Longitudinal evaluation of circulating angiogenic and antiangiogenic factors in normotensive pregnancies.

submitted in fulfillment of the requirements for the degree of Masters of Health Sciences:

Environmental Health in the Faculty of Health Sciences at

The Durban University of Technology.

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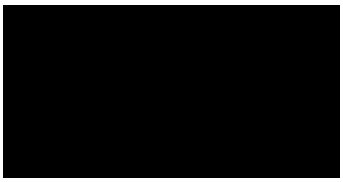
Supervisor: **Dr Nalini Govender**

Co-Supervisor: **Professor Poovendhree Reddy**

DECLARATION

The author hereby declares the content of this research project is the author's own unaided original work, except where specific indication is given to the contrary (by reference). This work has not been previously submitted to the Durban University of Technology (DUT) or any other University.



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Date: 29th March 2018

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 Department of Community Health Studies, Faculty of Health Sciences Durban University of Technology Durban, South Africa under the supervision and co-supervision of Dr N. Govender and Prof P Reddy respectively.

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DEDICATION

To my wife Pharm. Sofiat Yetunde Oguntade, my son Zayd Ogunlola,

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

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

"My prayer, my sacrifice, my living and my dying are for Allah, the Lord of all that exists"

Al-an'am 6:162

SUBMITTED MANUSCRIPT: APPENDIX 1

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- My utmost gratitude goes to Almighty Allah the Lord of all that exists, the sole reason for my existence. I thank Allah for guiding me through this masters program and for making my path through life easy. Alhamdulillah.

LIST OF ABBREVIATIONS

KZN	KwaZulu-Natal
VEGF	vascular endothelial growth factor
PlGF	placental growth factor
VEGFR-1	vascular endothelial growth factor receptor 1
VEGFR-2	vascular endothelial growth factor receptor 2
sFlt-1	soluble fms-like tyrosine kinase receptor 1
Eng	endoglin
sEng	soluble endoglin
TGF-β_1	transforming growth factor-beta 1
HIV	human immunodeficiency virus
ng	nanograms
SST	Serum Separator Gel Tube
Fig	figure
MCV	Mean Corpuscular Volume
pg	picograms
DUT	Durban University of Technology
NRF	National Research Foundation
UID	User Identification
UNAIDS	The Joint United Nations Programme on HIV/AIDS
µl	microliters
mL	milliliters
mM	millimolar

nm	nanometers
mmHg	millimetres mercury
BP	blood pressure
wks	weeks
g	grams
°C	degrees celcius
min	minute
sec	seconds
rpm	revs per minute
RT	room temperature
CHC	Community Health Center
ANOVA	analyses of variance
r	correlation coefficient
SD	standard deviation
mRNA	messenger ribonucleic acid
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assays
R&D	Research and development
ST	syncytiotrophoblast
HELLP	Haemolysis, Elevated Liver Enzymes and Low Platelets
KZNPA	KwaZulu-Natal Provincial Administration
EJOGRB	European Journal of Obstetrics & Gynaecology and Reproductive Biology

EC	endothelial cells
RBC	red blood cells
MMR	Maternal Mortality Ratio
WHO	World Health Organization
HDP	Hypertensive Disorders of Pregnancy

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Chapter 1

Introduction and Literature Review

1.1 Context

Most maternal deaths (99%) occur in developing countries, with more than half occurring in sub-saharan Africa (WHO, 2015). The 2015 WHO report has indicated that worldwide, approximately 830 women die from preventable causes that are related to complications from child birth and pregnancy on a daily basis (WHO, 2015). The maternal mortality ratio (MMR) in developing countries in 2015 was 239 per 100,000 live births versus 12 per 100,000 live births in developed countries (WHO, 2015). In South Africa, the primary obstetric problems include eclampsia, severe hypertension, haemolysis, elevated liver enzymes and low platelets (HELLP) and liver rupture (Saving Mothers Report, 2014).

Maternal deaths were reported in all sub categories of hypertensive disorders of pregnancy (HDPs) however, preeclampsia is reported as the major contributor to maternal mortality (Saving Mothers Report, 2014). Maternal deaths due to non-pregnancy related infections, obstetric haemorrhage and hypertension account for 65% of all preventable maternal deaths in South Africa, with hypertension accounting for 14.8% of all maternal deaths (South Africa, 2014). The overall maternal mortality rate (MMR) for hypertension in South Africa is 22.75/100,000 live births, with KwaZulu-Natal having a MMR of 14.02/100,000 live births (Saving Mothers Report, 2014).

In 2016, approximately 36.7 million people were living with HIV, of which 15.4– 20.3 million were women of reproductive age, of whom 11.5 million reside in eastern and southern Africa (UNAIDS 2016). In pregnancies complicated by HIV infection, an exacerbation of events such as recurrent miscarriages, preeclampsia, diabetes and preterm labor may occur (Wimalasundera *et al.,* 2002). Fourie *et al.,* (2011) reports that HIV infection contributes to the development of chronic arterial injury including endothelial damage, atherosclerosis and thrombosis (Fourie *et al.,* 2011). There is limited normotensive data regarding the maternal characteristics with angiogenic profiles across various stages of pregnancy, hence the rationale for our study to determine variation in angiogenic and antiangiogenic biomarkers in relation to maternal clinical characteristics, including HIV.

1.2 **Angiogenesis and placental growth in pregnancy**

Pregnancy is characterized by functional changes that begin immediately after conception and have widespread effects on every organ system in the body (Locktich, 1997). These physiological changes may alter normal biochemical function or imitate symptoms of disease (Locktich, 1997). It is thus important to differentiate between normal physiological changes and pathological processes during pregnancy. In utero, both vasculogenesis and angiogenesis contribute to the formation of new blood vessels (Risau, 1997). Vasculogenesis is defined as the differentiation of precursor cells called angioblasts into endothelial cells and the formation of a primitive network of vessels, whereas, angiogenesis is the growth of new capillaries from pre-existing blood vessels (Risau, 1997).

Pregnancy is dependent on both angiogenesis and pseudo-vasculogenesis (Kim, West and Byzova, 2013), and is characterized by remodeling of spiral arteries via the invasive fetal cytotrophoblasts (Andraweera, Dekker, Laurence and Roberts, 2012). Sprouting and intussusception characterize angiogenic mechanisms, in which intussusceptive angiogenesis refers to the enclosure of columns of interstitial cells into the lumen of already existing vessels (Risau, 1997). The successive growth of these interstitial columns and their stabilization causes vessel partitioning and vascular transformation (Risau, 1997). In contrast, sprouting angiogenesis consists of two sequential phases, namely; growth of new vessels and stabilization of new vessels (Benjamin et al, 1998). These phases prevent rapid regression and apoptosis of the immature capillaries (Benjamin *et al.,* 1998). The first stage is the dissolution of the basement membrane of the pre-existing blood vessel and the surrounding interstitial matrices, followed by endothelial migration in the space created in the direction of angiogenic factor, this stage is succeeded by endothelial proliferation behind the migration, lumen formation within the endothelial sprouts and consequent anastomoses and loop formation (Benjamin *et al.,* 1998). The stabilization phase in contrast, involves the inhibition of endothelial proliferation, regrowth of basement membrane, and invasion of the immature capillary with pericytes.

Angiogenesis during pregnancy is essential for both placental and fetal development (Huppertz, 2005). It is divided into folliculogenesis, decidualization, implantation, and embryo development; and involves cell development vis a vis cell differentiation and proliferation (Rizov, Andreeva and Dimova, 2017). Folliculogenesis enables maturation and development of the primordial follicles into a graafian follicle, thereby enabling

ovulation or destruction by atresia (Jones, 2006). These angiogenic processes are also influenced by various hormones, stem cells, cytokines, progenitor cells, growth factors and immune mediated cells.

1.2.1 **Angiogenic factors in placental development**

There are several angiogenic factors, including placental growth factor (PlGF), vascular endothelial growth factor (VEGF), endoglin (Eng) and transforming growth factors (TGFβ). Placental trophoblasts also synthesize and secrete the angiogenic PlGF and VEGF into the maternal vasculature, which support endothelial proliferation and survival and arterial remodeling (Kaufmann *et al.,* 2003, Andraweera, 2012, Espinozoa (2014). VEGF regulates angiogenesis and follicular maturation as well as endothelial integrity (Baumwell and Karumanchi 2007, Robinson *et al.,* 2009). The angiogenic action VEGF in promoting follicular development *in-vivo* is widely reported (Zimmermann, Xiao, Bohlen and Ferin 2002, Zimmerman *et al.*, 2001, Wulff *et al.*, 2002, Celik-Ozenci *et al.*, 2003, and Roberts *et al.*, 2007).

The invasion of the placental bed and utero-placental spiral arteries occurs between 8-18 weeks gestational age (Staff, Dechend and Pijnenborg, 2010). The spiral arteries are subsequently invaded by mononuclear extravillous foetal cytotrophoblasts via the uterine vasculature or the interstitium (Staff, Dechend and Pijnenborg, 2010). The spiral arteries are extensively remodeled by the invasive endovascular cytotrophoblast (Figure1.1) in their terminal decidual and inner myometrial segment (Lam, Lim and Karumanchi, 2005, Staff, Dechend and Pijnenborg, 2010). The invaded segments are

widely dilated and subsequently lose their smooth muscle, which reduces the pressure and velocity of utero-placental flow. This increase blood volume flow in the spiral artery blood minutely (Staff, Dechend and Pijnenborg, 2010).

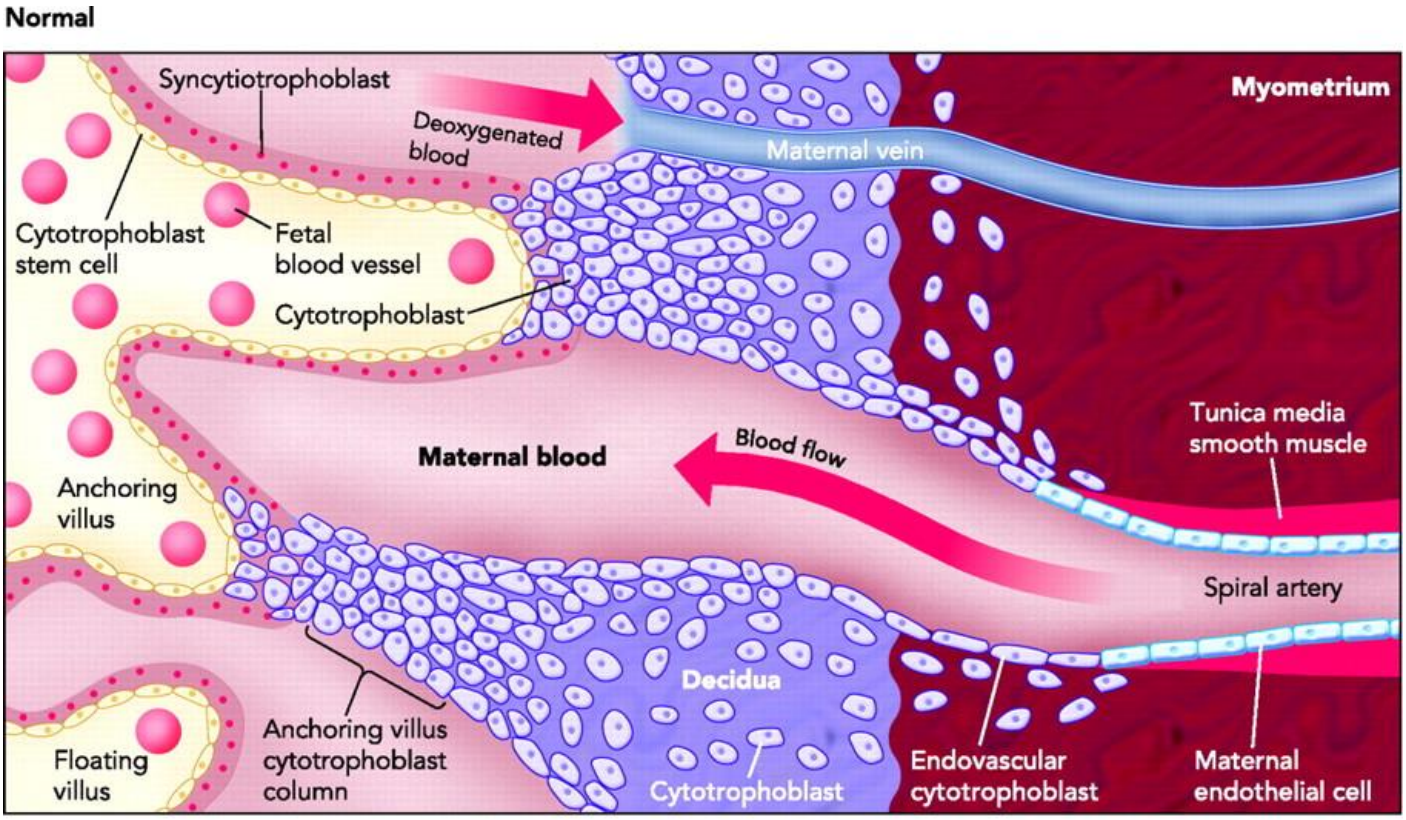

Figure 1.1 Placentation in Normal Pregnancy (Figure adapted from Lam, Lim and Karumanchi 2005)

However, the pro-angiogenic action is counteracted by the release of antiangiogenic factors (Iruela-Arispe and Dvorak, 1997). This has led to the concept of the angiogenic balance, and overexpression of either angiogenic or antiangiogenic factors controls active angiogenesis or endothelial dormancy, respectively (Iruela-Arispe and Dvorak, 1997).

The anti-angiogenic proteins such as soluble endoglin (sEng) and soluble fms like tyrosine kinase receptor (sFlt-1) are also released by the placenta (Maynard *et al.,*

2003, Levine *et al.,* 2006). Antiangiogenic sFlt-1 binds to and subsequently reduces the free unbound circulating levels of the pro-angiogenic factors VEGF and PlGF (Levine *et al.*, 2006). Angiogenic VEGF supports angiogenesis as well as endothelial integrity (Baumwell and Karumanchi, 2007). Similarly, sEng antagonizes the pro-angiogenic effects of TGFβ. These antiangiogenic factors are believed to function synergistically to interrupt endothelial integrity, maybe by opposing vasomotor and vasodilatory effects of nitric oxide (Tjoa, Levine and Karumanchi, 2007).

Angiogenesis has been said to be biphasic in human placenta, with peaks at 20 weeks gestation and at term, this is as a result of endothelial proliferation earlier in pregnancy and vascular remodeling at mid-gestation (Mayhew, 2002). The process of angiogenesis is regulated by at least three growth factor families, namely: VEGF, ephrins and angiopoietins (Gale and Yancopoulos, 1999). Angiogenesis is regulated by other factors, including: interleukin-8, Tumor Necrosis Factor α, transforming growth factors α and β, hepatocyte growth factor, angiogenin, fibroblast growth factors and members of the NOTCH family (a family of transmembrane proteins with repeated extracellular domains) (Yancopoulos *et al.*, 2000, Matsumoto and Claesson-Welsh, 2001, Ferrara, Gerber and LeCouter, 2003). Evidence indicates that angiogenesis involves the sequential activation of some receptors, including platelet derived growth factor receptor β, Tie1, Tie2, and by ligands in mural and endothelial cells (Ferrara, 2004). However, VEGF signaling represents a critical rate-limiting step in physiological angiogenesis (Ferrara, 2004).

1.2.1.1 **Vascular Endothelial Growth Factor (VEGF)**

PlGF and VEGF are angiogenic factors expressed by the placenta (Agarwal and Karumanchi, 2011). The VEGFs is a family of dimeric proteins which are structurally related, whose members include VEGF-A, VEGF-B, VEGF-C, VEGF-D and PlGF. These angiogenic proteins promote migration, proliferation and differentiation of endothelial cells as well as vascular permeability (Romero *et al.*, 2008). The function of VEGF is achieved by their interaction with high affinity receptor tyrosine kinases Flt-1 (VEGFR-1) and VEGFR-2 (Figure1.2) (Romero *et al.*, 2008).

sVEGFR-1 regulates vascular growth and is produced by the placenta. sVEGR-1 mRNA (messenger Ribonucleic Acid) is expressd in extravillous and villous trophoblast and sVEGFR-1 protein has also been expressed in villous culture as supernatant (Clark *et al.,* 1998). sVEGFR-1 has been expressed in serum of women with uncomplicated pregnancies (Clark *et al.*, 1998). Studies reported that sVEGFR-1 is expressed in pregnant (Clark *et al.*, 1998) as well as non-pregnant women (Barleon *et al.*, 2001). Barleon and colleagues (2001) revealed that sVEGFR-1 may contribute to the fine regulation of VEGF bioavailability in non-pregnant and pregnant and women (Barleon *et al.*, 2001). This fine regulation is important as constant low levels of VEGF are required for endothelial cell survival and proliferation and survival (Luttun and Carmeliet, 2003) sVEGFR-1 forms heterodimers with VEGF receptors and stops their signal transduction, this consequently regulates the bioavailability of VEGF (Barleon *et al.*, 2001).

1.2.1.2 **Placental Growth Factor (PlGF)**

PlGF is a major member of the VEGF family, it serves as a ligand for VEGFR-1 that enhances the angiogenic response of VEGF (Autiero *et al.*, 2003, Autiero *et al.*, 2003). This is accomplished by three propositions namely: a) availaibility of VEGF to bind VEGFR-2 due to displacement of VEGF from sVEGFR-1 by PlGF, b) PlGF activation of VEGFR-1, resulting in activation and transphophorylation of VEGFR-2 which cross reacts with VEGFR-1 and c) destabilization of inactive VEGFR-2 and sVEGFR-1 heterodimers by PlGF heterodimers, thereby causing more VEGFR-2 to be available to form heterodimers (Autiero *et al.*, 2003, Autiero *et al.*, 2003).

In non-pregnant women, PlGF concentrations are detectable at low plasma levels (44±4.7pg/mL), however higher plasma concentrations of PlGF are seen in pregnant women (Krauss, Pauer and Augustin, 2004). Normally, small amount of PlGF is released in endothelial cells, however higher amount of PlGF is released from endothelial cells when activated (Autiero *et al.*, 2003). PlGF is also produced by other cell types namely, inflammatory cells, bone marrow cells, neurons, tumor cells and vascular smooth muscle cells (Autiero *et al.,* 2003, Beck *et al.*, 2002, Iyer and Acharya, 2002 and Luttun *et al.*, 2002). Hypoxia may affect PlGF expression as demonstrated in an *in vitro* study which revealed that under hypoxic conditions, mRNA *PlGF* gene expression is reduced by 75% in isolated human syncytiotrophoblast (Shore *et al.*, 1997).

1.2.1.3 **Soluble Fms-like Tyrosine Kinase -1(sFlt-1)**

The anti-angiogenic sFlt-1 is a soluble form of VEGF receptor 1 (Wang, Rana and Karumanchi, 2009). It results from a regulated process during gene expression of Flt-1 receptor (mRNA) which is an endothelial receptor for VEGF and PlGF, this process is called “alternative splicing” (Wang, Rana and Karumanchi, 2009). sFlt-1 consists of an extracellular ligand binding domain of Flt-1, but lacks the transmembrane and intracellular signaling domain. The anti-angiogenic soluble endoglin (sEng) and sFlt-1 is released by the placenta (Maynard *et al.*, 2003, Levine *et al.*, 2006). Antiangiogenic sFlt-1 binds to and subsequently reduces the free unbound circulating levels of the pro-angiogenic factors VEGF and PlGF (Figure 1.2), thereby altering the angiogenic balance (Wang Rana and Karumanchi, 2009, Mutter and Karumanchi, 2008 Levine *et al.*, 2006). Free sFlt-1 is also capable of binding with both PlGF and VEGF, thereby neutralizing them, and decreasing PlGF and VEGF levels in maternal circulation (Lapaire, Shennan and Stefan, 2010).

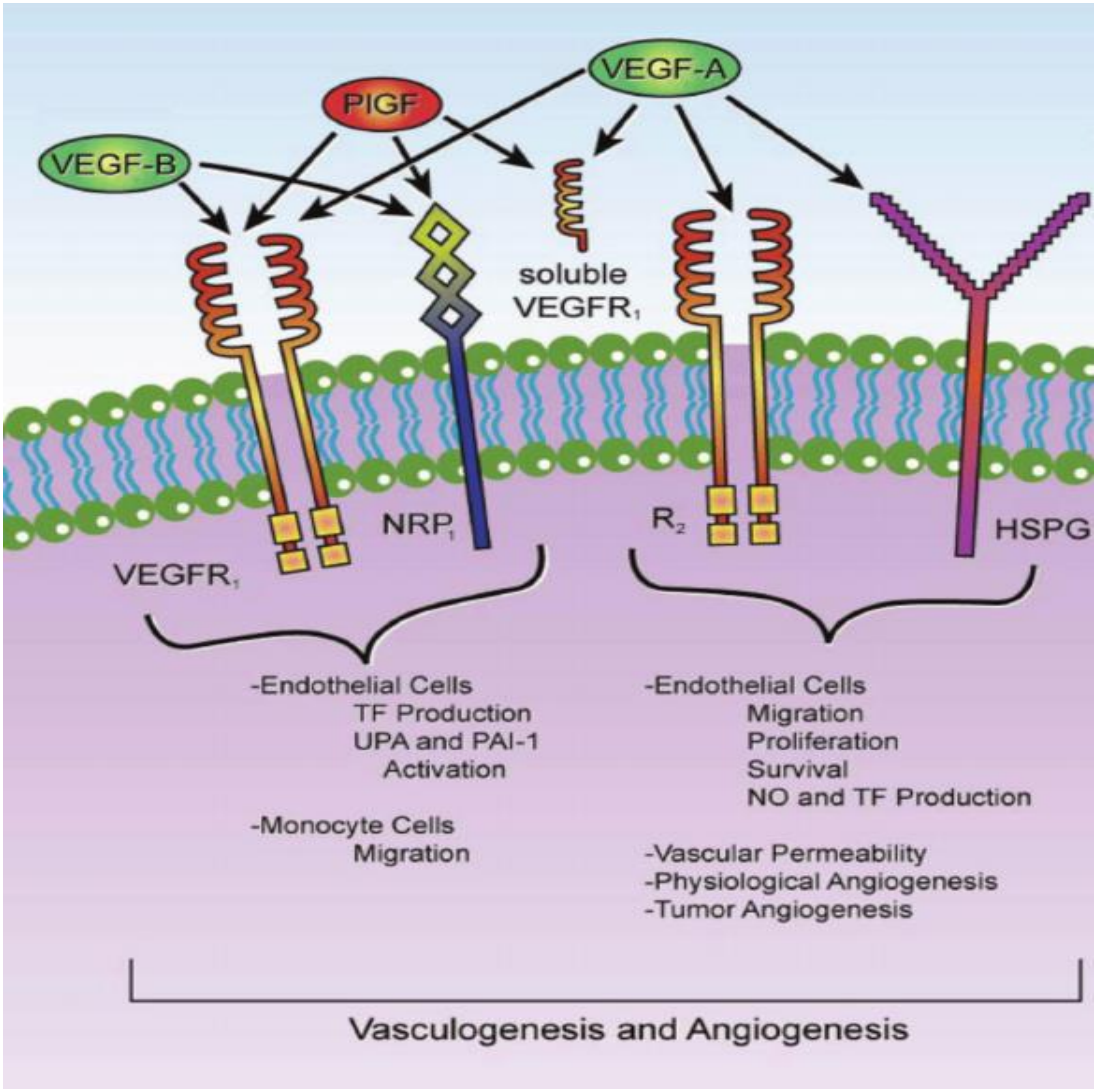

Figure 1.2 Role of VEGF, PlGF and sVEGFR1 in vasculogenesis and angiogenesis (Figure adapted from Alvarez *et al.*, 2011)

Studies have demonstrated that high sFlt-1/PlGF ratios to be associated with increased blood pressure in late pregnancies (Levine, 2006). Moreover, low serum levels of sFlt-1 are reported in normotensive pregnancies (Levine, 2006) whereas Baumann *et al.* (2008) highlighted increased sFlt-1 and sEng levels in the first trimester. Levine *et al.*, (2006) reported a mean sFlt-1 value of 1,643 pg/mL in normotensive pregnancies. Wolf and his colleagues (2005) reported higher sFlt-1 levels in nulliparous women in an

American cohort study (1998-2005), the study included 122 Hispanic and non-Hispanic white women who provided serum samples during both their first and second completed pregnancies (Wolf, 2005). An earlier study highlighted that nulliparity was associated with higher antiangiogenic profile in normotensive pregnancies at delivery (Staff *et al.*, 2009). South African studies have demonstrated lower serum levels of sFlt-1 and sEng in normotensive preganancies compared to pre-eclamptic pregnancies (Govender, Naicker and Moodley, 2013). These investigators further revealed higher sFlt-1 and sEng levels in the pre-eclamptic (HIV negative and positive) compared with normotensive pregnancies (Govender, Naicker and Moodley, 2013).

1.2.1.4 **Endoglin (Eng)**

Endoglin is a protein that normalizes the pro-angiogenic effects of TGF-β (Romero *et al.*, 2009). Endoglin is a trans-membrane homo-dimeric glycoprotein and it is considered to be the functional co-receptor for TGF β3 and TGF β1 (Lastres *et al.*, 1996). TGF-β causes movement and proliferation of endothelial cells (Goumans, 2002). Although the mechanism by which the soluble form of endoglin is generated remains unclear, shedding appears to be a potential source of circulating s-Eng in the maternal blood. Romero *et al.*, (2008) reported that endoglin and sVEGFR-1 increased with increasing gestational age.

Endoglin has anti-angiogenic effects and it is made up of two splice variants, S-endoglin (S, short) and L-endoglin (L, long). Both endoglin variants are expressed in high concentrations on the cell membrane of endothelial cells and syncitiotrophoblasts

(Chen, 2009). . L-endoglin is the most abundantly expressed isoform (Velasco *et al.*, 2008). Apart from the membrane-bound forms, endoglin can exist as a soluble form (sEng) (Fig. 2A). The Two endoglin isoforms S-Endoglin and L-endoglin differ in their length of their degree of phosphorylation and intracellular domain length. S-endoglin has a low level of phosphorylation and only contains 14 amino acids embedded in its intracellular domain, whereas L-endoglin has a high degree of phosphorylation, contains 47 amino acids in its cytoplasmic tail and is predominantly expressed in endothelial cells (Cheifetz *et al.*, 1992, Bellon *et al.*, 1993). Soluble forms of endoglin are formeded by the cleavage of the extracellular domain of endoglin by membrane-type metalloprotease-14 (MMP-14), MMP-14 may function as a naturally occurring antagonist for signaling of TGF-β (Venkatesha *et al.*, 2006). MMP-14 causes cleavage of endoglin at position 586, thereby releasing a soluble fragment representing almost the entire extracellular domain of endoglin (Hawinkels *et al.*, 2010). MMP-14 is expressed highly in malignant endothelial and epithelial cells. Both S-endoglin and L-endoglin isoforms have the ability to bind to their ligands and subsequently interact with Activin receptor-like kinase-1 (ALK-1) and Activin receptor-like kinase-5 (ALK-5); however, the two membrane-bound endoglin isoforms differ in their level of phosphorylation, affinity for each receptor, and capacity to regulate TGF-β-dependent responses (Blanco *et al.*, 2008). S- endoglin has anti-angiogenic effects, whereas L-endoglin has pro-angiogenic effects through induction of endogenous nitric oxide synthase (eNOS) expression. Thus, short-form endoglin contributes to the cardiovascular pathology associated with the gradual deterioration of function characteristic of cells. In addition, S-endoglin which inhibits TGF-β signaling, is thought to be cleaved from the cell membrane, thus, entering the systemic circulation, and may

represent a useful biomarker of inflammation, endothelial injury, activation, inflammation, and ageing of cells (Venkatesha *et al.,* 2006). Endoglin is also a co-receptor for transforming growth factors (TGFβ-1 and TGFβ-3), and modulates TGF β signaling in angiogenesis while regulating vascular tone (Chen, 2009, Grill *et al.*, 2009). sEng is a truncated form of endoglin and its anti-angiogenic function is by binding TGFβ1 to its receptor thereby interfering with the binding of TGFβ1, this subsequently affects vasodilatation in maternal blood vessels as well as nitric oxide production and capillary formation by the endothelial cells (Chen, 2009). Serum levels of sEng are stable throughout pregnancy (Chen, 2009), however when compared to a longitudinal study, the maternal endoglin concentration remained relatively stable until 25 weeks gestation after which there was an increase until term (Romero *et al.*, 2008). The antiangiogenic factors are believed to function synergistically to interrupt endothelial integrity, maybe by opposing vasomotor and vasodilatory effects of nitric oxide (Tjoa, Levine and Karumanchi, 2007). Thus angiogenic imbalance in pregnancy is believed to cause increased inflammation in the maternal vasculature and a generalized endothelial dysfunction, which is capable of inducing new onset of hypertension and proteinuria, characteristic of preeclampsia (Roberts *et al.*, 1989).

Maternal circulating concentrations of PlGF tend to be higher in normotensive pregnancies in contrast to the antiangiogenic factors sEng and sFlt-1 and the anti-angiogenic ratio sFlt-1/PlGF which are lower in normotensive pregnancies (Levine, 2006). There is limited normotensive data regarding the maternal characteristics with angiogenic profiles across various stages of pregnancy.

1.3 **Physiological changes in pregnancy**

1.3.1 **Haematological changes**

Plasma volume increases as pregnancy progresses (Rodger *et al.*, 2015). Approximately 50% of this elevation occurs by 34 weeks gestational age and is proportional to the birthweight of the neonate (Soma-Pillay *et al.*, 2016). However, the plasma volume expansion is greater than the increase in red blood cells, which subsequently reduces the haematocrit, red blood cell count and haemoglobin concentration, despite this haemodilution, the mean corpuscular haemoglobin concentration (MCHC) and the mean corpuscular volume (MCV) do not change typically (Sarma, 1990). The platelet count is however, reduced as pregnancy progresses, and remains within normal limits.

Nonetheless, in approximately 5‑10% women, there is a low platelet count by the end of gestation, occurring in the absence of any maternal pathology, thus, pregnant women are not considered thrombocytopenic until their platelet count is less than 100×10^9 cells/L (Soma-Pillay *et al.*, 2016). A physiological hypercoagulable state is seen in pregnancy which is as a result of major changes in the coagulation system during pregnancy to forestall haemorrhage and to ensure optimal haemostasis at delivery (Ramsay 2010). Pregnancy is also responsible for a 2-3 fold increase in iron requirement as a result of haemoglobin synthesis, fetal blood supply and enzyme production; In addition, a 10-20 fold increase in folic acid and a 2-fold increase in vitamin B_{12} is also reported (Soma-Pillay *et al.*, 2016).

1.3.2 **Cardiovascular changes**

Significant changes occur in the maternal cardiovascular system during early pregnancy and at 8 weeks' gestational age, there is already 20% increase in cardiac output, with peripheral vasodilatation being a key event (Soma-Pillay *et al.,* 2016). There is approximately 25-30% reduction in systemic vascular resistance due to peripheral vasodilation and a compensatory 40% increase in cardiac output. This increase in cardiac output is due to an increase in stroke volume and heart rate, although this accounts for a lesser contribution of increase in cardiac output (Soma-Pillay *et al.,* 2016). However, the maximal increase in cardiac output is noted around 20–28 weeks gestational age with a negligible reduction at term (Soma-Pillay *et al.*, 2016). Troisi and co-workers (2008) reported that blood pressure increases from mid-pregnancy to late pregnancy in normotensive pregnancies and this was associated with the antiangiogenic profile at delivery (Troisi *et al.*, 2008).

Soma-Pillay and colleagues (2016) also reported that there is a decrease in the blood pressure from first to second trimester but increases progressively to pre-pregnant levels in the third trimester (Soma-Pillay *et al.*, 2016). Mean arterial blood pressure is reduced in the first trimester, when there is an increase in cardiac output, reaching the lowest in mid-pregnancy (Clap *et al.*, 1988). This reduced cardiac output is linked with a reduction in utero-placental blood flow and subsequent reduction in placental perfusion, which could affect blood flow to the fetus (Soma-Pillay *et al.*, 2016). Despite the elevations in both stroke volume as well as total blood volume during pregnancy, the central venous pressure and pulmonary capillary wedge pressure do not increase significantly, however, pulmonary vascular resistance decreases significantly during

pregnancy (Soma-Pillay *et al.*, 2016). Robson *et al.*, (1989) also highlighted a relatively constant systolic blood pressure remained during pregnancy with a relative fall in diastolic blood pressure during the first half of pregnancy (Robson *et al.,* 1989).

Furthermore, the onset of labour is associated with increase in cardiac output, since uterine contractions result in auto-transfusion of about 300 to 500 ml of blood back into the maternal circulation and the sympathetic response to pain and anxiety further increases blood pressure and the heart rate (Soma-Pillay *et al.*, 2016). Post-delivery, there is an instantaneous increase in cardiac output by 60 – 80% due to relief of the pressure on the inferior vena cava by the gravid uterus and contraction of the uterus, which empties blood into the maternal circulation, this is followed by a rapid decline to pre-labour values within an hour of delivery with a corresponding increase in stroke volume and venous return due to transfer of fluid from the extra-vascular space (Soma-Pillay *et al.*, 2016). Cardiac output returns to pre-pregnancy values two weeks post-delivery (Soma-Pillay *et al.*, 2016).

High blood pressure during pregnancy contributes significantly to the risks of adverse neonatal consequences and maternal deaths (Langenveld *et al.,* 2010, Khan *et al.*, 2006). Even though the etiology of hypertensive disorders during pregnancy is not clearly delineated, maternal anthropometric measurements such as high body mass index (BMI) in pregnancy has been reported to increase the risk of pregnancy induced hypertension (PIH) or preeclampsia (Leeners *et al.*, 2006, Ramsay *et al.*, 2002, Mahomed *et al.*, 1998, Group HSCR, 2010).

Hypertensive disorders of pregnancy such as preeclampsia, however, are leading causes of maternal deaths in SA (South Africa, 2014). Normal pregnancy requires a balance between pro-angiogenic and anti-angiogenic factors for effective angiogenesis and placental development; however, preeclampsia is characterized by an excessive anti-angiogenic state (Redman, Sacks and Sargent, 1999). The inflammatory response in normal pregnancy is usually a low-grade inflammatory systemic response, this response is, however, further heightened in preeclampsia (Redman, Sacks and Sargent, 1999).

1.4 **Inflammatory response in normal pregnancy**

Localized inflammation is imperative during the menstrual cycle. The uterine endometrium contains cells of the innate immune system, which includes dendritic cells, macrophages, neutrophils, natural killer cells and mast cells (Romero *et al.*, 2007). These cells change progressively during the menstrual cycle, and a role for the innate immune system has been proposed in the mechanisms of menstruation (Romero *et al.*, 2007). Implantation is the process by which the blastocyst invades the endometrium, penetrates and adheres to it. This process is also associated with inflammatory changes deployed to ensure tissue remodeling that is required for successful placentation (Pijnenborg, 2006).

Parturition has also been considered a localized inflammatory process. The common terminal pathway of parturition consists of myometrial activation, cervical ripening which includes cervical dilatation/cervical effacement, and membrane/decidual activation (Romero *et al.*, 2007). The molecular and cellular components of inflammation play a

pivotal role in each of the components of the common terminal pathway of parturition and normal gestation is accompanied by an increase in the plasma concentrations of acute-phase reactants, including ceruloplasmin, fibrinogen, plasminogen activator inhibitor-1, and leukocytes (Romero *et al.*, 2007).

1.5 **Haemoglobin levels and Body Mass Index (BMI) during pregnancy**

Additional iron absorption is required in pregnancy because pregnancy causes an increase in plasma volume with a corresponding decrease in haemoglobin concentration, which is usually more pronounced in woman with multiple gestation and big babies (Steer, 2000). The fetal demand for iron causes increase in daily maternal iron requirements from 1 to 2.5 mg per day early in pregnancy and an increase maternal requirement up to 6.5 mg per day in the third trimester (Barrett *et al.*, 1994). There is an increase in the percentage of iron absorbed from food during normal pregnancy as pregnancy progresses with 7% of non-heme iron being absorbed at 12 weeks gestation and progressive increase to 36% at 24 weeks gestation and 66% at 36 weeks gestation as shown in stable isotope studies (Steer, 2000). These dramatic changes serve as adaptive mechanisms for healthy pregnant women to prevent anaemia despite the extra fetal iron demands of pregnancy (Barrett *et al.*, 1994).

However, if the pregnant woman's diet is deficient in iron as seen in developing countries, fetal demands can only be met by extra iron contribution from maternal stores. Iron deficiency anaemia can result from depletion of maternal iron stores in early pregnancy (Steer, 2000). Haemoglobin concentration in pregnant women who are not

given supplemental iron drops from 13.3g/dL in non-pregnant state to 11.0 g/dL at 36 weeks gestation. Failure of the plasma volume to expand adequately can lead to intrauterine growth restriction which causes a small for gestational age (SGA) neonate at birth (Taylor and Lind 1979). Several studies have shown that maternal haemoglobin levels were positively associated with PIH (Knottnerus *et al.*, 1990, Huisman and Aarnoudse, 1986, Rasmussen and Oian, 1998).

A review conducted by Yip in 2000 suggests that higher than normal haemoglobin concentrations should be regarded as a risk for possible complications in pregnancy. The mechanisms underlying the positive association of haemoglobin levels with blood pressure in pregnancy are poorly understood, but previous evidence suggested that elevated haemoglobin levels might impact hypertensive disorders in pregnant (Knottnerus *et al.*, 1990, Yip, 2000, Murphy *et al.,* 1986) as well as non-pregnant women (Gobel *et al.,* 1991) due to hemoconcentration i.e. increased blood viscosity, which is generally associated with both central obesity and overall adiposity (Brun *et al.*, 2011).

Furthermore, a population-based study, particularly on the association between haemoglobin levels and BMI during pregnancy, showed that Haemoglobin levels were significantly associated with BMI in 561 pregnant women (Rasmussen *et al.*, 2005). However, less is known about the combined effect of BMI and haemoglobin levels on blood pressure in pregnancy. The limited data of associations of biomarker levels with BMI, blood pressure, and HIV in normotensive pregnancies has involved measurements

from either late pregnancy or at time of delivery. It is unclear whether these associations could be detected throughout different stages of pregnancy. We explored the association of maternal characteristics (Blood Pressure, BMI, HIV, Haemoglobin levels) with pro-angiogenic (PlGF and VEGF) and anti-angiogenic factors (sFlt-1 and sEng), measured in three different stages of normotensive pregnancies.

Few studies (Dekker, 1999, Thadhani *et al.*, 1999, Wolf *et al.*, 2004) have evaluated the concentrations of angiogenic factors in the first trimester of healthy normotensive pregnant women. There is limited data on the relationships of angiogenic factors measured in pregnancy with maternal characteristics in normotensive pregnancies.

Faupel-Badger and colleagues (2011) demonstrate significant, independent associations between concentrations of specific angiogenic factors in early, normotensive pregnancies and selected maternal characteristics such as nulliparity, high BMI, and greater maternal age, which are also risk factors for the development of preeclampsia (Dekker, 1999, Thadhani *et al.*, 1999, Wolf *et al.*, 2004). A recent study of 182 singleton normotensive pregnancies reported that BMI at first prenatal visit was positively associated with first and second trimester sFlt-1 levels (Faupel-Badger *et. al.,* 2011).

In a recent study of 668 normotensive pregnancies by Mijal *et al.* (2011), 96% of the maternal serum samples were collected between 20 and 28 weeks gestation, it was found that there is an inverse association of sFlt-1 concentrations in the second trimester with BMI. A Norwegian cohort reported that angiogenic profile was not

associated with BMI at term in normotensive and preeclamptic pregnancies (Faupel-Badger *et al.*, 2011).

Moreover, limited SA studies exist on associations between maternal characteristics and the progression of both angiogenic and antiangiogenic factors in pregnancy. Whilst some studies have evaluated the natural history of the angiogenic balance during early pregnancy that remain uncomplicated, and how it varies with maternal and gestational factors (Wolf *et al.*, 2005, Troisi *et al.*, 2003), the data suggest that normal first pregnancies are characterized by increased levels of sFlt-1 compared with subsequent pregnancies. Our study therefore aims to determine the circulating levels of PlGF, sFlt-1and sEng throughout pregnancy in relation to various clinical parameters such as BMI, HIV status, blood pressure and haemoglobin Levels. We also sought to determine if maternal characteristics are associated with pro and anti-angiogenic profiles at the three different stages of normotensive pregnancies.

1.6 **Aim and objectives of this study**

This study aims to determine the circulating levels of PlGF, sFlt-1and sEng throughout pregnancy in relation to various clinical parameters (BMI, HIV status, blood pressure and haemoglobin Levels).

1.6.1 **The objectives of the study were:**

1. To determine the circulating concentrations of PlGF, sFlt-1and sEng in pregnant women across 3 stages of pregnancy using enzyme linked immunoassays.

2. To evaluate the associations between PlGF, sFlt-1, sEng and selected clinical parameters (such as BMI, HIV status, blood pressure and haemoglobin Levels).

Chapter 2

Materials and methods

2.1 Study design and location

This was a prospective study which was nested in a facility based descriptive observational cohort study of 372 participants who were followed at 3 different gestational periods during pregnancy. The study was conducted in a selected Primary Health Care Clinic (PHC) facility in Cato Manor, which is a resource poor community in the eThekwini District, KwaZulu-Natal (KZN). KwaZulu-Natal is one of the nine provinces of South Africa, with a total population of 10 449 300, accounting for 21,4% of the total population of South Africa. Thirty four percent of the total population of KwaZulu-Natal resides within the eThekwini district (Durban) (Department of Health, 2010). The eThekwini District is one of the 11 health districts of KwaZulu-Natal located on the south-east of South Africa. The district is divided into three sub-districts namely, south, north and west and the district health services are jointly provided by the Provincial Department of Health and the Local Government (eThekwini municipality) authority, with the former contributing 60% and the latter 40%.

There are eight Community Health Centers (CHCs); (seven provincial and one shared between the two health authorities) and 102 PHC facilities. Of these, 43 are provincial and 59 are managed by the local authority. There are 3 gateway clinics and 28 mobile units within the 102 PHC clinics, of which 12 are provincial and 16 are local authority. However, not all PHC clinics in the eThekwini district provide maternity services. A total of 58 Municipality PHC clinics and 41 KwaZulu-Natal Provincial Administration (KZNPA) PHC clinics provide antenatal care services.

2.2 **Study sampling and ethical approval**

Convenient sampling was utilized to recruit the participants reporting at clinic for their first antenatal visit. Forty-six (n=46) pregnant women who agreed to participate in the biomarker analyses study were followed up from their first antenatal visit until delivery. This subset was part of the larger maternal cohort study (n=372). (Institutional research ethical clearance, IREC 045/14, Appendix 2) was obtained from the Institutional Research Ethics Committee, Durban University of Technology. Permission to conduct research at Cato Manor PHC facility was granted by the KwaZulu-Natal Department of Health (HRKM 234/14, Appendix 3). Participation was voluntary and no coercion was used to recruit participants. Prospective participants were verbally informed of the study by the research nurse, recruitment personnel and through advertisements placed in the clinic via the Medical Research Council Flagship Advertisement banner (Appendix 4).

All pregnant women who showed interest in participating were given a letter of information and consent available in English (Appendix 5). In the instance where the participant was illiterate, the letter of consent was read to them in their own language. The study population included pregnant women aged 18 - 45yrs who presented for their first antenatal visit at the PHC before 24 weeks of pregnancy and who consented to participate in the study. Those who agreed to participate were required to donate 3 vials of blood at three different stages of pregnancy. Due to logistical constraints and dropouts only 46 participants donated blood during all 3 trimesters and these participants were included in this study. All of the initial participants who presented with chorioamnionitis, chronic hypertension, eclampsia, abruptio placentae, intra-uterine death, chronic diabetes mellitus, gestational diabetes, chronic renal disease, connective tissue disease, treatment with aspirin, warfarin, non-steroidal anti-inflammatory drugs,

lipid lowering or anti-hypertensive disease, systemic lupus erythematous, sickle cell disease and anti-phospholipid antibody syndrome; thyroid disease, cardiac disease and active asthma requiring medication during pregnancy and pre-existing seizure disorders were excluded from this study. Identification of these disorders ensured that the study data remained uncontaminated by extraneous influences. Screening for the above disease was done using the chart review tool and the clinic antenatal record.

2.3 **Data collection**

The participants were required to complete an epidemiological questionnaire in English (Appendix 6) or IsiZulu. A trained research assistant used the chart review tool (Appendix 7) to collect demographic and clinical data at three gestational stages of the pregnancy (10 –20 weeks, 22–30 weeks and 32–38 weeks). The chart review tool was used to capture relevant demographic and clinical data linked to current and previous pregnancies, current or previous illnesses (including TB and HIV infection), blood pressure, haemoglobin concentration and urinalysis data. A birth and postnatal outcomes questionnaire (Appendix 8) was used to capture the postnatal data.

2.4 **Collection of serum**

Three vials of maternal venous blood samples (10 mL each) were obtained from each participant at 3 gestational periods at the PHC clinic. Blood was collected in Serum Separator Gel Tube (SST) between 10-20 weeks gestation, 22-30 weeks gestation and 32-38 weeks gestation. Blood samples were centrifuged within 2 hours after collection at 3,500 rotations per minute for 10 minutes at 4^{o}C. Serum was then collected and carefully aliquoted into labelled cryo-tubes and stored at -80^{o}C without thaw until analysis (R&D Systems, Minneapolis, USA)

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

2.5.1 Principle of Enzyme-linked immunosorbent assay (ELISA)

The quantikine immunoassay kit (R&D Systems, Minneapolis, USA) was used to measure the levels of all biomarkers under study. This kit utilised the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for soluble vascular endothelial growth factor receptor-1/ soluble Fms-like tyrosine kinase-1 and polyclonal antibodies specific for Eng and PlGF was pre-coated onto microplates. Standards and samples are pipetted into the wells and any analyte present was bound by the immobilised antibody (R&D Systems, Minneapolis, USA).

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

2.5.2 Placental Growth Factor (PlGF), soluble Fms-like tyrosine kinase-1 (sFlt-1) and Endoglin (Eng)

2.5.2.1 Preparation of samples, standards and kit reagents

All reagents and standards were prepared according to the manufacturer's manual (R&D Systems, Minneapolis, USA). Frozen serum samples and refrigerated reagents

were brought to room temperature before use. The wash buffer was warmed to room temperature and mixed gently until all crystals dissolved in the concentrate. The concentrated wash buffer (20 mL) was diluted with deionized water to prepare 500mL of wash buffer. The substrate solution color reagents A and B were mixed together in equal volumes within 15 minutes of use and protected from light.

2.5.2.2 **Preparation of Human Endoglin Standard/CD105 and conjugate**

Endoglin standard was reconstituted with 1.0 mL of distilled water. This reconstitution produced a stock solution of 100 ng/mL. The standard was mixed to ensure complete reconstitution and allowed to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. 900µL of Calibrator Diluent RD5K was pipetted into the 10 ng/mL tube and 500µL into the remaining tubes. The stock solution was then used to produce a dilution series of 10ng/mL, 5 ng/mL, 2.5 ng/mL, 1.25 ng/mL, 0.625 ng/mL, 0.313 ng/mL and 0.156 ng/mL. Each tube was vortexed and mixed thoroughly before the next transfer. The 10 ng/mL standard served as the highest standard whilst the Calibrator Diluent served as the zero standard (0 ng/mL). Samples were diluted according to a dilution ratio of 1:5, with 20 µL of sample diluted in 80 µL of calibrator diluent. A total quantity of 100 µL of sample, control and standard was added to each well.

2.5.2.3 **Human Placental Growth Factor (PlGF) Standard Preparation**

Human PlGF standard was reconstituted with 1.0 mL of RD6-11 calibrator diluent. This reconstitution produced a stock solution of 1000 pg/mL. The standard was mixed to ensure complete reconstitution and allowed to sit for a minimum of 15 minutes with

gentle agitation prior to making dilutions. 500 µL of Calibrator Diluent RD6-11 was pipetted into each tube. The stock solution was then used to produce a dilution series of 1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL and 15.6 pg/mL. Each tube was mixed thoroughly before the next transfer. The 1000 pg/mL standard served as the high standard. The RD6-11 calibrator diluent served as the zero standard (0 pg/mL). A dilution ratio of 1:2 was used, with 50 µL of sample diluted into 50 µL of calibrator diluent. A total quantity of 100 µL of sample, control and standard was added to each well.

2.5.2.4 **Human Soluble Vascular Endothelial Growth Factor Receptor 1 (VEGFR-1/Flt-1) Standard Preparation**

Human VEGFR-1 standard was reconstituted with 1.0 mL of distilled water. This reconstitution produces a stock solution of 20,000 pg/mL. The standard was mixed to ensure complete reconstitution and allowed to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. 500µL of calibrator diluent RD6-10 was pipetted into each tube. The stock solution was then used to produce a dilution series of 2000 pg/mL, 1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL and 31.3 pg/mL Each tube was mixed thoroughly before the next transfer. The 2,000 pg/mL standard served as the high standard. The RD6-10 Calibrator Diluent served as the zero standard (0 pg/mL). A dilution ratio of 1:10 was used, adding 10 µL of sample, control or standard to each well and 90 µL of calibrator diluent to each well. A total quantity of 100 µL of sample, control and standard was added to each well.

2.5.3 **Immunoassays**

All reagents and samples were brought to room temperature before use. Samples, controls and standards were assayed in triplicate and also in three different stages of pregnancy, inter-plate and intra-plate variability taken into account. All angiogenic measures show substantial between-person variation. Circulating serum levels of all biomarkers (PlGF, sFlt-1 and Eng) were quantitatively evaluated using the ELISA technique according to the manufacturer's protocol (R&D Systems, Minneapolis, MN).

100 µL of Assay Diluent RD1S was added to each well. Standard, control or sample was added per well according to the dilutions stated above and covered with adhesive strip and incubated in a 96-well plate pre-coated with a capture antibody directed against PlGF, sVEGFR-1/sFlt-1 and sEng for 2hrs at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500±50 rpm.

Each well was then aspirated and washed, repeating the process three times for a total of four washes. Washing was by filling each well with wash buffer using a squirt bottle. After the last wash, any remaining wash buffer was removed by decanting, the plate was inverted and blotted against clean paper towels. This was followed by the addition of 200 µL of each biomarker conjugate into each well, covered with a new adhesive strip and incubated for 2 hours at room temperature on the shaker. The aspiration/wash process was repeated. This was then followed by the addition of 200 µL of substrate solution into well. The plates were incubated for 30 minutes at room temperature on the benchtop and protected from light. Fifty (50) µL of stop solution was then added to each well and a color change from blue to yellow was observed in each well. Plates were gently tapped to ensure thorough mixing so that final color obtained was uniform in

appearance. The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm and a wavelength correction set to 540 nm.

2.6 **Data analysis**

After data collection, the data was captured using Microsoft excel, after recoding and cleaning procedures through range checking and spot checking, the data was transferred into STATA version 11 for statistical analysis. The dependent (outcome) variable of the study were the levels of biomarkers (PlGF, sFlt-1 and Eng). The independent variables included age, parity, HIV status, and BMI. Descriptive statistics was done to provide a summary of the data. Continuous Data was expressed as mean ± SD, Medians + Inter-Quartile Ranges (IQR) and frequency distributions as appropriate depending on statistical distribution. Data is presented as means and SD for parametric data or median (inter-quartile range) for non-parametric data. Normality was evaluated graphically and data was analysed using ANOVA and T- tests, as appropriate to compare data sets.

The Pearsons chi-squared test was used to evaluate bivariate associations between demographic and clinical variables stratified by HIV status. HIV status was evaluated as a binary, HIV Positive (+) vs HIV Negative (-) variable. Biomarker concentrations was evaluated as a continuous variable. Pearson's coefficient correlation was used to assess interrelatedness between concentrations of sFlt-1, sEng and PlGF during three different stages of pregnancy. Continuous variables were summarized by mean and SD, and pair-wise comparisons between groups were made. Categorical variables were

summarized using frequency measures, and comparisons between groups were made using Fisher's exact test or chisquared test, as appropriate. We compared the geometric means of Endoglin, sFlt-1, PlGF, and their ratio in each of 3 gestational age windows (10-20, 22-30, and 32-38 weeks) and dichotomized biomarker concentration by HIV status. Differences between data from women who are HIV positive and HIV Negative were tested using the t-test and Mann-Whitney test for normally and non-normally distributed continuous variables respectively. A p-value ≤ 0.05 was considered statistically significant at a 95% confidence level throughout the study.

Chapter 3

Results

3.1 Clinical Characteristics

There were 46 participants enrolled at 10-20, 22-30 and 32-38 weeks' gestation, respectively, however, none of these participants developed preeclampsia. The demographic and clinical characteristics of the study population across the three gestational periods are presented in Table 3.1. The mean age of the women was 25.76 (SD± 5.01) years whilst gestational age ranged between 10-38 weeks. All participants were of African origin and approximately 85% of the participants were single, 8.70% reported that they used alcohol whilst 4.35% reported smoking during pregnancy. The proportion of women who consumed alcohol and smoked during pregnancy was too small to do any further comparisons. The medians of BMI, systolic blood pressure (SDP) and diastolic blood pressure (DBP) across the three gestational periods ranged from 27.00 to 28.71kg/m^2, 101.5-110 mmHg and 60-70 mmHg, respectively. The mean haemoglobin concentrations across the three gestational periods ranged from 10.62 to 10.83 g/dL. BMI, haemoglobin, SBP and DBP increased progressively during pregnancy. Approximately 28.26% of the participants were nulliparous whilst 71.74% were either primiparous or multiparous. Most of the study participants were HIV positive (82.61%) and the mean birth weight was 3.01 (0.40) kg. All study participants that were HIV+ were on highly active antiretroviral therapy (HAART) (100%).

Table 3.1 Clinical characteristics of maternal cohort with normotensive pregnancies (n=46)

Maternal Characteristics	Mean ±SD or n (%)
Maternal age at delivery (yrs)	25.76 (5.01)
Smoking	2 (4.35)
Alcohol	4 (8.70)
Blood pressure (Median, range mmHg)	
Systolic	
10-20 weeks	101.5 (11)
22-30 weeks	108 (10)
32-38 weeks	110 (10)
Diastolic	
10-20 weeks	60 (8)
22-30 weeks	68 (9)
32-38 weeks	70 (14)
BMI (Median, range Kg/m^2)	
10-20 weeks	25.38 (5.79)
22-30 weeks	27.00 (6.57)
32-38 weeks	28.71 (6.61)
Haemoglobin g/dL	
10-20 weeks	10.62 ± 1.39
22-30 weeks	10.76 ± 1.45
32-38 weeks	10.83 ± 1.50
Parity	
Nulliparous	13 (28.26)
Primiparous/multiparous	33 (71.74)
HIV Status	
Positive	38 (82.61)
Negative	8 (17.39)
Birth Weight (kg)	3.01 ± 0.40
Positive on HAART	38 (100)

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

The median serum concentrations of PlGF and sFlt-1 at each defined gestational period is illustrated in Figure 3.1 and Table 3.2. The median concentration of PlGF was 301.52 (201.71) pg/mL at 10–20 weeks, in contrast to the median concentration at 22 – 30 weeks [median 642.48 (374.27) pg/mL]. There was in an increase in the median PIGF levels at 32 – 38 weeks [713.53 (534.28) pg/mL].

The median concentration of sFlt-1 at 10-20 weeks was 1169.17 (824.98) pg/mL, which was lower in comparison with that observed at 22-30 weeks [1244.14 (936.62) pg/mL]. The concentration however, increased at 32-38 weeks with median of 1358.07 (870.87) pg/mL.

The medians of the angiogenic and antiangiogenic factors measured during pregnancy and the trends across the three gestational periods are reported in Table 3.2., Figure 3.1 and 3.2 shows the serum concentrations of PlGF (pg/ml), sFlt-1 (pg/ml) and sEng respectively during pregnancy. As expected, the median of PlGF concentration increased with increasing gestational age, with a corresponding increase in sFlt-1 concentration whilst sEng remained constant in the 10-20 and 22-30 weeks gestational period but increased in the third trimester. Both antiangiogenic ratios (sFlt-1/PlGF and sFlt-1+sEng/PlGF) were higher during the 1st half of pregnancy. The index of vascular disturbance, sFlt-1/PlGF however dropped by almost 50%, at 30 weeks' gestation in contrast to the sFlt-1+sEng/PlGF. Lower anti-angiogenic ratios of sFlt1/PlGF and (sFlt1 + sEng)/PlGF in the 22-30 weeks gestational period were noted compared with the 10-20 weeks gestational period due to the increase in PlGF from 10-20 and 22-30 weeks gestational period.

Table 3.2 Angiogenic and antiangiogenic factors in normotensive pregnancies (n=46)

Gestational period (wks)	Angiogenic factor	Antiangiogenic factor		Antiangiogenic ratios	
	PlGF pg/mL	sFlt-1 pg/mL	sEng (ng/mL)	sFlt-1/PlGF (pg/mL)	(sFlt1 + sEng)/PlGF) (pg/mL)
10-20 wks	301.52 (201.71)	1169.17 (824.98)	3.94 (1.65)	5.23 (11.3)	8.97 (19.41)
22-30 wks	642.48 (374.27)	1244.14 (936.62)	3.94 (1.65)	2.20 (2.24)	5.03 (5.56)
32-38 wks	713.53 (534.28)	1358.07 (870.87)	5.33 (3.06)	1.81 (2.71)	5.99 (7.01)

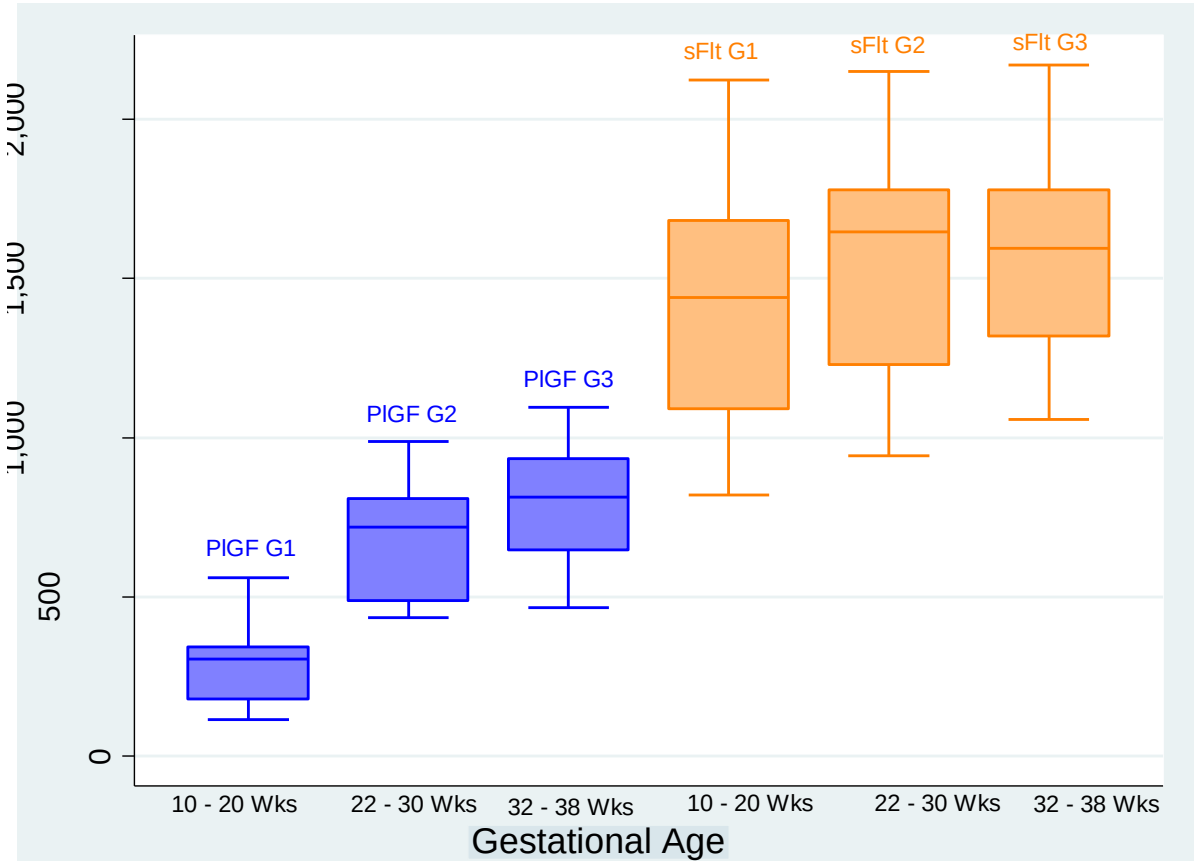

Figure 3.1 Angiogenic and antiangiogenic biomarker (PlGF & SFlt-1) serum concentrations stratified by gestational age and comparison between the three gestational periods (n=46), measured in pg/mL

Similarly, the mean endoglin concentration at 10 – 20 weeks was 5.22 (1.12) ng/mL, which was higher in comparison with that observed at 22 – 30 weeks [4.47 (1.05) ng/mL]. There was however an elevation in the concentration levels [6.21 (1.86) ng/mL] at 32-38 weeks gestational period.

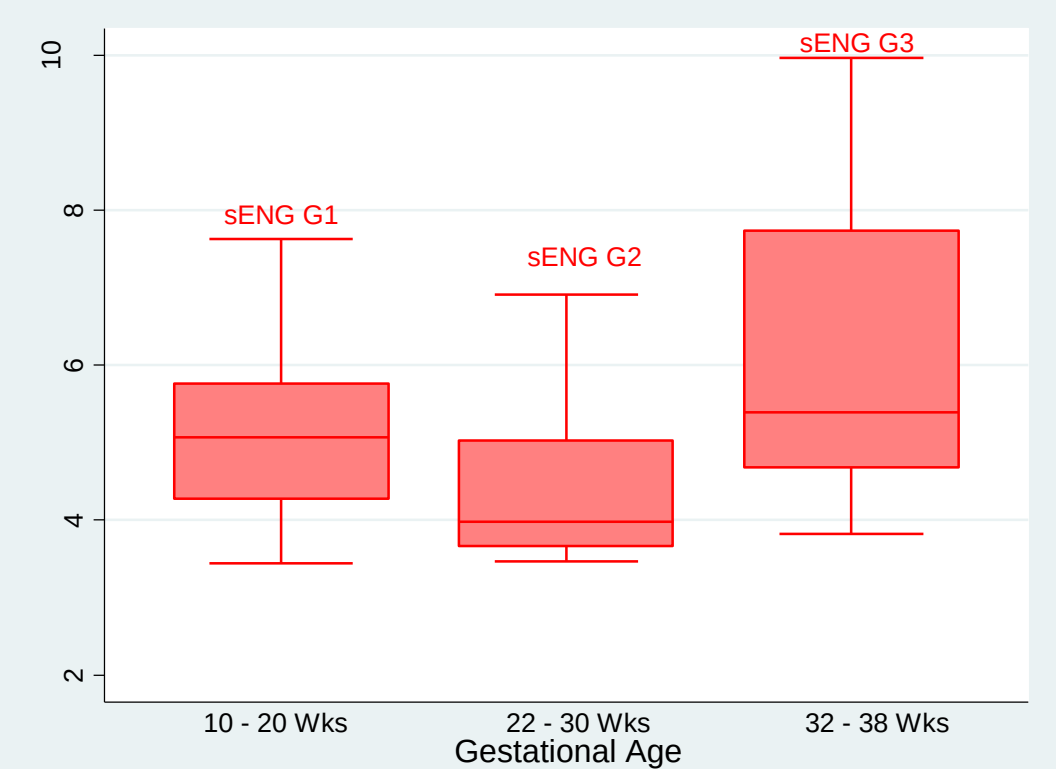

Figure 3.2 Anti-angiogenic biomarker (sENG) serum cncentrations stratified by gestational age and comparison between the three gestational periods (n=46).

The Pearson's correlation coefficient was computed to assess the relationship between the clinical characteristics and biomarker concentrations (Table 3.3, 3.4 and 3.5). There was a slight correlation between BMI; systolic BP; DBP; haemoglobin and the biomarker concentration across the three gestational periods. A positive correlation was noted between haemoglobin level at 10-20 weeks and biomarker concentrations (Table 3.3, 3.4 and 3.5). A significant correlation (p=0.04) was shown between BMI and sFlt-1 levels at 22-30 weeks gestational period.

3.3 **Angiogenic and antiangiogenic factors stratified by clinical characteristics**

Table 3.3 shows median levels of angiogenic and antiangiogenic factors stratified by BMI and parity. Median PlGF levels in multiparous women increased progressively

throughout pregnancy. However, in nulliparous women, there was an increase in serum PlGF levels between 10-30 weeks with a reduction of 130pg/ml at 32 weeks' gestation (Table 3.3) Nulliparous women had higher sFlt-1 concentrations than primi/multiparous women in the 10-20 and 32-38 weeks gestational period, resulting in a higher anti-angiogenic sFlt-1/PlGF ratio, however in the third trimester, higher sFlt-1 concentrations with a corresponding increase in sFlt-1/PlGF ratio were observed in nulliparous women when compared to their parous counterparts. Of note was the reduction in serum sEng levels at 22-30 weeks' gestation and an elevation at 32 weeks. In the 10-20 and 22-32 weeks gestational period, sEng concentrations were inversely associated with BMI in the overweight (BMI≥25) and normal (BMI≤24.9) BMI group while a positive association was observed between BMI and sEng levels in the third trimester. However, PlGF increased with increasing BMI in both overweight women and women with normal BMI while sFlt1 had inverse association with BMI in the overweight group.

Table 3.3Angiogenic and antiangiogenic factors stratified by clinical characteristics in normotensive pregnancies (n=46)

Clinical factor	Angiogenic	Antiangiogenic		Antiangiogenic ratios	
	PlGF (pg/mL)	sFlt-1 (pg/mL)	sEng (ng/mL)	sFlt1/PlGF	(sFlt1 + sEng)/PlGF)
Parity					
Nulliparous					
10-20wks	244.08 (57.44)	1457.98 (288.82)	5.08 (0.00)	6.94 (1.71)	19.09 (3.17)
22-30wks	757.13 (114.65)	1244.14 (0.00)	4.01 (0.07)	2.00 (0.20)	5.55 (2.01)
32-38wks	627.04 (86.49)	1594.77 (383.85)	5.13 (0.20)	1.99 (0.18)	5.99 (1.86)
Primi/Multiparous					
10-20wks	307.58 (6.06)	956.87 212.30)	5.08 (0.00)	4.01 (1.22)	22.31 (0.05)
22-30wks	569.02 (73.46)	1260.31 (16.17)	3.85 (0.10)	2.21 (0.01)	8.94 (1.38)
32-38wks	713.53 (0.00)	1210.92 (147.16)	5.33 (0.00)	1.73 (0.08)	8.24 (0.39)
BMI					
>25.00					
10-20wks	236.73(187.62)	1290.76(832.90)	4.77(1.68)	6.94(11.59)	7.24(25.28|)
22-30wks	272.30(198.95)	1228.58(895.67)	3.94(1.73)	2.21(2.89)	5.55(6.47)
32-38wks	705.56(588.1)	1210.92(920.63)	5.32(3.05)	3.82(7.23)	5.92(6.7)
<24.9					
10-20wks	316.28(210.78)	1055.51(925.90)	5.52(1.87)	4.63(7.21)	11.18(16.68)
22-30wks	752.81(305.25)	1702.48(1079.18)	4.18(1.29)	2.19(0.52)	2.49(4.37)
32-38wks	891(471.19)	1502.57(585.79)	5.67(1.43)	2.02(1.05)	7.05(2.37)

3.4 Angiogenic and antiangiogenic factors in normotensive pregnancy stratified by HIV status.

The medians of selected clinical characteristics and angiogenic levels, stratified by HIV status is shown in Table 3.4. The associations of the maternal characteristics, angiogenic and antiangiogenic factors were mostly similar in both HIV+ and HIV– participants across pregnancy with a significant association observed in the sFlt-1+sEng/PlGF ratio among the two groups for the 10-20 weeks gestational period. (p=0.04). Our study revealed elevations in serum sFlt-1 levels as pregnancy progressed in HIV negative participants however, a downward trend was observed in HIV positive mothers as pregnancy progressed. The mean haemoglobin concentration was slightly

higher in HIV negative participants (11.78 g/dL) compared to HIV positive participants (10.63 g\dL). This trend was also observed for neonatal birth weight, the HIV negative mothers gave birth to babies with higher birth weight [(3.16 (0.35kg)] than the HIV positive mothers [(2.98kg, (0.41kg)]. Diastolic blood pressure (DBP) was also higher in HIV negative mothers (74mmHg) than in HIV positive mothers (70 mmHg).

The medians of angiogenic (PlGF) and anti-angiogenic (sFlt-1 and Eng) profiles stratified by gestational age and HIV status are also shown in Table 3.4. HIV status had no effect and showed no association with the concentration of biomarkers. However, it was noted that the concentrations of sFlt, PlGF and Endoglin increased progressively with increasing gestational age in both HIV+ and HIV- participants. The concentration of endoglin however, decreased from 5.34 (1.10) ng/mL to 4.79 (1.43) ng/mL at 22 – 30 weeks' gestational period among HIV positive participants. Additionally, an elevation in the concentration of endoglin from 4.33 (0.96) ng/mL at 10-20 weeks to a median of 4.41 (0.98) ng/mL at 22-30 weeks gestational period was observed among HIV-participants (Table 3.4).

Table 3.4 Angiogenic and antiangiogenic factors in normotensive pregnancy stratified by HIV status (n=46)

Clinical characteristics	HIV –ve	HIV +ve
Blood Pressure (mmHg, Mean (SD)		
Systolic	113 (12)	111 (7)
Diastolic	74 (10)	70 (8)
PlGF (pg/mL, Median, range)		
10-20wks	240.41 (61.52)	308.08 (6.56)
22-30wks	771.40 (128.92)	608.09 (34.39)
32-38wks	483.86 (229.67)	721.50 (7.97)
sFlt-1(pg/mL, Median, range)		
10-20wks	819.69 (349.48)	1246.43 (77.27)
22-30wks	1335.78 (91.64)	1244.14 (0.00)
32-38wks	1663.97 (305.22)	1230.05 (128.03)
sEng (ng/mL, Median, range)		
10-20wks	5.02 (0.06)	5.08 (0.00)
22-30wks	3.88 (0.06)	4.01 (0.07)
32-38wks	5.67 (0.35)	5.32 (0.01)
sFlt/PlGF (Median, range)		
10-20wks	4.00 (1.23)	5.36 (0.13)
22-30wks	2.0 (0.2)	2.21 (0.01)
32-38wks	2.69 (0.88)	1.81 (0)
sFlt-1+sEng/PlGF (Median, range)	**19.09 (3.17)**	**22.31 (0.06)***
10-20wks	5.55 (2.01)	8.15 (0.59)
22-30wks	5.51 (2.34)	8.24 (0.39)
32-38wks		
BMI (kg/m^2, Mean, SD)	29.26 (3.16)	29.83 (7.32)
Haemoglobin (g/dL, Mean, SD)	11.78 (0.87)	10.63 (1.54)
Birth weight (kg, Mean, SD)	3.16(0.35)	2.98 (0.41)

***p<0.05 was considered statistically significant**

3.5 **Correlation analyses of circulating factors**

Haemoglobin, BMI SBP, and DBP were measured during visits when samples were collected to measure angiogenic and antiangiogenic factors. Pearson's correlations between the clinical characteristics mentioned and angiogenic/antiangiogenic factors

are presented in Table 3.5. Despite the notable correlations observed in angiogenic profile for some clinical factors at the defined gestational intervals in our study, statistically significant correlations were undetectable for most factors. Weak inverse associations were noted between maternal age and PlGF at 22-32 and 32-38 weeks' gestation respectively, in contrast to sEng. Maternal age was however, positively correlated (statistically significant) with the antiangiogenic ratio (sFlt-1/PlGF) at 32-38 weeks' gestation. A positive correlation of BMI (<24.99kg/m2) with antiangiogenic factor sEng, antiangiogenic ratios (sFlt-1/PlGF; (sFlt-1 + sEng)/PlGF), was observed at 32-38 weeks gestation, but it was not statistically significant. In the overweight group (BMI>25), higher BMI had an inverse correlation with sFlt-1 anti-angiogenic level in 10-20 weeks gestational which was statistically significant (p=0.04). Despite the minimal increase noted in both systolic and diastolic blood pressure throughout the defined gestational intervals, third trimester PlGF and sFlt-1 levels were inversely correlated with third trimester systolic blood pressure ($r = -0.38$, $p = 0.04$ and $r = -0.47$ $p=0.01$ respectively), however, third trimester sFlt-1/PlGF levels were positively correlated with third trimester diastolic Blood Pressure ($rs = 0.50$, $p = 0.03$).

Table 3.5 Pearson's correlations between clinical characteristics and angiogenic/antiangiogenic factors in normotensive pregnancies (n=46)

***p<0.05 was considered statistically significant.**

Clinical factor	Angiogenic factor	Antiangiogenic factors		Antiangiogenic ratios	
	PlGF (pg/mL)	sFlt-1 (pg/mL)	sEng (ng/mL)	sFlt1/PlGF	(sFlt1 + sEng)/PlGF
Maternal age (years)					
10-20wks	0.08	-0.29	0.10	-0.37	-0.30
22-30wks	-0.11	-0.09	0.02	-0.07	0.01
32-38wks	-0.13	0.13	-0.10	**0.61***	0.10
Parity					
Nulliparous					
10-20wks	0.02	0.25	-0.08	0.11	-0.04
22-30wks	0.19	0.04	0.13	-0.12	-0.24
32-38wks	0.14	0.27	0.01	-0.21	-0.29
Multiparous					
10-20wks	0.02	-0.25	0.08	-0.10	0.04
22-30wks	-0.19	-0.04	-0.13	0.11	0.24
32-38wks	-0.14	-0.27	-0.01	0.22	0.29
BMI (kg/m^2)					
<24.9					
10-20wks	0.09	-0.02	0.41	-0.43	-0.29
22-30wks	-0.36	-0.31	-0.22	0.19	0.25
32-38wks	-0.03	0.19	0.41	0.85	0.79
>25.00					
10-20wks	0	-0.47*	-0.06	-0.19	0.05
22-30wks	0.08	-0.25	-0.27	-0.16	0.32
32-38wks	0.04	-0.20	-0.14	-0.33	0.08
Blood Pressure (mmHg)					
Systolic					
10-20wks	0.07	0.08	0.11	-0.10	-0.10
22-30wks	0.17	-0.19	-0.12	-0.19	-0.14
32-38wks	**-0.38***	**-0.47***	-0.11	0.29	0.37
Diastolic					
10-20wks	-0.09	0.10	0.03	0.01	-0.07
22-30wks	-0.04	-0.11	0.28	-0.02	0.12
32-38wks	-0.29	0.15	-0.24	**0.50***	0.12
Haemoglobing/dL					
10-20wks	-0.31	0.17	0.14	0.23	0.18
22-30wks	-0.17	-0.17	0.06	0.11	0.15
32-38wks	-0.01	0.04	-0.21	-0.16	-0.41
Birth weight (g)					
10-20wks	0.24	-0.13	-0.22	-0.24	-0.17
22-32wks	-0.38*	-0.31	0.08	0.06	0.29
32-38wks	0.21	-0.03	0.04	-0.18	0.03

Chapter 4

Discussion

This study examined the concentrations of angiogenic and antiangiogenic factors across three gestational periods of normotensive pregnancies. We also explored the potential relationships between proangiogenic and antiangiogenic factors with maternal characteristics in normotensive pregnancies. The results of our study demonstrate independent associations between angiogenic and antiangiogenic biomarker concentrations of PlGF, sFlt-1 and sEng across three stages of normotensive pregnancies and selected maternal characteristics namely: SBP, DBP, haemoglobin, BMI, and parity.

Our data support a previous American cohort study conducted by Wolf and his colleagues which showed higher sFlt-1 levels in nulliparous women (Wolf *et al.*, 2005). These results were later replicated in 2011 amongst a Hispanic population (Faupel-Badger *et al.*, 2011). These authors found that nulliparous women had higher sFlt-1 levels ($p = 0.001$) and consequently a higher mean sFlt-1/PlGF ratio ($p = 0.003$) in the second trimester when compared to parous women (Faupel-Badger *et al.*, 2011). Nulliparity was also associated with higher antiangiogenic profile where angiogenic proteins were measured in normotensive pregnancies at delivery (Staff *et al.*, 2009).

A slight variation was noted for sEng levels between the HIV negative and HIV positive groups, however, a more prominent pattern was noted throughout the defined gestational intervals. Whilst increased concentrations were notable at 32-38 weeks' gestation, there was a significant decline at 22-30 weeks' gestation.

In the overweight group (BMI≥25), BMI was inversely associated with greater sFlt-1 concentrations in the 10-20 weeks gestational period, this contradicts a recent study of 182 singleton normotensive pregnancy that reported that BMI at first prenatal visit was positively associated with first and second trimester sFlt-1 levels (Faupel-Badger *et al.*, 2011). This may be due to BMI categories established using tertiles, whereas in our study we assessed BMI as a categorical variable using the Centers for Disease Control and Prevention (CDC) BMI guidelines.

In the third trimester, our study did not show statistically significant association between BMI and biomarker levels, which was supported by results from an American cohort, which showed that the angiogenic profile was not associated with BMI at term in normotensive pregnancies (Faupel-Badger *et al.*, 2011). Our study confirms several previously reported associations between pro-angiogenic and anti- angiogenic biomarkers and maternal characteristics, including lower sFlt-1 levels among multiparous women and the inverse relation between all markers and maternal BMI (Thadhani *et al.*, 2004, Staff *et al.*, 2009, Law *et al.*, 2010). Studies comparing BMI at the initial prenatal consult and first and second trimester sFlt-1 levels and sFlt-1/PlGF ratio, revealed a positive correlation (Fauper-Badger *et al.*, 2011). This is inconsistent with our data, however, a major limitation in our study is the late antenatal access/admission of our pregnant women. Within the context of SA, antenatal care is poorly accessed during the 1st trimester, which is the reason for defining our gestational intervals as it is. It is possible that some of our second trimester data is colluded in this gestational age grouping. The results of our study indicate a synergistic effect between increased BMI and haemoglobin on blood pressure

as pregnancy progresses. However, additional research is warranted to clarify the biochemical mechanism of interaction, and its clinical role in pregnancy. We also found an inverse association of sFlt-1 concentrations in maternal serum with BMI in the 22-30 weeks gestational period, which correlates with a recent study of 668 normotensive pregnancies (Mijal *et al.,* 2011).

Our study revealed elevations in serum sFlt-1 and sEng levels, irrespective of HIV status with a slight decrease in the sEng levels in the 22-30 weeks gestational period. These serum elevations are sustained throughout the pregnancy in normotensive pregnant women, suggestive of their utility as biomarkers for disease detection and management. Findings from this study also revealed that all biomarker concentrations evaluated were similar in HIV positive and HIV negative normotensive pregnant women.

Our study further demonstrates a progressive increase in systolic and diastolic blood pressure in normotensive pregnancies as pregnancy progresses, irrespective of the HIV status which may be due to the physiological and hormonal changes in pregnancy (Table 3.4). Studies exploring a similar relationship in normotensive pregnancies, suggests that elevations in blood pressure at term may be ascribed to increases from the second to third trimesters (Zhong *et al.*, 2012, Troisi *et al.*, 2008 and Levine *et al.*, 2006). However, our study reported no significant difference in biomarker levels across various gestational periods based on HIV status. Our study also revealed that all studied biomarker concentrations were similar in HIV positive and HIV negative normotensive pregnant

women whereas, a study by Govender, Naicker and Moodley (2013) showed slightly higher circulating levels of sFlt-1 and sEng in HIV negative pregnancies (preeclamptic and normotensive), compared to HIV positive pregnancies.

Normal pregnancy causes altered immune sensitivity, thereby affecting maternal resistance against infection and foetal tolerance (Mahmoud *et al.*, 2003 and Wimalasundera *et al.,* 2002), the immune deficiency caused by HIV together with the normal immune changes of pregnancy may affect biomarker concentrations in pregnancy (De Groot *et al.*, 2003, Mattar *et al.*, 2004, and Lapaire, Shennan and Stepan, 2010). These findings may inform the selection of covariates included in models using pro-angiogenic and anti-angiogenic biomarkers and also suggest new avenues for research examining the contribution of these factors in the etiology of adverse pregnancy outcomes. A reduction in maternal serum levels of PlGF, endoglin and sFlt-1 may result in maternal endothelial dysfunction which eventually could lead to the spectrum of preeclampsia, HELLP syndrome.

Our study prospectively evaluated the relationship between selected proangiogenic, antiangiogenic factors and maternal characteristics (BMI, haemoglobin and blood pressure and HIV status). The data from our study potentially highlights a clinical use of these angiogenic/antiangiogenic factors as reference values for comparison of normotensive pregnancies with that of pregnancies complicated by HDPs. Our study revealed elevations in serum sFlt-1 and endoglin levels, irrespective of HIV status. These serum elevations are

sustained throughout the pregnancy in normotensive pregnant women, suggestive of their utility as biomarkers for disease detection and management. Previous studies report an increased risk for preeclampsia development in healthy nulliparous women in response to elevated systolic and diastolic blood pressures (Sibai *et al.*, 1993, Levine *et al.*, 2006). However, systolic and diastolic pressures at measured at 13-21 weeks' gestation in pre-eclamptic women with high levels of sFlt-1 was reported to be similar to those in normal, uncomplicated pregnancies (Levine *et al.*, 2006), indicative that among those who have preeclampsia, there are some who have healthy endothelial cells and that preeclampsia development occurs in response to increased circulating sFlt-1 levels or mainly due to their increased susceptibility to vascular disorders. Thus, it is possible that even slight increases in blood pressure during the early mid trimester can result in vascular damage.

Our results demonstrate an increase in PlGF level around 32-38 weeks' gestation and concur with other longitudinal studies (Levine et al, 2004, Romero et al, 2008, Chaiwaporinga *et al.*, 2005). However, the increase appeared earlier in our study, at weeks 22–30 similar to Palm et al (2009), compared to week 32 (Chaiwaporinga *et al.*, 2004) and weeks 33–36 (Levine et al, 2004). The rising levels observed might be caused by a relative placental ischemia due to an increasing myometrial tone and more uterine contractions at the end of pregnancy and thus be a sign of uterine preparation for delivery (Bdolah, Sukhatme and Karumanchi, 2004). Moreover, it is possible that the modest expression of PlGF during the 10-20 weeks' gestational interval in our study may be attributed to the preliminary vasculogenic phases and thereafter progressively increases

after 22 weeks ‘gestation since there is a switch from branching angiogenesis to nonbranching angiogenesis (Andraweera *et al.*, 2012).

The associations between maternal age, gestational age, blood pressure, BMI and parity and pro-angiogenic and anti-angiogenic concentrations was also evaluated in our study for adverse pregnancy outcomes. Our findings were consistent with previous studies that evaluated maternal age, gestational age, blood pressure, BMI and parity (Wang, Rana and Karumanchi, 2009, Sibai, 2005).

Troisi *et al.*, (2008) also illustrated a positively associated correlation between blood pressure and maternal endoglin concentrations, with a reciprocal effect on PlGF concentrations in normotensive pregnancies. In our study, the elevated blood pressure observed within the cohort may be ascribed to the increased levels of sFlt-1 and sEng. Our data correlates with that reported by Noori *et al.*, (2010), who reported higher sFlt-1 and sEng levels with elevated blood pressure as pregnancy progressed. This elevation in blood pressure from mid- to late gestation is probably related to the raised anti-angiogenic profile at delivery (Troisi *et al.*, 2008). An association was also noted between increasing maternal age and an elevated anti-angiogenic profile in normotensive pregnancies. However, due to the limited data, a larger epidemiological study with a larger sample size is warranted.

The levels of PlGF in our study increased as pregnancy progressed, which is consistent with several others who reported a rise in PlGF midway through pregnancy (Thadhani *et*

al., 2004, Levine *et al.*, 2004 and Romero *et al.*, 2008). Others reported a minimal change in sFlt-1 levels at mid-gestation which subsequently increased later in pregnancy (Romero *et al.*, 2008, Levine *et al.*, 2004) whilst Endoglin levels increased with increasing gestational age (Romero *et al.*, 2008 and Levine *et al.*, 2006). The increased PlGF and constant sEng levels between first and second trimester of pregnancy have been shown in other populations (Levine *et al.*, 2006, Levine *et al.*, 2004) including Massachusetts General Hospital (MGH) Obstetric Maternal Study (MOMS), a prospective cohort study that recruited 9930 pregnant women between 1998 and 2005 (Rana *et al.*, 2007). Our findings were also in agreement with the results from a study by Faupel-Badger *et al.*, (2011) where they examined 182 women with singleton normotensive pregnancies and reported that PlGF concentrations increased from first to second trimester (Faupel-Badger *et al.*, 2011). In our study, lower anti-angiogenic ratios of sFlt1/PlGF and (sFlt1 + sEng)/PlGF in the 22-30 weeks gestational period was noted compared with the 10-20 weeks gestational period due to the increase in PlGF from 10-20 and 22-30 weeks gestational period, which was also reported by a recent American study (Faupel-Badger *et al.*, 2011).

Earlier studies have also explored the relationship between maternal age, parity and biomarker levels (Thadhani *et al.,* 2004, Law *et al.,* 2010 and Staff *et al.,* 2009), however inconsistent data have been produced. Associations between maternal age and first trimester levels of PlGF and sFlt-1 have been inconsistent (Thadhani *et al.* 2004 and Law *et al.*, 2010), however, PlGF level was associated with maternal age only at delivery (Staff *et al.*, 2009). In contrast, bivariate analyses demonstrated lower PlGF and higher sFlt-1

concentrations among nulliparous women, irrespective of gestational age at measurement (Thadhani *et al.*, 2004, Staff *et al.*, 2009, and Law *et al.*, 2010). These data were consistent with findings of our study.

Furthermore, blood pressure, BMI and concentrations of haemoglobin increased with increasing gestational age throughout pregnancy among our participants. It is possible that haemoglobin increased with increasing gestational age because pregnant women were given haematinics throughout pregnancy which subsequently caused an increase in haemoglobin levels. In addition, a negative correlation was found between sFlt-1 and BMI at 22-30 weeks gestational period (r=-0.31).

The mechanisms influencing the interaction between haemoglobin and BMI on increased blood pressure are unclear. Based on the association of elevated blood viscosity with increasing body mass or increased haemoglobin levels (Knottnerus *et al.*, 1990, Yip, 2000, Murphy *et al.*, 1986 and Gobel *et al.*, 1991), this study supports the notion that increased BMI and haemoglobin may have a combined effect on increased blood pressure via elevated blood viscosity during normal pregnancy. Anaemia in pregnancy is defined as an reduction in haemoglobin levels (< 11g/dL) in peripheral blood (Buseri et al, 2008). However, the WHO classifies anaemia into mild anaemia (10.0 - 10.9 g/dL), moderate anaemia (7.0 - 9.9 g/dL) and severe anaemia (<7.0 g/dL) (WHO, 2011). Our study thus demonstrates the presence of mild anaemia in the HIV positive group. It is possible that the commencement of HAART in HIV pregnant women, irrespective of their CD4 counts has most likely reduced the HIV related mortality and morbidity (Sebitloane, Moodley and Sartorius, 2017). In our study, lower haemoglobin levels were noted in the HIV positive group (Table 3.4), who are also on HAART.

Sebitloane and co-workers suggests that HAART has the potential to lower the prevalence and severity of anaemia. It is reported that mitochondrial toxicity and irregular reticulocyte counts are a common effect of the antiretroviral drug, zidovudine, which exacerbates the risk of anaemia (Sebitloane, Moodley and Sartorius, 2017). However, an earlier study reports no difference in anaemia prevalence in those on HAART in comparison to those who received zidovudine alone during pregnancy (Nundlall *et al.*, 2014). This area of study however, requires further investigation.

Our study demonstrated that BMI is positively associated with sFlt-1 levels during the 22-30 weeks gestational period and inversely associated with endoglin levels. This correlates with studies done by Faupel-Badger and colleagues, who demonstrated that sFlt-1 levels at first antenatal visit is positively associated with maternal serum sFlt-1 levels and sFlt-1/PlGF ratio in the first and second trimester (Faupel-Badger *et al.,* 2011). In contrast, an inverse association was observed for endoglin level.

The results of our study indicate a synergistic effect between increased BMI and haemoglobon on blood pressure as pregnancy progresses. However, additional research is warranted to clarify the biochemical mechanism of interaction, and its clinical role in pregnancy. We also observed consistent inverse associations between BMI and all studied biomarker levels in normotensive pregnancies. An exception to this was the positive association observed for sFlt-1 level at 10-20 weeks gestation. This inverse correlation noted between biomarker levels and BMI may be attributed to dilution effects which also observed when Maternal Serum Alpha-Feto Protein crosses from the fetus

(Kim *et al.,* 1996 and Louis *et al.,* 1996) or placenta into the maternal circulation (e.g. corticotrophin releasing hormone) (Louis *et al.,* 1996 and Chen *et al.,* 2010).

Nulliparity is a documented risk factor for preeclampsia development, however, it is unclear why nulliparous women exhibit higher angiogenic/antiangiogenic profiles (Duckitt and Harrington, 2005). Earlier reports have suggested that a higher ischemic placental environment is found in nulliparous women since their spiral arteries have not been formerly remodeled (Karumanchi and Bdoloah, 2004). Hence, this increased hypoxic environment increases placental production of sFlt-1 and its subsequent release into the maternal circulation and influences a greater risk of preeclampsia development in first pregnancies (Karumanchi and Bdoloah, 2004). The elevated antiangiogenic state observed in our study at the 3rd gestational interval (32-38 weeks) (Table 3.2) is consistent with that reported by other studies (Bdolah *et al.*, 2014). These investigators emphasize the moderately anti-angiogenic state in circulation during late third trimester in nulliparous women. Thus, a modified angiogenic profile may be correlated nulliparity in pregnancy.

A limitation in our study is the small number of participants due to loss to follow-up, which reduced statistical power to explore the associations of clinical characteristics and circulating angiogenic profiles. This limited power may have resulted in a lack of statistical significance for some real differences in angiogenic profiles by clinical characteristics. However, we did observe some significant correlations that should be confirmed in larger studies.

Conclusion

In conclusion, this study confirms several previously reported associations between pro-angiogenic and anti- angiogenic biomarkers and maternal characteristics, including lower sFlt-1 levels among multiparous women and the inverse relation between all markers and maternal BMI. Examining profiles in women according to HIV Status, in this study, there was no significant difference in the trend of biomarker levels across various gestational periods. Our data suggest that the angiogenic levels during pregnancy may be differentially adjusted based on the varying physiological maternal and fetal demands. The use of the anti-angiogenic profiles in evaluating the biophysical and cardiovascular burden during pregnancy enhances the identification of a multifactorial group of HDPs, potentially reducing cardiovascular mortality in pregnant women (Verlohren *et al.,* 2017).These findings can inform the selection of covariates included in models using pro-angiogenic and anti-angiogenic biomarkers and also suggest new avenues for research examining the contribution of these factors in the etiology of adverse pregnancy outcomes.

Clinical perspective

A reduction in maternal serum levels of PlGF, endoglin and sFlt-1 may result in maternal endothelial dysfunction which eventually could lead to the spectrum of preeclampsia, eclampsia and HELLP syndrome. This study evaluated prospectively the relationship between selected proangiogenic, antiangiogenic factors and maternal characteristics (BMI, haemoglobin and blood pressure and HIV status). Placental growth factor, endoglin and sFlt-1 levels in normotensive pregnancy as shown in this study may provide a clinical

reference to compare normotensive pregnancies with those that may be complicated by HDPs.

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Appendices

Appendix 1

Introduction

Despite the significant advancement in maternal morbidity and mortality in both developed and low and middle income countries (LMIC), there is still estimated 289000 maternal mortalities worldwide (Arendt, 2016). Whilst prospective mothers enter their pregnancies feeling excited, uncertainties regarding the health of their babies still prevail during pregnancy.

Pregnancy is dependent on angiogenesis and pseudo-vasculogenesis (Kim, 2013), and characterized by spiral arterial remodeling by the invasive fetal cytotrophoblasts (Andraweera, 2012). Placental trophoblasts also synthesize and secrete the angiogenic PlGF and VEGF into the maternal vasculature, which support endothelial proliferation and survival and arterial remodeling (Kaufmann *et al.* 2003, Andraweera, 2012, Espinozoa (2014). During pregnancy, the bioavailability of both VEGF and PLGF is controlled by the anti-angiogenic factor soluble fms-like tyrosine kinase-1 (sFlt-1), which is a splice variant of the VEGF receptor 1 (Clark *et al.* 1998, Mutter & Karumanchi, 2008). This angiogenic regulation is imperative for a successful pregnancy (Romero *et al.,* 2010), however, excess release of these anti-angiogenic factors into the maternal circulation result in angiogenic imbalances and subsequent hypertensive disorders of pregnancy (HDP), including preeclampsia (PE) (Llurba *et al.,* 2015; Verhloern et al. 2017), coronary artery disease (Matsumuto *et al.,* 2013) and heart failure (Hammadah *et al.*,). Definitions of these disease entities rely largely on clinical evaluations, such as measurement of blood pressure and proteinuria, which can occasionally be imprecise, especially in predicting HDP related adverse outcomes (Zhang *et al.,* 2001).

Preeclampsia is characterized by increased circulating levels of sFlt-1 which inhibit angiogenic signaling and subsequently induce endothelial dysfunction (Roberts *et al.,* 1989). The predictive role of sFlt-1, sEng and PlGF in the clinical diagnosis of preeclampsia development is widely explored and correlates to its symptomatic onset and gravity (Levine et al 2004; Palmer *et al.,* 2017; Ohkuchi 2010, Jantarasaengaram et al, 2012, Verhloren et al 2017, Kusanovic *et al.,* refs). Increased antiangigenic ratios as a measure of vascular dysregulation is highlighted by several studies (Sundrani *et al.,* 2013; Troisi et al 2008; Staff et al 2009). In addition, some have shown strong correlations between blood pressure/maternal and increased sFlt-1 to PlGF levels in mid to late gestation in both normal and preeclamptic pregnancies [Troisi et al 2008, Staff et al 2009]. Placental delivery is no longer an option for PE resolution since epidemiological evidence confirms the onset of various long-term cardiovascular (ref) and metabolic (ref) diseases, long after the dissolution of preeclampsia symptoms (Jim and Karumanchi, 2017). Despite the improved understanding of PE and the positive laboratory investigations, delayed childbearing has raised worry regarding HDPs as it intensifies their risk. It is suggested that certain maternal factors including BMI, maternal age, and nulliparity, may be correlated with angiogenic balance even in uncomplicated pregnancies [Troisi et al 2008, Staff et al 2009].

However, limited data exist on these correlations in normotensive pregnancies from a SA perspective and thus we aimed to produce a reference range that can used to discriminate those that may be at risk of developing PE. We also investigated the association of selected maternal characteristics identified as risk factors for preeclampsia, with the angiogenic and antiangiogenic factors measured during defined gestational intervals of pregnancies that remained normotensive through delivery. The circulating concentrations of sFlt-1, sEng and PlGF throughout pregnancy may contribute to the development of a reference range for black South African pregnant women.

Methods

This prospective and longitudinal study was conducted at a primary health care facility in Durban, South Africa between . Following institutional (IREC 045/14) and regulatory (HRKM 234/14) permission, blood samples were collected from nulliparous women aged 15-45 years, through convenient sampling. Pregnant women (n=46) were enrolled at 10-20, 22-30 and 32-38 weeks of gestation respectively. Gestational interval was defined into these intervals based on convenience, since the first antenatal visit of most pregnant mothers seeking primary health care in SA is later in pregnancy. The study group was stratified by HIV status into HIV positive (HIV+) and HIV negative (HIV-) pregnant women. Inclusion criteria were 18 years or older, HIV positive or HIV negative pregnant women. Exclusion criteria included chronic diabetes, chronic hypertension, gestational diabetes, connective tissue disorder, cardiac disease, sickle cell disease, antiphospholipid antibody syndrome, chorioamnionitis, unknown HIV status, and those unable to provide informed consent.

Blood samples were centrifuged at 3500 rpm for 5 min in yellow top gel tubes serum samples were aliquoted and stored at -80ºC until analysis. The clinic records of all participants were used to collect demographic data including maternal age, weight and blood pressure, parity, HIV status, gestational age at delivery, diagnosis of preeclampsia at the time of delivery, maternal haemoglobin levels and birth weight. Serum concentrations sFlt-1 (1:5), sEng (1:5) and PlGF (1:2) were measured in triplicate by ELISA according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). The reference ranges of serum levels of sFlt-1, PlGF and sFlt-1/PlGF ratio were constructed. Selected maternal factors including age, parity, tobacco and alcohol use, blood pressure, hemaglobin levels and body mass index (BMI)and pregnancy outcomes including gestational age at delivery and birth weight were evaluated for potential associations with maternal serum concentrations of sFlt-1, sEng and PlGF.

Statistical Analysis

All statistical analyses were conducted using STATA Statistics version 20. Data were assessed for their distribution by the Shapiro–Wilk test. The homogeneity of variance between groups was assessed by Levene's test for equality of error variances. Descriptive statistics were utilized and outcome variables are presented as means and median (interquartile range) where appropriate. Kruskal–Wallis post hoc tests were used to compare the means of clinical parameters. Pearson's correlation coefficient was computed to assess the relationship between serum expression of PlGF,

sFlt-1 and sEng and maternal factors. A probability level of $p < 0.05$ was considered statistically significant.

Results

There were 46 participants enrolled at 10-20, 22-30 and 32-38 weeks' gestation, respectively. Of these participants, none developed preeclampsia. The demographic and clinical characteristics are presented (Table 1). The mean maternal age at delivery was 25 years, whilst the mean neonatal birth weight was 3.01 ± 0.40kg. There were only four participants who consumed alcohol (8.7%) and two (4.35%) who used tobacco during pregnancy thus, these variables were excluded from further analysis. Of the total sample, 28.26% were nulliparous and 82% were HIV positive. Both systolic and diastolic blood pressure increased slightly throughout the defined gestational intervals.

The median circulating levels of PlGF, sFlt-1 and sEng throughout the defined gestational intervals are presented (Table 2, Figure 1 and 2). The levels of both PlGF and sFlt-1 seems to rise throughout pregnancy. Soluble endoglin on the other hand remained fairly constant until 30 weeks' gestation and thereafter increased by almost 4fold. Both antiangiogenic ratios (sFlt-1/PlGF and sFlt-1+sEng/PlGF) however was higher during the 1st half of pregnancy. The index of vascular disturbance, sFlt-1/PlGF however dropped by almost 50%, at 30 weeks' gestation in contrast to the sFlt-1+sEng/PlGF. Median levels of serum PlGF, sFlt-1 and sEng throughout the defined gestational intervals, stratified by parity and BMI are presented (Table 3). Median PlGF levels in multiparous women increased progressively throughout pregnancy however, in nulliparous women, there was an increase in serum PlGF levels between 10-30 weeks with a reduction of 130pg/ml at 32 weeks' gestation (Table 2). Similarly, increased median serum levels of sFlt-1 in multiparous women were noted between 10-30 weeks with a decline at 32 weeks' gestation, with similar sEng patterns being observed in multiparous women. However, the median serum levels of sFlt-1and sEng in nulliparous women declined between 22-30 week's gestation and increased at from 32 weeks onwards. Of note, is the reduction in serum sEng levels at 22-30 weeks' gestation and an elevation at 32 week's (Table 3).

Median PlGF levels increased progressively throughout pregnancy, irrespective of BMI being < 24.99 or > 25.00kg/m2, while sEng levels decreased at 22-30 weeks and increased at 32 weeks' gestation (Table 2). Concentration levels of sFlt-1 decreased consistently throughout pregnancy amongst those with BMIs > 25kg/m2, with a great increase noted at 22-30 weeks' gestation amongst those with BMIs <24.99 kg/m2 however levels declined at 32 weeks' gestation. The medians of selected clinical characteristics and angiogenic levels, stratified by HIV status, are presented (Table 4). Both systolic and diastolic blood pressure was greater in the HIV negative compared to the HIV positive participants. Median PlGF levels increased during 10-30 weeks' gestation but dropped at 32-38 weeks' gestation in the HIV -ve group whereas a progressive elevation was noted throughout the defined gestational intervals in the HIV +ve group. Higher PlGF levels, however, were observed in the HIV +ve group at 10-20 and 32-38 weeks' gestation respectively, in comparison to the HV -ve groups. Median levels for sFlt-1 increased progressively throughout the defined gestational intervals in the HIV –ve group, with a slight reduction shown in

the HIV +ve group. However, higher sFlt-1 levels were noted in the HIV +ve group at 10-20 weeks, with a prominent reduction at 22-30 and 32-38 weeks' gestation respectively, in comparison to the HIV-ve groups.

A slight variation was noted for sEng levels between the HIV –ve and HIV +ve groups, however, a more prominent pattern was noted throughout the defined gestational intervals. Whilst increased concentrations were notable at 32-38 weeks' gestation, there was a significant decline at 22-30 weeks' gestation. BMI however, remained unchanged in both the HIV–ve and HIV +ve groups (Table 4) while, the medians for haemoglobin levels and birth weight was slightly higher in the HIV –ve compared to the HIV +ve participants.

The correlations between the maternal serum levels of angiogenic/antiangiogenic factors of the defined gestational intervals and selected clinical characteristics is presented (Table 5). Despite the notable correlations observed in angiogenic profile for some clinical factors at the defined gestational intervals in our study, statistically significant correlations were undetectable for most factors (Table 5). Weak inverse associations were noted between maternal age and PlGF at 22-32 and 32-38 weeks' gestation respectively, in contrast to sEng. Maternal age was however, positively correlated (statistically significant) with the antiangiogenic ratio (sFlt-1/PlGF) at 32-38 weeks' gestation. Weak correlations were demonstrated between parity and aternal serum levels of angiogenic/antiangiogenic factors of the defined gestational intervals. A positive correlation of BMI (<24.99kg/m2) with antiangiogenic factor sEng, antiangiogenic ratios (sFlt-1/PlGF; (sFlt-1 + sEng)/PlGF), was observed at 32-38weeks gestation, but not statistically significant.

Despite the minimal increase noted in both systolic and diastolic blood pressure throughout the defined gestational intervals, an inverse and significant correlation was observed of systolic blood pressure and PlGF and sFlt-1 at 32-38 weeks' gestation. A positive and statistically significant correlation was observed between diastolic blood pressure and the antiangiogenic ratio (sFlt-1/PlGF) at 32-38 weeks' gestation. In addition, an inverse and statistically significant association was observed between birth weight and PlGF concentration at 22-30 weeks' gestation.

Discussion

This longitudinal study demonstrates the fluctuating angiogenic and antiangiogenic profile (PlGF, sFlt-1, sEng, ratios sFlt-1:PlGF and sFlt-1+sEng/PlGF) throughout specific gestational intervals in normotensive pregnant women. The antiangiogenic ratio sFlt-1/PlGF ratio is suggestive of higher sFlt-1 and lower PlGF levels and is accentuated as a dependable predictor of HDPs including PE than either protein alone (Levine et al 2006). Due to the growing association between circulating angiogenic anti-angiogenic factors in HDPs, it is necessary to comprehend their role across gestation and their association with maternal factors even in normotensive pregnancies. Hence, these profiles documented in our study may assist as a reference against which these angiogenic factors can be deliberated in complicated pregnancies. The clinical usefulness of angiogenic/anti-angiogenic profiles as predictors of HDP, such as preeclampsia arose in response to the revolutionary antiangiogenic study conducted by Maynard and coworkers (Maynard *et al.*, 2003).

Our study explored the combination of maternal risk factors and angiogenic profiles throughout the defined gestational intervals to establish a reference range in normotensive pregnancies. The clinical value of our data will assist in predicting the development of diseases such as PE in those pregnancies that may be at risk, and permitting timeous treatment, and subsequently improve both maternal and neonatal outcomes. Whilst our findings reflect a nominal yet visible increase in both systolic and diastolic blood pressure throughout the defined gestational intervals, it is impossible for any potential pathology to exist among normotensive women whose blood pressure does not increase further. Changes in diastolic blood pressure, was positively correlated with the maternal antiangiogenic ratio (sFlt-1/PlGF) at 32-38 weeks' gestation. All other correlations between diastolic blood pressure changes and PlGF, sFlt-1, and sEng did not reach statistical significance in these pregnancies (Table 5). The mean systolic blood pressure measurements were inversely correlated with PlGF and sFlt-1levels at 32-38 weeks' gestation. All other negative correlations were weak and did not reach statistical significance (Table 5). Similar angiogenic data was previously reported among normotensive pregnancies (Levine et al. 2006, Troisi *et al.,* 2008).

Previous studies report an increased risk for PE development in healthy nulliparous women in response to elevated systolic and diastolic blood pressures (Sibai *et al.,* 1997, Levine *et al.,* 2004). However, systolic and diastolic pressures at measured at 13-21 weeks' gestation in pre-eclamptic women with high levels of sFlt-1 was reported to be similar to those in normal, uncomplicated pregnancies (Levine *et al.,* 2006), indicative that amid those who have preeclampsia, there are some who have healthy endothelial cells and that PE development occurs in response to increased circulating sFlt-1 levels or mainly due to their increased susceptibility to vascular disorders. Thus, it is possible that even slight increases in blood pressure during the early mid trimester can result in u vascular damage. An earlier study documents the development of PE at much lower sFlt-1 concentrations in women with pregestational hypertension or diabetes mellitus, suggestive that these women enter pregnancy with pre-existing vascular disease (Cines *et al.,* 1998). Thus, variations in sFlt-1 during pregnancy may be valuable for PE identification and prediction in those who lack the conventional risk factors.

Both the angiogenic PlGF and anti-angiogenic factor sFlt-1 increased with advancing gestational age. Both PlGF and sFlt-1 increased during from 10-20 weeks and peaked between 30-38 weeks (Table 2). In contrast, sEng levels remained fairly constant around 10-30 weeks but peaked at 32-38 weeks' gestation. Our results which demonstrate an increase around 32-38 weeks' gestation concur with other longitudinal studies (Levine et al, 2004, Romero et al, 2008, Chaiwaporinga *et al.,* 2005). However, the increase appeared earlier in our study, at weeks 22–30 similar to Palm et al (2009), compared to week 32 (Chaiwaporinga *et al.,* 2005) and weeks 33–36 (Levine et al, 2004). The rising levels observed might be caused by a relative placental ischemia due to an increasing myometrial tone and more uterine contractions at the end of pregnancy and thus be a sign of uterine preparation for delivery (Bdolah et al 2004). Moreover, it is possible that the modest expression of PlGF during the 10-20 weeks' gestational interval in our study may be attributed to the preliminary vasculogenic phases and thereafter progressively increases after 22 weeks 'gestation since there is a switch from branching angiogenesis to nonbranching angiogenesis (Andraweera *et al.,* 2012). Furthermore, the rise in serum sFlt-1 levels as gestation progresses observed in our study, is consistent with that observed in several studies (Sundrani 2013, Palm, Romero, Espinozoa 2007),

suggestive that this elevation occurs in response to increasing placental ischemia and oxidative stress occurring during pregnancy (Palm, Redman & Sargen 2009]. This link to oxidative stress is corroborated by many Bukhari *et al.,* 2011; Mihailovic´ *et al.,* 2000; Ozkan 2011. It is also possible that this increased sFlt-1 concentration may indicate angiogenic restriction, and controlled permeability of blood vessel (Hastings et al, 2003; Ni *et al.*, 1997).

More recently, studies show that those who developed HDPs were initially asymptomatic at the time of blood sampling, ratifying that the rise of the antiangiogenic ratio heralds the clinical onset of the disease (Forest el al., 2014, Villa *et al.*, 2013; Leanos-Miranda *et al.*, 2012). It is therefore possible that late-onset PE development was probably too far-off to cause a rise the anti-angiogenic factors since collection of blood samples occurred prior to 32 weeks' gestation.

Our data suggests a correlation of maternal age with antiangiogenic ratio at 32-38 weeks' gestation

Which is consistent with that documented by Staff et al 2009. However, our data shows no link between these proteins with birth weight or haemoglobin levels, indicative that the circulating angiogenic alterations may have little or no effect on neonatal wellbeing, or that our sample size was too small.

However, our data has shown that hemaglobin levels were lower in the HIV +ve groups in comparison to the HIV –ve groups, despite not reaching significance. Anaemia in pregnancy is defined as when the haemoglobin level in peripheral blood is < 11g/dL (Buseri et al, 2008). In our study, the mean haemoglobin level was 11.78 and 10.63 g/dL in HIV negative and positive women respectively. Anaemia is classified according to WHO (Regil *et al.*, 2011; Grewal, 2010) into mild anaemia (10-10.09 g/dL), moderate anaemia (7.9.9) and severe aanaemia (<7g/dL). Thus, reduced levels of haemoglobin in pregnant women is correlated with a greater predisposition to maternal and perinatal mortality and low birth weight (Stevens *et al.*, 2013). Our study thus demonstrates the presence of mild anaemia in the HIV positive group. The recent commencement of HAART in HIV pregnant women, irrespective of their CD4 counts, is most likely to reduce the HIV related mortality (Sebitloane 2017). In our study, lower haemoglobin levels were noted in the HIV positive group, who are also on HAART. A recent report however, suggests that HAART has the potential to lower the prevalence and severity of anaemia (Sebitloane 2017). It is reported that mitochondrial toxicity and irregular reticulocyte counts are a common effect of the antiretroviral drug, zidovudine, which exacerbates the risk of anaemias (Sebitloane 2017). However, an earlier study report no difference in anaemia prevalence in those on HAART in comparison to those who received zidovudine alone during pregnancy (Nundlall *et al.*, 2014). This area of study however, requires further investigation.

In our study, the median serum levels of sFlt-1and sEng in nulliparous women declined between 22-30 week's gestation and increased at from 32 weeks onwards. Of note, is the reduction in serum sEng levels at 22-30 weeks' gestation and an elevation at 32 week's (Table 2). Nulliparity is a documented risk factor for PE development, however, it is unclear why nulliparous women exhibit higher angiogenic/antiangiogenic profiles (Duckitt and Harrington, 2005). Earlier reports have suggested that a higher ischemic placental environment is found in nulliparous women since their

spiral arteries have not been formerly remodeled (Karumanchi and Bdoloah, 2004). Hence, this increased hypoxic environment increases placental production of sFlt-1 and its subsequent release into the maternal circulation and influences a greater risk of PE development in first pregnancies (Karumanchi and Bdoloah, 2004). The elevated antiangiogenic state observed in our study at the 3rd gestational interval (32-38 weeks) is consistent with that reported by Bdolah *et al.,* (2014). These investigators emphasize the moderately anti-angiogenic state in their circulation during late third trimester in nulliparous women. Thus, it is possible that a modified angiogenic profile in nulliparous women is a possible epidemiological linkage between increased PE development and nulliparity. More recent data suggest that the sFlt-1:PlGF ratio at 36 weeks' gestation, pooled with maternal characteristics, may be clinically valuable in extrapolating the risk of PE development at term in unselected nulliparous women (Sovio *et al.,* 2017). This method of assessment may advance both maternal and perinatal outcomes.

Similarly, BMI is believed to be correlated with angiogenic balance even in uncomplicated pregnancies (Troisi et al 2008, Staff et al 2009). However, in our study the sFlt-1 levels decreased consistently throughout pregnancy amongst those with BMIs > 25kg/m2, however, a significant negative correlation was observed at -10-20 weeks. Studies comparing BMI at the initial prenatal consult and first and second trimester sFlt-1 levels and sFlt-1/PlGF ratio, revealed a positive correlation (Fauper-Badger *et al.,* 2011). This is inconsistent with or data, however, a major limitation in our study is the late antenatal access/admission of our pregnant women. Within the context of SA, antenatal care is poorly accessed during the 1st trimester, which is the reason for defining our gestational intervals as is. It is possible that some of our 2nd trimester data is colluded in this gestational age grouping. Although, Mijal *et al.,* (2011) also report and inverse correlation between second trimester circulating sFlt-1 levels with BMI in normotensive pregnancies (Mijal *et al.,* 2011).

Conclusion

Since circulating concentrations of angiogenic and anti-angiogenic factors change with gestational age as shown in our study and various others (refs), the longitudinal evaluations of sVEGFR-1, PlGF and sEng levels in a normotensive population may be useful in estimating the risk for HDPs such as PE.

Our data suggest that the angiogenic levels during pregnancy may be differentially adjusted based on the varying physiological maternal and fetal demands. The use of the anti-angiogenic profiles in evaluating the biophysical cardiovascular burden during pregnancy enhances the identification of a multifactorial group of HDPs, potentially reducing cardiovascular mortality in pregnant women (Verhloren et al 2017). Our study however, has limitations in that we had a small sample size, which reduced statistical power to explore the associations of clinical characteristics and circulating angiogenic profiles, which may have resulted in a lack of statistical significance for some real differences in angiogenic profiles by clinical characteristics. However, we did observe some significant correlations that should be confirmed in larger studies.

Acknowledgements

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Disclosure

We declare that there is no potential conflict of interest.

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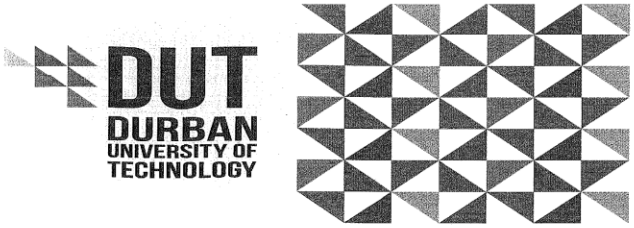
Appendix 2



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www.dut.ac.za

15 July 2014

IREC Reference Number: **REC 34/14**

Prof M N Sibiya
Department of Nursing
Faculty of Health Sciences
DUT

Dear Prof Sibiya

A multi-staged multi-disciplinary health care approach in reducing maternal morbidity and mortality rates in a selected district in KwaZulu-Natal

I am pleased to inform you that Provisional Approval subject to piloting of the data collection tools has been granted to your proposal REC 34/14.

The Proposal has been allocated the following Ethical Clearance number **IREC 045/14**. Please use this number in all communication with this office.

Approval has been granted for a period of one year, before the expiry of which you are required to apply for safety monitoring and annual recertification. Please use the Safety Monitoring and Annual Recertification Report form which can be found in the Standard Operating Procedures [SOP's] of the IREC. This form must be submitted to the IREC at least 3 months before the ethics approval for the study expires.

Any adverse events [serious or minor] which occur in connection with this study and/or which may alter its ethical consideration must be reported to the IREC according to the IREC SOP's. In addition, you will be responsible to ensure gatekeeper permission.

Please note that any deviations from the approved proposal require the approval of the IREC as outlined in the IREC SOP's.

Please note that you may continue with validity testing and piloting of the data collection tools. Research on the proposed project may not proceed until IREC reviews and approves the final documents. If there are no changes to the data collection tools, kindly notify IREC in writing.

Appendix 3

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

Health Research & Knowledge Management sub-component
10 – 103 Natalia Building, 330 Langalibalele Street
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Tel.: 033 – 3953189
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Email.: hrkm@kznhealth.gov.za
www.kznhealth.gov.za

Reference : HRKM 234/14
Enquiries : Mr X Xaba
Tel : 033 – 395 2805

Dear Prof MN Sibiya

Subject: Approval of a Research Proposal

1. The research proposal titled **'A multi-staged multi-disciplinary health care approach in reducing maternal morbidity and mortality rates in a selected district hospital in KZN'** was reviewed by the KwaZulu-Natal Department of Health.

   The proposal is hereby **approved** for research to be undertaken at Cato Manor for a period of three years.

2. You are requested to take note of the following:
   a. Make the necessary arrangement with the identified facility before commencing with your research project.
   b. Provide an interim progress report and final report (electronic and hard copies) when your research is complete.

3. Your final report must be posted to **HEALTH RESEARCH AND KNOWLEDGE MANAGEMENT, 10-102, PRIVATE BAG X9051, PIETERMARITZBURG, 3200** and e-mail an electronic copy to hrkm@kznhealth.gov.za

For any additional information please contact Mr X. Xaba on 033-395 2805.

Yours Sincerely

[redacted]

Dr E Lutge
Chairperson, Health Research Committee
Date: 11/09/14.

Appendix 4

DUT DURBAN UNIVERSITY OF TECHNOLOGY

FACULTY OF HEALTH SCIENCES

PROOF

MRC SOUTH AFRICA

APPROVED
DA RAJPAL
[redacted]
27/08/2015

Would you like to participate in our study?

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PROOF

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- Health assessments
- Antenatal care
- Dietary and nutritional advice

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PROOF

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www.dut.ac.za

[redacted]

Appendix 5

Letter of information

Welcome to our research study. Thank you for taking time to consider agreeing to participate in the study.

Title of the Study: A multi-staged multi-disciplinary health care approach in reducing maternal morbidity and mortality rates in a selected district in KwaZulu-Natal.

Principal Investigator: Prof MN Sibiya, D Tech: Nursing.

Co-investigators: Dr P Reddy, PhD and Prof T Puckree, PhD.

Briefly Introduction and Purpose of the Study: Maternal mortality rates continue to be an issue of concern despite improved healthcare. The province of KwaZulu-Natal suffers the highest rate of maternal deaths in South Africa. The purpose of this study is to focus on improving maternal health.

Outline of the Procedures: If you agree to take part in the study, I kindly request you to complete a form which will have a few questions regarding your pregnancy in general. Completion of this form should last for approximately fifteen to twenty minutes. The questions asked will be simple and straight forward information. You are also kindly requested to donate some blood specimens. Research assistants who are professional nurses will withdraw blood samples from you and these blood samples will be used only for this research study.

Discomforts to the Subject: There is no risk or discomfort that will because by partaking in the study.

Benefits: This study may contribute to a reduction in maternal morbidity and mortality rates by providing antenatal and nutritional interventions.

Reason/s why the Subject May Be Withdrawn from the Study: You will be allowed to opt out from the study or withdraw at any time should you wish to do so.

Remuneration: You will not be expected to pay anything for taking part in the study, and also no payment will be given to you for taking part in the study.

Confidentiality: All the information will be kept in strict privacy. Your name will not be written on the field notes with your responses. The information gathered will only be used for the purpose of this study.

Research-related Injury: The nature of the study does not have any risk of injury to you.

Persons to Contact in the Event of Any Problems or Queries:

Principal Investigator: Prof MN Sibiya, Durban University of Technology Tel: 031-373 2606

Co-investigators: Dr P Reddy, Durban University of Technology Tel: 031-373 2609

Co-investigators: Prof T Puckree, Durban University of Technology Tel: 031-373 2704

Institutional Research Ethics administrator, Durban University of Technology Tel: 031-373 2900

Complaints can be reported to the DVC: TIP, Prof F. Otieno on 031-373 2382 or dvctip@dut.ac.za.

CONSENT

Statement of Agreement to Participate in the Research Study:

I,…….(*Full name)*…….……..…. (*ID number*), have read this document in its entirety and understand its contents. Where I have had any questions or queries, these have been explained to me by ……..to my satisfaction. Furthermore, I fully understand that I may withdraw from this study at any stage without any adverse consequences and my future health care will not be compromised. I, therefore, voluntarily agree to participate in this study.

Name (print) ……..……

Signature: ……Date: ……..……..

Researcher's name (print): ……..…….

Researcher's signature: ……..……..Date :.....

Witness name (print): …….

Witness signature: …….....Date: ……..……..

Appendix 6

DUT DURBAN UNIVERSITY OF TECHNOLOGY

STUDY ID

2. EPIDEMIOLOGY 1ST TRIMESTER

Thank you for agreeing to be part of this study and for taking the time to fill out this questionnaire with us.

Please read this before starting.

- It's your choice whether or not to do the survey.
- Your answers will be kept **confidential**.
- Whether or not you answer the questions will **not** affect your health care or any benefits you may get.
- You can skip questions you don't want to answer.
- Please put a cross (X) next to your chosen answer.

Recruitment Assistant Name Date	
What is your marital status?	☐1 Married ☐2 Living together ☐3 Single ☐4 Divorced ☐5 Separated ☐6 Widow ☐7 Other _____
Just before I became pregnant… **(Please tick only one)**	☐1 I wanted to have a baby ☐2 I had mixed feelings about having a baby ☐3 I did not want to have a baby
When you got pregnant with your new baby, were you trying to get pregnant?	☐1 Yes ☐2 No
Was this pregnancy forced?	☐1 Yes If yes, please comment; ☐2 No
Have you/your partner at any time during the last year used the following methods to avoid becoming pregnant? ***(Fill in all that apply)***	☐1 Implant ☐2 Injection ☐3 Pill ☐4 Traditional methods ☐5 Condom

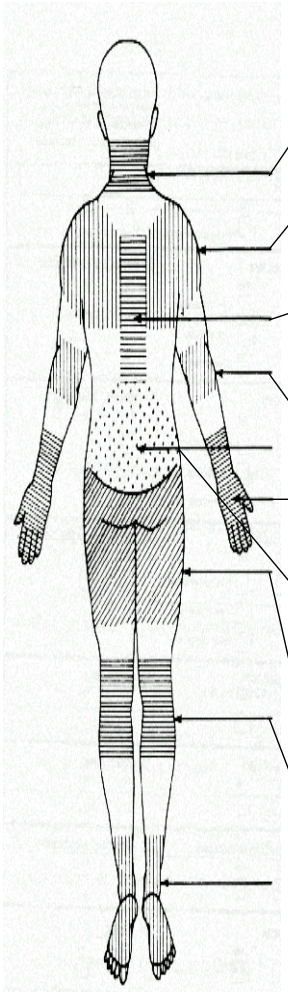
	☐6 IUD ☐7 Withdrawal ☐8 Spermicides (foam, suppositories, cream) ☐9 Safe period ☐10 Withdrawal ☐11 No such methods ☐88 Other If other, please specify:
Did you use any home remedies to stop your pregnancy?	☐ Yes ☐ No If yes, please specify:
What were your reasons for not using a contraceptive? **Check all that apply.**	☐1 I didn't mind if I got pregnant ☐2 I thought I could not get pregnant at that time ☐3 I had side effects from the birth control method I was using ☐4 I had problems getting birth control when I needed it ☐5 I thought my husband or partner or I was sterile (could not get pregnant at all) ☐6 My husband or partner didn't want to use anything ☐ Religious purposes/beliefs ☐7 Other _____
What age did you become sexually active?	Age:
What age did you first use contraception?	Age:
Have you ever heard of emergency contraception (EC) or the morning after pill (MAP) before?	☐1 Yes ☐2 No
Have you ever used EC/MAP before?	☐1 Yes ☐2 No
If Yes, how many times have you used EC/MAP in the year previous to you falling pregnant?	☐1 Once ☐2 2-4 times ☐3 5-10 times ☐4 over 10 times Other_____
What are the time frames to use EC?	☐1 within 12 hours of sexual intercourse ☐2 within 24 hours of sexual intercourse ☐3 within 3 days of sexual intercourse ☐4 within a week of sexual intercourse ☐5 Other _____ ☐ I don't know
How many months pregnant were you when you discovered you were pregnant?	_____months
How many months pregnant were you when you had your first antenatal visit?	_____months _____I don't know
Did you receive antenatal care as soon as you found out you were pregnant?	☐1 Yes – From where? ☐2 No – Please comment why not?
Did any of these things keep you from getting antenatal care at all or as early as you wanted? (You may choose more than one reason, if applicable)	☐1 I couldn't get an appointment when I wanted one. ☐2 I had no money for transportation to get to the clinic. ☐3 I had too many other things going on. ☐4 I was trying to hide this pregnancy from my family ☐5 I didn't know that I was pregnant ☐6 I didn't want anyone else to know I was pregnant ☐7 I didn't want antenatal care ☐8 I could not take time off from work ☐9 I had no one to take care of my other children

How many times have you been pregnant (Include all pregnancies that ended in abortion, miscarriage or stillbirth)	_____pregnancies
Did you experience any of the following with your ***previous pregnancies*** (if yes, then state number of times next to it)	☐$_1$ Abortion Number ___ ☐$_2$ Miscarriage Number___ ☐$_3$ Stillbirth Number ___ ☐$_4$ Early neonatal deaths Number___ ☐$_5$ Ectopic Pregnancy Number___
How many live children do you currently have?	_____ Ages_____ _____
Have you had any of the following problems during ***previous pregnancies?*** *(Tick all that apply.)*	☐ Serious nausea and vomiting ☐ Hypertension ☐ Threatened abortion ☐ Diabetes during pregnancy ☐ Sugar in urine ☐ Problems with incontinence ☐ Bleeding from the vagina ☐ Other:
Do you know what a pap smear is?	☐$_1$ Yes ☐$_2$ No
Have you ever had a pap smear?	☐$_1$ Yes ☐$_2$ No
How often have you had a pap smear?	☐$_1$ Once a year ☐$_2$ Once in 2 years ☐$_3$ Once in 3-5 years ☐$_4$ Don't remember
Why is it important to have a pap smear?	_____ _____
Select all the conditions that you were treated by a doctor for **before you became pregnant?**	☐ Diabetes ☐ Heart problems ☐ High cholesterol ☐ High blood pressure ☐ Hypothyroidism ☐ Hyperthyroidism ☐ Anaemia ☐ Ulcers – if so, please state where… ☐ Cancer ☐ TB ☐ Endometriosis ☐ Ovarian cysts ☐ Epilepsy ☐ HIV ☐ Stress – please comment… ☐ Depression/Anxiety ☐ Fainting attacks ☐ Fits ☐ Headaches ☐ Neck pain ☐ mid back pain (pain between the shoulders) ☐ Low back pain ☐ Pelvic pain (pubic or groin) ☐ Hip pain ☐ Knee pain

	☐ Foot and ankle pain ☐ Shoulder pain ☐ Elbow pain ☐ Wrist and hand pain		
Are you currently taking any medication?	☐$_1$ Yes ☐$_2$ No		
If yes, what is the medication for, where did you get it from and how often are you taking it?	Medication for?	Where from?	How often?
	_____ _____ _____ _____ _____ _____ _____	_____ _____ _____ _____ _____ _____ _____	_____ _____ _____ _____ _____ _____ _____
Did you use medications before becoming pregnant?	☐$_1$ Yes ☐$_2$ No		
Please indicate which of these medications you have used (at a frequency of more than once) over the 12 months **prior to your pregnancy?**	☐ Antibiotics ☐ Pain Killers ☐ Immune Boosters ☐ ARV ☐ TB Drugs ☐ Isihlambezo ☐ Chronic medication ☐ Traditional Medication ☐ Other Please Specify_____		
Are you currently taking any of the medications indicated above?	☐$_1$ Yes ☐$_2$ No How often? _____		
Have you used any recreational drugs prior to your pregnancy?	☐$_1$ Yes ☐$_2$ No How often? _____		
Do you use any of these drugs now during your pregnancy?	☐ Dagga ☐ Wonga ☐ heroine ☐ cocaine ☐ sugars ☐ other…		
Have you been smoking/using snuff while pregnant?	☐ No ☐ Sometimes ☐ Daily		
Do you smoke?	☐ No ☐ Yes If yes, how many _____ cigarettes per week _____ cigarettes per day		
Have you ever had alcohol?	☐$_1$ Yes ☐$_2$ No		
How often did you drink alcohol in the 3 months before you became pregnant?	☐ Approximately 6-7 times a week ☐ Approximately 4-5 times a week ☐ Approximately 2-3 times a week ☐ Approximately once a week ☐ Approximately 1-3 times a month		

	☐ Less than once a month ☐ Never
How often do you consume alcohol during this pregnancy?	☐ Approximately 6-7 time a week ☐ Approximately 4-5 times a week ☐ Approximately 2-3 times a week ☐ Approximately once a week ☐ Approximately 1-3 times a month ☐ Less than once a month ☐ Never
What type of alcohol do you usually drink? *(Fill in one or several boxes.)*	☐ Homemade/traditional beer ☐ Purchased traditional Beer ☐ Wines ☐ Ciders ☐ Spirits (*vodka, gin, whisky, liqueur)* ☐ Other specify
How many hours sleep do you currently get a night?	☐ Less than 4 hours per night ☐ 4 – 8 hours per night ☐ All night
Are you experiencing any pregnancy related cravings?	☐ Yes ☐ No Please specify? _____
Do you do any of the following activities on a daily basis?	☐ Carry water ☐ Walk long distances ☐ Gardening work ☐ House work ☐ Manual labour/lifting ☐ Carry Children Other:_____
Do you currently have musculoskeletal (bone/muscle/joint) pain?	☐ Never ☐ Rarely ☐ Sometimes ☐ Very Often ☐ Always
Did you experience pain in a previous pregnancy?	☐ Yes ☐ No If yes, where?
Only answer the following questions if you have indicated that you have musculoskeletal pain **during pregnancy**	
Has the pain prevented you from spending time with your family and friends?	☐ Yes ☐ No
Has the pain made you feel concerned or worried about your health?	☐ Yes ☐ No
Has the pain made you feel sad or down?	☐ Yes ☐ No
Are you able to cope with the pain?	☐ Yes ☐ No

How have you treated your pain?	☐ Medication: specify_____ ☐ Just living with it ☐ Other comment:



Have you experienced MSK pain in any of the areas in the during your pregnancy:		When did the pain starts	Is your pain:	Has your pain interfered with your ability to perform your daily activities, such as gardening, house work etc.	Has the pain affected your ability to work?
Neck	☐ Yes ☐ No	☐ 2 weeks ago ☐ 3-8 weeks ago ☐ 3-6 months ago ☐ + 6 months	☐ Mild ☐ Moderate ☐ Severe	☐ Yes ☐ No If yes comment _____	☐ Yes ☐ No If yes comment _____
Shoulders	☐ Yes ☐ No	☐ 2 weeks ago ☐ 3-8 weeks ago ☐ 3-6 months ago ☐ + 6 months	☐ Mild ☐ Moderate ☐ Severe	☐ Yes ☐ No If yes comment _____	☐ Yes ☐ No If yes comment _____
Upper Back	☐ Yes ☐ No	☐ 2 weeks ago ☐ 3-8 weeks ago ☐ 3-6 months ago ☐ + 6 months	☐ Mild ☐ Moderate ☐ Severe	☐ Yes ☐ No If yes comment _____	☐ Yes ☐ No If yes comment _____
Elbows	☐ Yes ☐ No	☐ 2 weeks ago ☐ 3-8 weeks ago ☐ 3-6 months ago ☐ + 6 months	☐ Mild ☐ Moderate ☐ Severe	☐ Yes ☐ No If yes comment _____	☐ Yes ☐ No If yes comment _____
Wrist/ Hands	☐ Yes ☐ No	☐ 2 weeks ago ☐ 3-8 weeks ago ☐ 3-6 months ago ☐ + 6 months	☐ Mild ☐ Moderate ☐ Severe	☐ Yes ☐ No If yes comment _____	☐ Yes ☐ No If yes comment _____
Low Back	☐ Yes ☐ No	☐ 2 weeks ago ☐ 3-8 weeks ago ☐ 3-6 months ago ☐ + 6 months	☐ Mild ☐ Moderate ☐ Severe	☐ Yes ☐ No If yes comment _____	☐ Yes ☐ No If yes comment _____
Hips/ Thighs	☐ Yes ☐ No	☐ 2 weeks ago ☐ 3-8 weeks ago ☐ 3-6 months ago ☐ + 6 months	☐ Mild ☐ Moderate ☐ Severe	☐ Yes ☐ No If yes comment _____	☐ Yes ☐ No If yes comment _____
Knees	☐ Yes ☐ No	☐ 2 weeks ago ☐ 3-8 weeks ago ☐ 3-6 months ago ☐ + 6 months	☐ Mild ☐ Moderate ☐ Severe	☐ Yes ☐ No If yes comment _____	☐ Yes ☐ No If yes comment _____
Ankles/ Feet	☐ Yes ☐ No	☐ 2 weeks ago ☐ 3-8 weeks ago ☐ 3-6 months ago ☐ + 6 months	☐ Mild ☐ Moderate ☐ Severe	☐ Yes ☐ No If yes comment _____	☐ Yes ☐ No If yes comment _____

Thank you for taking the time to complete this questionnaire.

Appendix 7

CHART REVIEW

Study ID : _____

CRITERIA	
Age	
Race	
Parity	
Gravida	
HIV status	
History previous pregnancy	
• Anemia	
• Congenital abnormalities	
• Eclampsia	
• PPH	
• APH	
• Other. State……..	
Chronic medical conditions	
• HIV and AIDS	
• TB	
• Diabetes	
• Hypertension	
• Other, State……..	
Current antenatal data:	
• Last normal menstrual period	
• Estimated Delivery Date	
• Gestational age at booking (weeks)	
• Current gestational age	
• Ultrasound done during this pregnancy	
• Maternal height	
• Weight	
• BP (each visit)	
• Maternal Heart examination	
• Urinalysis test	
• Hemoglobin test	
• Rh factor test	
• Calcium supplements given	
• Folic supplements given	
• Date for 2nd Trimester Visit	
• Date for 3rd Trimester Visit	

Appendix 8

DUT DURBAN UNIVERSITY OF TECHNOLOGY

STUDY ID

7: BIRTH OUTCOMES & POSTNATAL QUESTIONNAIRE

Delivery

Date of delivery: .....

Mode of delivery: Normal | C/section | Assisted

Place of delivery: Clinic | Hospital | Home | On the way to hospital/clinic

Complications during Labour: No | Yes cify: .....

Birth weight

APGAR (1 min)

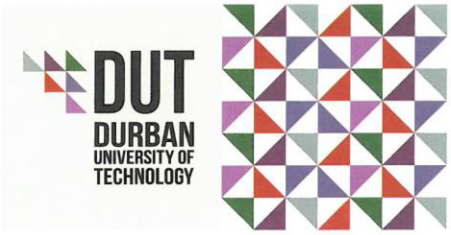
APGAR (5min)

	YES	NO
Condition of mother Are you feeling well post-delivery? Are you still taking prenatal vitamins? Have you been tested for HIV? Are you on PMTCT programme?		
Condition of baby at birth Was your bay delivered at term? Was your baby kept in an incubator post-delivery? Is your baby alive? Did your baby cry well at birth (state apgar score if known) Did your baby require being resuscitated immediately post-delivery? Was your baby admitted into nursery post-delivery? Is your baby on PMTCT programme?		

Post natal check-up (mother) Have you had a post natal check-up: 1. Within three days 2. 6 Weeks post delivery 3. Any other time since delivery		
Child care How are you feeding baby? 1. breastfeeding only, 2. supplementing with formula, 3. formula feeding only Are you having difficulty with Breast feeding? Please explain……		
Post natal check-up (baby) Have you had a post natal check-up for your baby: 1. Within three days 2. 6 Weeks post delivery 3. Any other time since delivery		
Immunisation status Has your baby received immunizations 1. At birth 2. Six Weeks Post Delivery		
Signs of illness Have you had any of the following problems since delivery: 1. Breathing problems, 2. Pain with urination, 3. Fever or chills, 4. Vaginal discharge, 5. Vomiting, 6. Diarrhea, 7. Excessive tiredness, 8. Abdominal pain, 9. Depression 10. Bleeding longer than 4 weeks		
Social habits Do you smoke cigarettes? Are you taking alcohol? Are you taking any dependency producing drugs?		
Emotional Health **1.** Do you feel like you are under stress? 2. Have you been having any mood swings? 3. Are you mentally depressed?		
Family support Do you need help with child care? Are you getting any assistance from your family Are you satisfied with the assistance that you are getting?		

Current Medication Are you currently being treated for any of the following conditions? 1. Infections, 2. TB 3. HIV, 4. Chronic Medical Conditions, 5. Mental Health Problems. 6. Was the medicine you are taking 7. Prescribed by a doctor, 8. Over-the counter medications, 9. Herbal or alternative medicines		

Appendix 9



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www.dut.ac.za

17 October 2016

IREC Reference Number: **REC 102/16**

Dr M O Ogunlola
Flat 619
London House
354-358 West Street
Durban
4001

Dear Dr Ogunlola

Circulating levels of pro-angiogenic and anti-angiogenic biomarkers as predictors for development of preeclampsia in a cohort of pregnant women aged 15-45 years in KwaZulu-Natal

The Institutional Research Ethics Committee acknowledges receipt of your notification regarding the piloting of your data collection tool.

Kindly ensure that participants used for the pilot study are not part of the main study.

In addition, the IREC acknowledges receipt of your gatekeeper permission letter.

Please note that FULL APPROVAL is granted to your research proposal. You may proceed with data collection.

Yours Sincerely,

[redacted]

Professor J K Adam
Chairperson: IREC