRANSFORMING GROWTH FACTOR BETA

1.5.1 Transforming growth factor-β superfamily

The transforming growth factor-β family consists of three interrelated homo-dimeric isoforms denoted TGF-β1, TGF-β2 and TGF-β3, these factors bind to cell surface receptors to exert their biological action (Lyall et al., 2001). TGF-β family members are synthesized as pro-propeptide precursors which are processed and secreted as homodimers or heterodimers(Sporn and Roberts, 1989). Latent TGF-β, consists of TGF-β dimer, and a latency-associated peptide (LAP). This small latent protein complex binds with the latent TGF-β binding proteins (LTBP) and is anchored in the extracellular matrix. TGF-β is activated when a large protein complex is dissociated from extracellular matrix via proteolytic cleavage (Figure 2). Most ligands of this family signal through transmembrane serine/threonine kinase receptors and Smad proteins to regulate cellular functions (Figure 3)(Pennison and Pasche, 2007). Alterations of certain components of the TGF-β-signaling pathway may result various pathologies such as cancer, tissue fibrosis, cardiovascular pathology and autoimmune diseases (Kastin, 2013)
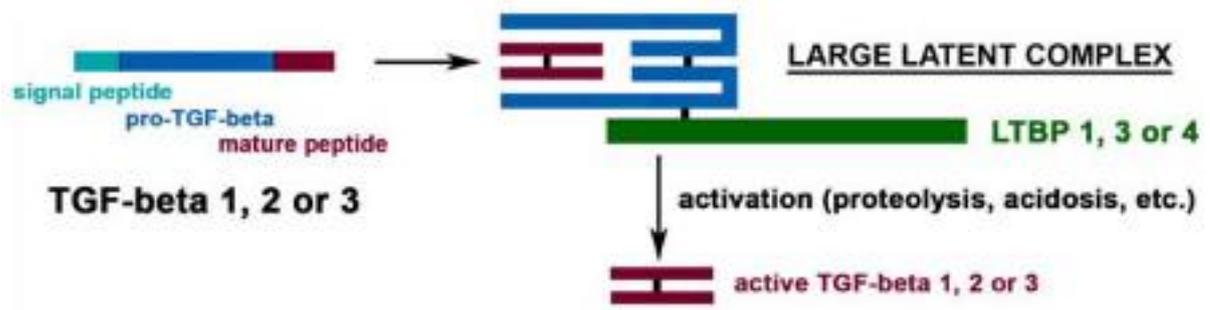
Figure 2: The precursor form of TGF beta consists of a mature TGF beta attached to a latency associated peptide (LAP). Enzymatic digestion of LAP or binding to integrins releases the active form(adopted from (Dobolyi,2012)

1.5.2 TGF-β Receptors

TGF-β binds to the receptor Tβ-RII; this binding might be enhanced by the presence of Tβ-RIII (Blobe et al., 2000). These receptors are serine/threonine kinase receptors which have a cysteine rich extracellular domain, a transmembrane domain and a cytoplasmic serine/threonine rich domain. After binding to TGF-β, Tβ-RII recruits and phosphorylates Tβ-RI, leading to activation of Smad 2 and Smad 3 by phosphorylation (Massagué et al., 2005). This process is inhibited by Smad 7. Activated Smad 2 and Smad 3 form heterodimers with Smad 4 and translocate to the nucleus (Massagué et al., 2005). Together with co-activators, co-repressors and other transcription factors, the Smad complex regulates gene expression (Figure 3).
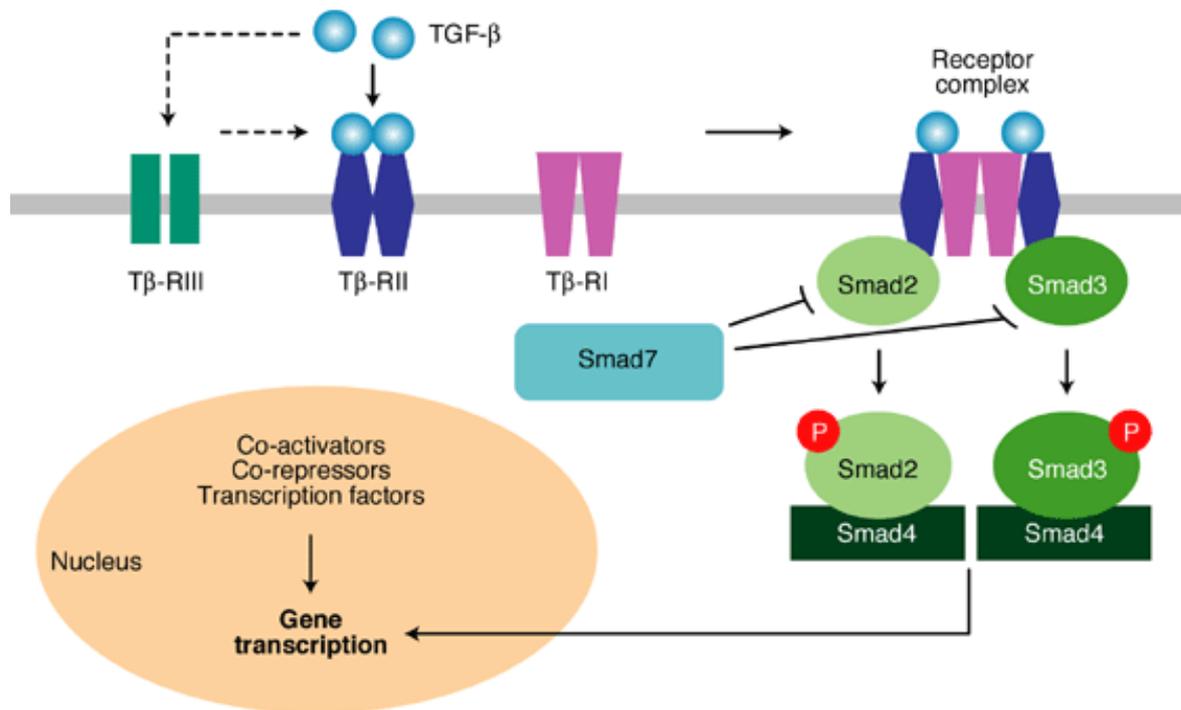
Figure 3: The transforming growth factor signalling pathway (adopted from (Pinzani and Marra, 2001)

1.5.3 TGF-β Function

Almost every cell in the body, including epithelial, endothelial, hematopoietic, neuronal, and connective-tissue cells, produce TGF-β (Blobe et al., 2000). TGF-β is known to play an important role in the physiology of a successful pregnancy (Inan et al., 2002). The biological action of TGF-β is facilitated in one of two ways namely autocrine or a paracrine manner (Inan et al., 2002). TGF-β1 is normally a protective, anti-inflammatory cytokine, but its overproduction may have serious pathogenic effects. TGF-β1 overexpression has been associated with many hypertensive disorders, it is suggested to be an important mediator of angiotensin II-induced hypertensive damage (Inan et al., 2002). The TGF-β family are reported to elevate the release of reactive oxygen species (ROS) by various cells, causing a down regulation of glutathione, the most profuse intracellular free thiol and a

vital antioxidant (Liu and Gaston Pravia., 2010). ROS, in turn, boosts the assembly and reactivity of TGF-β through activation of latent TGF-β (Liu and Gaston Pravia., 2010).

1.5.4 TGF-β isomer expression in pregnancy

TGF-βs are produced mainly by the decidua and mediate several cellular processes such as implantation, trophoblast invasion, angiogenesis and endothelial growth in pregnancy (Figure 4) (Poniatowski et al., 2015). TGF-β3 is the most common in the placenta and umbilical cord when compared to β-1 and β-2, it is known to stimulate collagen and sulfonated glycosaminoglycan synthesis by fibroblasts. TGF-β3 has been suggested to play a role in pre-eclampsia pathogenesis by altering the umbilical cord structure and responsiveness (Inan et al., 2002). Former studies reveal that TGFβ-1 and TGFβ-2 levels are increased in patients with pre-eclampsia, moreover evidence suggests that over expression of TGFβ-3 during early gestation results in trophoblast hypo-invasion in pre-eclampsia (Li, 2014).

TGF-βs are implicated as good biomarkers for pre-eclampsia development, however some investigators report indistinguishable differences in TGF-β levels in pre-eclamptic and normotensive women (Huber *et al*., 2002). The inconsistency in results demonstrate the need for further investigations to clarify expression particularly in HIV associated pre-eclampsia.
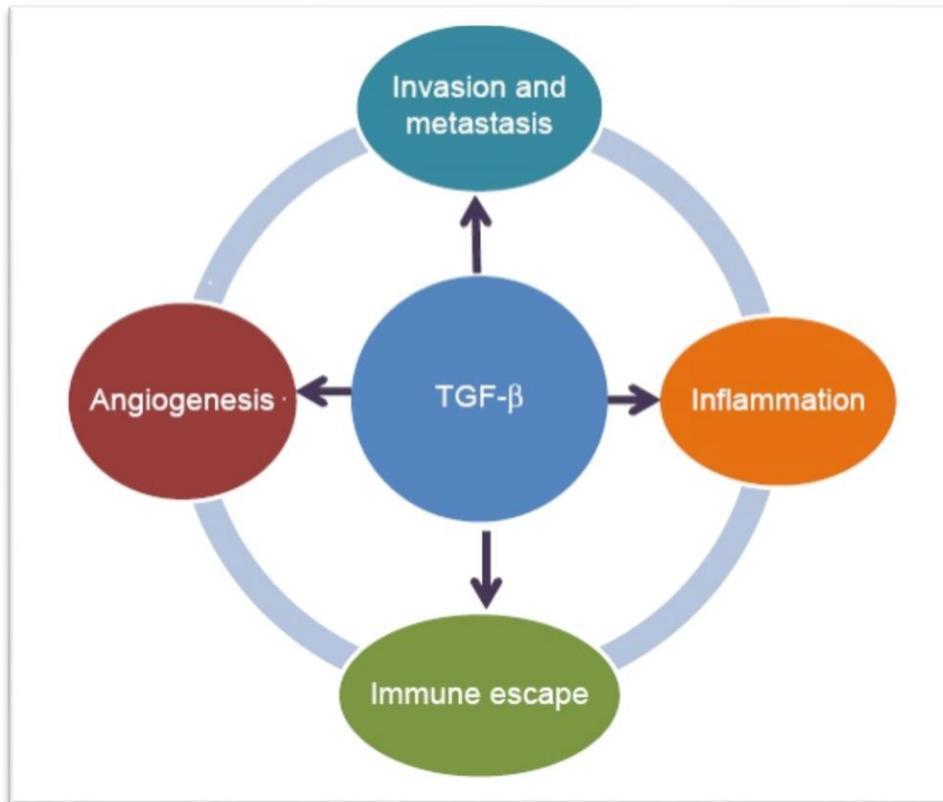
Figure 4: The role of TGF beta in inducing angiogenesis, inflammation, invasion and immune escape (adopted from (Herbertz et al., 2015))

1.6 AIM

The aim of this study is to determine TGF-β1-3 levels in serum of HIV associated pre-eclampsia

1.6.1 *Objectives*

- To determine the concentration of TGF-β 1-3 based on pregnancy type (normotensive vs pre-eclampsia)

- To determine the concentration of TGF-β 1-3 based on HIV status (HIV infected vs HIV uninfected)

- To compare and contrast the concentration of TGF-β 1-3 across all study groups

CHAPTER 2

Submitted manusript

Serum Transforming growth factor βeta 1-3 in HIV associated pre-eclampsia

Serum Transforming growth factor βeta 1-3 in HIV associated pre-eclampsia

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Abstract

Objective: The evident role of Transforming growth factor βs in pre-eclampsia has driven our interest to assess serum level expression of TGF-β1, β2 and β3 in pre-eclamptic and normotensive pregnant women in order to determine if they may be predictive biomarkers for pre-eclampsia development. *Method:* This retrospective study comprised of 76 participants, consisting of 38 pre-eclamptic pregnant women who were compared to 38 normotensive controls. The pre-eclamptic and normotensive groups were further subdivided into 19 cases of HIV positive and 19 of HIV negative. *Results:* There was no significant difference in maternal serum levels of TGF-β1 and β2, however β3 differed significantly between the HIV infected and HIV uninfected groups (p>0.05). *Conclusion:* We report no significant difference in TGF-β1 and β2 serum levels between the pre-eclamptic and normotensive groups, however there was a significant difference in β3 levels amongst HIV positive compared to negative women indicating that TGF-β3 is a potential biomarker for HIV associated pre-eclampsia.

Key words: HIV, pre-eclampsia, TGF-beta1, TGF-beta 2, TGF-beta 3

Introduction

South Africa has the highest HIV prevalence in the world, with 19.1% of South African women in their reproductive age being HIV positive and are 6 times more likely to die than HIV negative women (Africa and Health, 2013). Approximately 29.5% of antenatal attendees are living with HIV (Pattinson, 2014) Also, hypertension in pregnancy is one of the leading causes of maternal mortality in South Africa (14.8%), of which pre-eclampsia (PE) accounts for majority of these deaths (Pattinson, 2014). Worldwide, PE complicates approximately 3-8% of all pregnancies and is associated with considerable maternal and perinatal morbidity and mortality (Bergström et al., 1991, Faupel-Badger et al., 2011, Suy et al., 2006, Sansone et al., 2016) .

The transforming growth factor (TGF) superfamily includes inhibin, activin and morphogenic proteins which are dimeric and structurally related (Pepper, 1997, Godkin and Dore, 1998). In pregnancy, these cytokines are produced by decidual cells and play a crucial role in pregnancy success (Bergström et al., 1991). To initiate signalling these cytokines bind to type I and type II cell surface receptors as well as endoglin, a co-receptor (Perucci et al., 2014). Their functional ability to control cell-cell adhesion, cell migration and tissue remodelling has resulted in researchers proposing their modulatory role in implantation, trophoblast invasion, angiogenesis and endothelial growth in pregnancy (Africa and Health, 2013, Perucci et al., 2014). Studies have also implicated conflicting roles of the different isomers TGF-β1 and TGF-β2 but not TGF-β3 in trophoblast invasion (Simpson et al., 2002).

To-date the expression of circulating TGF-β is controversial and inconsistent due to varying sample types and detection methods (Li et al., 2014). Also, to our knowledge, there is a lack of data on TGF-β in the duality of HIV infection and pre-eclampsia. Therefore, the

aim of our study was to determine the expression of TGF-β1, β2 and β3 in serum of HIV associated pre-eclampsia and normotensive pregnant women.

Methods

Institutional approval: Post institutional ethics approval (BE256/12), to conduct the study at a large referral hospital situated in Umlazi, KwaZulu-Natal was obtained from the hospital manager and from the Department of Health, South Africa. A volume of 3.5 ml (serum) was collected by venepuncture in a separating tube (BD367957-SST, Becton Dickinson, USA) without anticoagulant and stored at -80°C. Furthermore, institutional permission to conduct this study on these retrospectively collected samples was obtained (BE212/17).

Study population: Patient demographics were recorded by a clinical research nurse at the antenatal clinic. The patients information recorded include; maternal age, gestational age, parity and HIV status. The serum samples were collected between 2013 and 2014 and were stored for 2 years prior to analysis. Participants consisted of 76 pregnant women divided into four groups *i.e.* HIV negative normotensive pregnant (n=19), HIV positive normotensive pregnant (n=19), HIV negative pre-eclamptic (n=19) and HIV positive pre-eclamptic (n=19). Inclusion criteria for the pre-eclamptic group was a persistent systolic blood pressure and diastolic blood pressure of >140 mmHg and 90 mmHg respectively, as well proteinuria of at least 1+ on dipstick analysis. The exclusion criteria for all the study groups included non-Black ethnicity, women with chronic hypertension, chronic renal disease, gestational diabetes, cardiac failure and chronic diabetes.

Bio-Plex multiplex method: Maternal serum samples were centrifuged at 3000 rpm for 10 min at 4°C and the supernatant was used for the quantification of TGF-β 1, 2 and 3 using

the Bio-Plex Pro TGF-β assay kit (Catalogue number-10024984; Bio-Rad Laboratories, Inc., USA). The experiment was conducted according to the manufacturer's instructions. Standards were diluted at 1:4. Five µl of HCL acid (1N) was added to 25µl of the sample to activate TGF-β. Five µl of base (1.2N NaOH/0.5M HEPES) was also added to neutralize the sample followed by dilution of the samples (1:16 dilution series). Capture antibodies directed at TGF-β1, 2 and 3 were covalently attached to the coupled magnetic beads, subsequently the coupled beads reacted with the serum sample containing the TGF-β isoforms. Following several wash steps to remove unbound protein, the biotinylated detection antibody was added to form a complex. The final detection complex was completed by the addition of streptavidin-phycoerythrin conjugate which served as a reporter. The sample was read using the Bio-Plex® MAGPIX ™ Multiplex Reader (Bio-Rad Laboratories Inc., USA). Analysis was performed using the Bio-Plex Manager™ software version 4.1.

The known concentration (pg/ml) of each analyte was used to generate a standard curve for each cytokine TGF-β isoform from which the concentration of the unknown samples was interpolated. Inter- and intra-plate variability were determined with CV <20% and (Obs /Expected) *100 between 70-130% (r=0.8, p=0.05).

Statistical analysis: The data obtained from the Bio-Plex reader was pre-processed and analysed using Graph Pad Prism 5 (California, USA). Non-parametric tests were performed, and the data is presented as median and interquartile range whilst the parametrically distributed data are presented as mean and standard deviation. A two-way t-test was used to compare TGF-β expression across the study groups (confidence interval: 95%). Clinical and demographical data was analysed using SPSS software version 24 and level of significance set at p <0.05.

Results

Patients demographics: Clinical and demographic data from the normotensive and pre-eclamptic groups are displayed in Table 1. As expected the systolic and diastolic blood pressure were significantly higher in pre-eclampsia compared to the normotensive group ($p<0.0001$). Similarly, gestational age at delivery was significantly lower in pre-eclampsia compared to the normotensive group ($p<0.0001$). Moreover, the birth weight of babies in the pre-eclamptic group was significantly lower than that of the normotensive group *i*.e., 3.27 ± 0.322 kg *vs* 2.16 ± 0.92 kg respectively ($p<0.0001$). However, maternal age was similar between the normotensive *vs* pre-eclamptic women [Mann-Whitney test; $p< 0.2153$].

TGF-β 1, 2 and 3 expressions

The concentration of TGF-β 1, 2 and 3 are outlined in Table 2.

TGF β1: Based on pregnancy type (normotensive *vs* pre-eclampsia) and regardless of HIV status there was no significant difference between the groups ($p=0.3921$). Furthermore, based on HIV status TGF-β 1 expression was similar between HIV positive vs HIV negative groups ($p=0.6312$). Moreover, there was no significant difference observed across all the groups ($p=0.7634$).

TGF-β2: Based on pregnancy type and regardless of HIV status, there was no significant difference between the normotensive and pre-eclampsia group ($p=0.9265$). Similarly, based on HIV status there was no significant difference between the HIV positive *vs* the HIV positive groups ($p=0.5655$). Also, no significant difference was observed across all the groups ($p=0.9146$).

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TGF-β3: Based on pregnancy type and regardless of HIV status, there was no significant difference in β3 levels between normotensive and pre-eclampsia patients ($p=0.0678$), however based on HIV status there was a significant difference in β3 between HIV positive vs HIV negative groups ($p=0.0461$). No statistical significant difference was noted across all the study groups ($p=0.6822$).

Correlation: Correlation analysis was performed within the pre-eclampsia group and a positive correlation was found between TGF-β1 and TGF-β2 (r=0.8525, $p<0.0001$); TGF-β1 and TGF-β3 (r=0.8381, $p<0.0001$) and TGF-β2 and TGF-β3(r=0.7333, $p<0.0001$).

Tables

Table1: Patient demographics and clinical characteristics of normotensive and pre-eclamptic pregnant women

Characteristics	Normotensive (n=38)	Pre-eclampsia (n=38)	*p* values
Maternal age (years)	25.26±6.08	26.18±5.02	0.2153(ns)
Gestational age (weeks)	38.25±2.23	33.81±4.40	<0.0001
Maternal weight(kg)	72.50±15.07	75.56±16.96	<0.0001
Systolic blood pressure(mmHg)	118.3±11.09	161.9±17.71	<0.0001
Diastolic blood pressure (mmHg)	73.50±11.15	101.7±13.69	<0.0001
Birth weight (kg)	3.27±0.32	2.16±0.92	<0.0001

Data given as mean ± standard deviation

Table 2: Serum transforming growth factor β1-3 across all the study groups

	Normotensive pregnant		Pre- eclampsia		
	HIV negative	HIV positive	HIV negative	HIV positive	*p* value
TGF-β1(pg/ml)	4.106±5.109	3.078±2.673	3.74±0.54	3.738±2,234	0.7634
TGF-β2 (pg/ml)	0.176±0.128	0.152±0.085	0.163±0.086	0.160±0.117	0.9146
TGF-β3 (pg/ml)	0.032±0.015	0.028±0.011	0.028±0.008	0.026±0.024	0.6822

Data represented as mean ± standard deviation

Figures

A

B

C

Figure 1

A

B

C

Figure 2

A

B

C
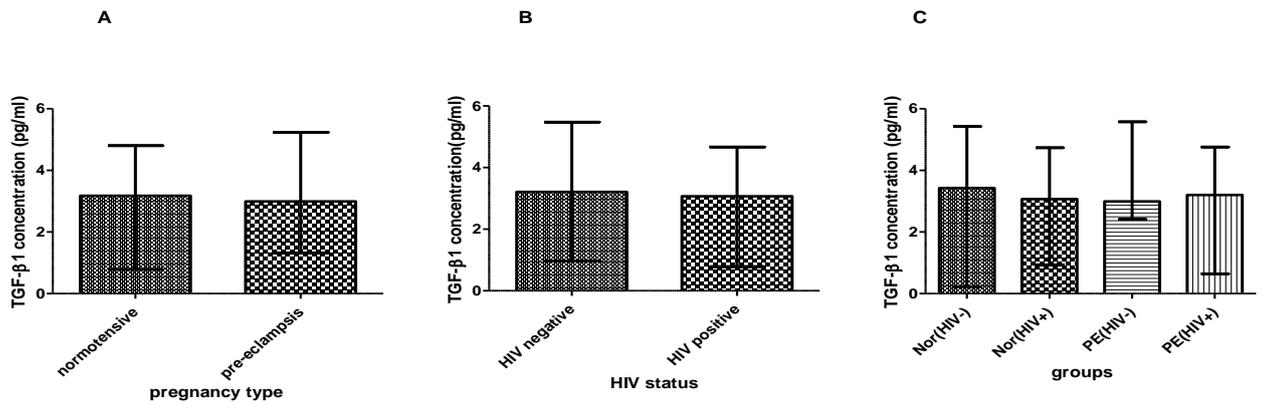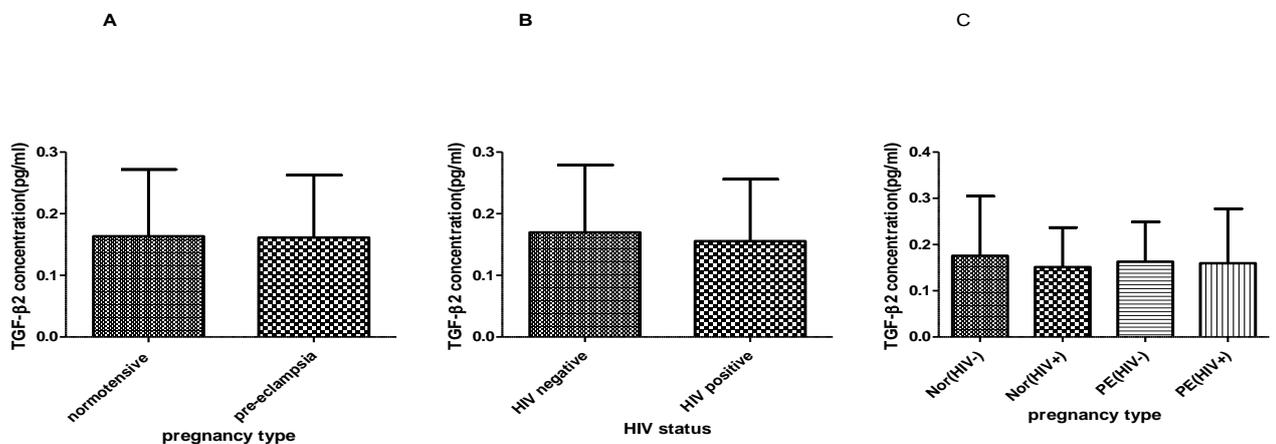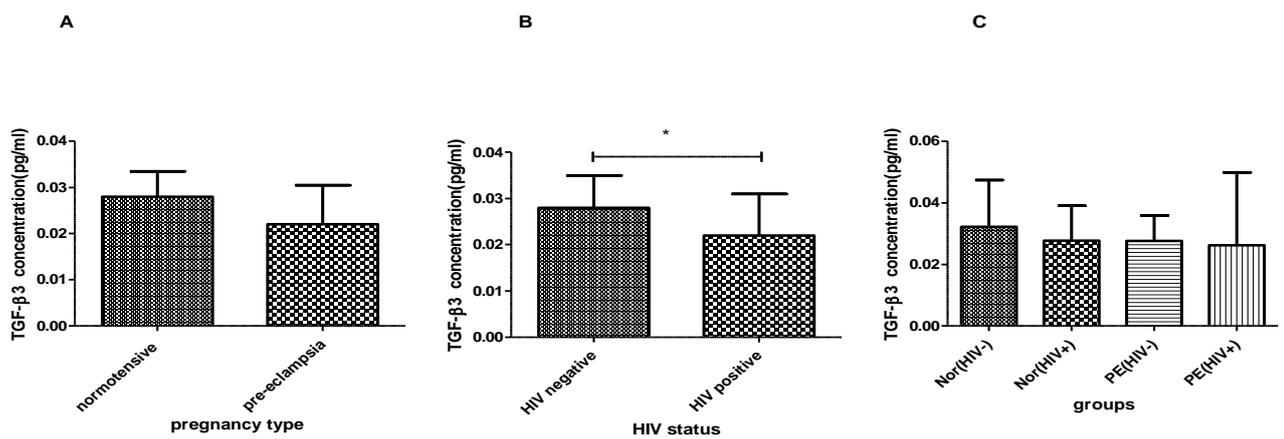
Figure 3

Discussion

In this study, as expected a significantly higher systolic and diastolic blood pressure ($p<0.0001$) was noted in the pre-eclamptic group. Also, maternal age was similar between the pre-eclampsia (PE) and normotensive pregnant group. Moreover, gestational age at delivery and birth weight were significantly lower in the pre-eclamptic group compared to normotensive pregnancy. It is well documented that PE significantly increases the risk of iatrogenic preterm birth for maternal indications (Xiong et al., 2002).

This study demonstrated a non-significant difference in serum TGF-β1, TGF-β2 and TGF-β3 expression based on pregnancy type (normotensive *vs* pre-eclampsia). These findings are in accordance with the work of Huber and co-workers (2002) who reported similar serum TGF-β1 expression between pre-eclamptic and normotensive pregnant women. In contrast, Yusrawati *et al.* (2017) reported a decline of TGF-β1 expression in PE compared to normal pregnancy.

The slight downwards trend of TGF-β1 expression in PE compared to normotensive pregnancies corroborates the abnormal trophoblast invasion theory of PE. It is widely accepted that TGF-β1 decreases trophoblast invasion via its effect on matrix metalloprotease and tissue inhibitors of metalloproteases and integrins (Cheng et al., 2013, Yusrawati et al., 2017).

Our results are also supported by other reports demonstrating similar serum TGF-β1 and TGF-β2 between pre-eclamptic and normotensive pregnancies (Szarka et al., 2010, Perucci et al., 2014, Huber et al., 2002). Surprisingly, the TGF-β1 co-receptor soluble Endoglin (sEng) is elevated in sera of women with pre-eclampsia resulting in disruptions between TGF-β1 and its receptors (Stepan et al., 2007).

Pre-eclampsia is known as a hypoxic state with high antioxidant production (Cohen et al., 2015). TGF-β is known to elevate the cellular release of reactive oxygen species (ROS), causing a down regulation of glutathione, the most profuse intracellular free thiol and a vital antioxidant (Liu and Pravia, 2010). ROS, in turn, boost the assembly and reactivity of TGF-β through activation of latent TGF-β (Liu and Pravia, 2010).

Since TGF-β3 is driven by low oxygen tension, it is the main regulator of early differentiation events that limit trophoblast invasion (Caniggia et al., 2000). When TGF-β3 is over expressed in the placenta as observed in PE, trophoblast differentiation is inhibited

thereby compromising its invasion (Caniggia et al., 1999). Moreover, TGF-β3 stimulates collagen and sulphated glycosaminoglycan biosynthesis thereby affecting cell migration (Inan et al., 2002).

In this study, in contrast to TGF-β1 and TGF-β2, TGF-β3 expression was downregulated in the HIV positive group. TGF-β is a powerful cytokine that suppresses the immune system driving disease progression in HIV-infected persons via both non- and profibrotic machinery. It suppresses T-cell proliferation and inhibits its signalling (George et al., 2015, Patel et al., 2014). Since this response aids in virion amplification, it is surprising that plasma and serum TGF-β levels in this study decreased in HIV infection [Izadi et al., 2015]. However, it must be noted that all HIV positive women in our study were on highly active anti-retroviral therapy (HAART), hence the immune reconstitution may reflect TGF activity and vice versa. Not only does this cytokine promote immunosuppression, it directly affects targets cells of both the adaptive and innate immune systems (Izadi et al., 2015, Patel et al., 2014). Sustained elevations in circulating TGF-β1 are believed to contribute not only to immunosuppression and progression to AIDS, but also to residual immunosuppression in virally suppressed persons (Zeng et al., 2011).

HIV-1 proteins may also contribute to the production of TGF-β1, in fact transactivation of transcription (Tat) induces TGF-β1 synthesis (Reinhold et al., 1999), whilst HIV-1 glycoprotein (gp)160 induces significant TGF-β mRNA expression (Hu et al., 1996). A twofold increase in plasma TGF-β1 was reported in HIV positive patients compared to controls (Wiercińska-Drapalo et al., 2004). However, in this study we report a non-significant decline of serum TGF-β1. It is plausible to attribute the similarity in TGF-β1 and TGF-β2 expression in our study to (HAART), the standard of care regimen in South Africa. Following antiretroviral therapy, TGF-β1 compromises repopulation of the T cell population (Zeng et al., 2011). However, no correlation between use of HAART and TGF-β1 expression has been confirmed (Wiercińska-Drapalo et al., 2004) .

HAART-induced nephrotoxicity is associated with increased expression of TGF-β in patients with diffuse fibrosis and sclerosis (Bartoli, 2016). Moreover, antiretroviral therapy stimulates hyperlipidaemia and pro-inflammatory cytokines that cause oxidative stress, endothelial damage, and hypercoagulability, these are all distinctive features of pre-eclampsia (Liu and Pravia, 2010). In contrast, HAART has been associated with immune reconstitution (Powis et al., 2013) , However former studies report conflicting findings on

the use of HAART and its association with PE development. A study in the UK conducted by Suy and co-workers which included mostly Caucasians on HAART showed that the use of HAART during pregnancy was associated with PE development when compared to non-treated women, however a study by Haeri *et al*. (2009) found that PE was less likely to develop among HIV infected women on HAART compared to uninfected women.

Former studies from our group have demonstrated elevated TGF-β1 in platelet depleted plasma in pre-eclampsia compared to the normotensive pregnant and non-pregnant groups (Khedun et al., 2002). Nonetheless, Khan *et al*. (2012) specified that circulating serum is a massive reservoir for bioactive TGF-β [Khan et al., 2012]. They demonstrated that majority of serum TGF-β occur as a latent form advocating an endocrine role. Latent TGF-β complex contains bioactive free TGF-β which binds to α2-macroglobulin, a key serum protein(McCaffrey et al., 1989). Considering the conflicting findings obtained when evaluating TGF-β1, β2 and β3 serum levels as well as HAART use among women with HIV associated pre-eclamptic pregnancies, larger studies are required to clarify the role of TGB-β in HIV associated PE.

Ethnicity, smoking, body mass index as well as PE severity may influence circulating TGB-β expression in pre-eclamptic and normotensive pregnancies (Feizollahzadeh et al., 2012). Also, discrepancies in assay method, patient classification and experimental settings contribute to variations [Keelan and Mitchell, 2007]. Our study group represented a homogenous population in terms of ethnicity, primigravidae and PE (early onset); however, we did not stratify by other risk factors.

In conclusion, we report no significant difference in TGF-β1 and β2 serum levels between the pre-eclamptic and normotensive pregnancy women, however TGF-β3 levels differed significantly amongst HIV positive compared to HIV negative women. These findings support the predictor test value of TGF-β3 as a biomarker for HIV associated pre-eclampsia.

Disclosure
The authors report no conflict of interest

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CHAPTER 3

SYNTHESIS

Pre-eclampsia is a multifactorial syndrome that complicates approximately 3-8% of all pregnancies globally (Venkatesha et al., 2006, Faupel-Badger et al., 2011). Despite being a major contributor to maternal mortality the precise events leading to PE pathogenesis remain unclear (Stepan et al., 2007). Pre-eclampsia is clinically characterized by hypertension and proteinuria, usually presenting during the third trimester of pregnancy although it is believed that they develop during the first trimester as a result of abnormal placentation (Venkatesha et al., 2006). Studies conducted over the past few decades have enlightened us extensively on the role that placental cytokines play in pre-eclampsia pathophysiology, however, there is great controversy (Keelan and Mitchell, 2007). Moreover, the association between HIV and pre-eclampsia is also debatable as investigators have reported conflicting results indicating that further research is necessary for validation (Suy et al., 2006, Wimalasundera et al., 2002, Mattar et al., 2004, Hall, 2007). The aim of this study was to determine TGF-β 1-3 levels in serum of HIV associated pre-eclampsia.

The present study demonstrates no significant difference in serum TGF-β 1-3 expression based on pregnancy type (normotensive vs pre-eclampsia). These results are in accordance with the findings of Huber *et al*., (2002) who also reported no difference in expression between pre-eclamptic and normal pregnant women. However, Naicker *et al*., (2002) demonstrated a significant increase in β1 levels in PE compared to normotensive pregnancies, while Yuswarati *et al*., (2017) reported the opposite, stating that β1 is significantly lower in PE. The lack of reproducibility in the results may be due to the use of different sample types and detection methods.

The determination of TGF-β expression in biological samples is debatable as suitability of the different sample types (serum vs plasma) and anticoagulants (citrate or EDTA) is questionable (Kropf et al., 1997). A comparison between serum and plasma concentrations reported a higher concentration of TGF-β in serum than plasma, this is likely due to platelet degranulation during the clotting process; moreover, cross-reactivity between TGF-β1 and other factors such as interleukin-1 and fibroblast growth factor may contribute to differences (Neta et al., 1992, Kropf et al., 1997). Nonetheless, Khan *et al*, 2012 has specified that circulating serum has a massive reservoir for bioactive TGF-β (Khan et al., 2012). They further demonstrate that the majority of serum TGF-β occur as a latent form advocating an endocrine role. Latent TGF-β complex contain bioactive free TGF-β which binds to α2-macroglobulin, a key serum protein (McCaffrey et al., 1989).

Endoglin (Eng) is a co-receptor of TGF-β1 and TGF-β2 and is expressed mainly by syncytiotrophoblast cells. Endoglin aids TGF beta signalling via its interaction with TGF-β receptors I and II (Dağdeviren et al., 1998, Cheifetz et al., 1992). This pro-angiogenic factor further mediates vasodilation a nitric oxide dependent process. Soluble endoglin (sEng) however inhibits TGF-β1 signalling by disrupting binding of Eng resulting in decreased vasodilation subsequently resulting in inhibition of signalling (Jerkic et al., 2004, Venkatesha et al., 2006).

Nevertheless, pre-eclampsia is associated with reduced trophoblast invasion and non-physiological transformation of myometrial spiral arteries with resultant low capacitance and high resistance (Naicker *et al*., 2003; Naicker *et al*., 2013). This creates a hypoxic micro-enviroment with high antioxidant levels (Cohen et al., 2015). TGF-β is known to elevate the cellular release of reactive oxygen species (ROS), causing a down regulation of glutathione, the most profuse intracellular free thiol and a vital antioxidant (Liu and Gaston

Pravia., 2010). ROS, in turn, boosts the assembly and reactivity of TGF-β through activation of latent TGF-β (Liu and Pravia 2010).

The present study further demonstrates no significant difference in TGF-β2 levels between pre-eclamptic and normotensive pregnant women irrespective of the HIV status. Former studies have demonstrated an upregulation in TGF-β2 levels in women with severe pre-eclampsia, correlated with increased uric acid and serum creatinine implicating, unfortunately we did not stratify our patients by severity of the condition (Kiran et al., 2012)

In this study we additionally reported a significant difference in TGF-β3 levels between the HIV positive and negative groups. In this study, in contrast to TGF-β1 and TGF-β2, TGF-β3 expression was significantly downregulated in the HIV positive groups. TGF-β is a powerful cytokine that suppresses the immune system driving disease progression in HIV-infected persons via both non- and profibrotic machinery. It suppresses T-cell proliferation and inhibits T-cell signalling (Patel, Khan *et al*. 2014, George, Lewis *et al*. 2015). Since this response aids in virion amplification, it is surprising that plasma and serum TGF-β levels in this study decrease in HIV infection (Izadi, Asadikaram *et al*. 2015). Not only does this cytokine promote immunosuppression, it directly affects targets cells of both the adaptive and innate immune systems (Patel, Khan *et al*. 2014, Izadi, Asadikaram *et al*. 2015). Sustained elevations in circulating TGF-β1 are believed to contribute not only to immunosuppression and progression to AIDS, but also to residual immunosuppression in virally suppressed persons (Zeng, Smith *et al*. 2011).

HIV-1 proteins may also contribute to the production of TGF-β, in fact transactivation of transcription (Tat) induces TGF-β synthesis (Reinhold, Wrenger *et al*., 1999), whilst HIV-1 glycoprotein (gp) 160 induces significant TGF-β mRNA expression (Hu, Oyaizu *et al*.

1996). A twofold increase in plasma TGF-β1 was reported in HIV positive patients compared to controls (Wiercińska-Drapalo, Flisiak *et al*., 2004). However, in this study we report a non-significant decline of serum TGF-β1. It is plausible to attribute the similarity in TGF-β1 and TGF-β2 expression in our study to HAART, a standard of care regimen in South Africa. Following antiretroviral therapy, TGF-β1 compromises repopulation of the T cell population (Zeng, Smith *et al*., 2011). However, no correlation between use of HAART and TGF-β1-3 expression has been confirmed (Wiercińska-Drapalo, Flisiak *et al*., 2004).

It is currently debatable whether HIV infection may influence the development of pre-eclampsia. Some authors have suggested that HIV plays a protective role against PE development due to opposing immune responses (Mattar et al., 2004). Before the routine use of highly active antiretroviral therapy (HAART), pre-eclampsia was infrequent in untreated HIV infected pregnant women, also the incidence of pre-eclampsia in HIV infected women was known to be lower than in the general population (Wimalasundera et al., 2002). It has been theorized that HAART will supposedly increase the risk of pre-eclampsia by causing a direct toxicity on the liver consequently affecting renal processes which are thought to underlie the development of pre-eclampsia in HIV uninfected pregnancies moreover it has been proposed that the immune restoration effect of HAART further contributes to the development of pre-eclampsia.

This study demonstrated no significant difference in TGF-β1 and β2 levels between the pre-eclamptic and normotensive groups irrespective of HIV status, however TGF-β3 levels differed significantly between the HIV positive and HIV negative groups implicating that TGF-β3 can be used as a biomarker for HIV associated pre-eclampsia.

The study limitations included a small sample size due to sample availability therefore it was difficult to find significant relations amongst the study groups.

CHAPTER 4

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APPENDIX

UNIVERSITY OF KWAZULU-NATAL

INYUVESI YAKWAZULU-NATALI

10 May 2017

Ms ZS Mtshali (211527135)
Discipline of Optics and Imaging
School of Laboratory Medicine and Medical Sciences
zamamtshali1992@gmail.com

Dear Ms Mtshali

Protocol: Pre-eclampsia aetiology TGFß1-3 in serum of HIV associated pre-eclampsia.
Degree: MMedSc
BREC reference number: BE212/17

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 20 March 2017.

The conditions have now been met and the study is given full ethics approval and may begin as from 10 May 2017.

This approval is valid for one year from 10 May 2017. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

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

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

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

The sub-committee's decision will be RATIFIED by a full Committee at its next meeting taking place on 13 June 2017.

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

Yours sincerely

Prof V Rambiritch
Deputy Chair: Biomedical Research Ethics Committee

cc co-investigator: naidooraj@ukzn.ac.za
cc postgraduate administrator: dudhrajhp@ukzn.ac.za

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