Quantification of Circulating Cell Free Fetal DNA and Cell Free Total DNA in Normal Pregnancy and in Pregnancy-Related Hypertension in Black South African Women

By

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Supervisor

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PREFACE

The study described in this dissertation was carried out by Mr. Simeon Eche and has not been submitted in any other form to another University. This study was carried out in the School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa from July 2015 to July 2016 under the supervision of Prof Irene Mackraj and Prof Jagidesa Moodley.

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Date 11th November 2017______
(215000273)

Professor Irene Mackraj

Supervisor _______________________
Date___________________________

Professor Jagidesa Moodley

Co-supervisor _______________________
Date___________________________
DECLARATION

I, Mr. Simeon Eche declare as follows:

(i) The research reported in this dissertation, except where otherwise indicated is my original work.
(ii) This dissertation has not been submitted for any degree or examination at any other University.
(iii) This dissertation does not contain other person’s data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
(iv) This dissertation does not contain other persons writing, unless specifically acknowledged as being sourced from other researchers. Where other sources have been quoted, then: Their words have been rewritten but the general information attributed by them has been referenced.

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Signed: ___________________________ Date: 11th November 2017
DEDICATION

This research project is dedicated to:

- God almighty, my source of inspiration and strength.
- My late mother; Mrs Patricia Adah.
- My late class mate and sister, Oteh Anyalewa Gloria.
ACKNOWLEDGEMENTS

I am grateful to God for His guidance, divine inspiration, and Wisdom that brought the existence of this research work.

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I wish to specially express my deep and profound gratitude to my family for their love and support all through my study.

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PUBLICATION

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# ABBREVIATIONS AND SYMBOLS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>BREC</td>
<td>Biomedical Research Ethics Committee</td>
</tr>
<tr>
<td>Bsh 1236I (BSTUI)</td>
<td>Restriction enzyme</td>
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<tr>
<td>cfDNA</td>
<td>Cell Free DNA</td>
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<td>cffDNA</td>
<td>Cell Free Fetal DNA</td>
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<td>cftDNA</td>
<td>Cell Free Total DNA</td>
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<td>DSCR3</td>
<td>Down Syndrome Critical Region 3</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetra-acetic Acid</td>
</tr>
<tr>
<td>EOPE</td>
<td>Early Onset Preeclampsia</td>
</tr>
<tr>
<td>GE</td>
<td>Genomic Equivalence</td>
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<tr>
<td>GH</td>
<td>Gestational Hypertension</td>
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<td>HDP</td>
<td>Hypertensive Disorder of Pregnancy</td>
</tr>
<tr>
<td>HELLP</td>
<td>Hemolysis Elevated Liver Enzyme and Low Platelet</td>
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<tr>
<td>LOPE</td>
<td>Late Onset Preeclampsia</td>
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<tr>
<td>MI</td>
<td>Milliliter</td>
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<tr>
<td>MPE</td>
<td>Mild to Moderate Preeclampsia</td>
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<td>NIPD</td>
<td>Non-Invasive Prenatal Diagnosis</td>
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<td>PIH</td>
<td>Pregnancy Induced Hypertension</td>
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<td>PE</td>
<td>Preeclampsia</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>RASSF1A</td>
<td>Ras Association Domain Family - 1 isoform A</td>
</tr>
<tr>
<td>RhD</td>
<td>Rhesus D</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real Time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>sENG</td>
<td>Soluble Endoglin</td>
</tr>
<tr>
<td>sFlt-1</td>
<td>Soluble Fms-like Tyrosine Kinase-1</td>
</tr>
<tr>
<td>SPE</td>
<td>Severe Preeclampsia</td>
</tr>
<tr>
<td>SRY</td>
<td>Sex-determining Region Y</td>
</tr>
<tr>
<td>TSPY</td>
<td>Testis Specific Protein-Y</td>
</tr>
<tr>
<td>T-test</td>
<td>A type of statistical test</td>
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<tr>
<td>µl</td>
<td>Microliter</td>
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<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<tr>
<td>VEGF-R1</td>
<td>Vascular Endothelial Growth Factor Receptor-1</td>
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<tr>
<td>≥</td>
<td>Greater and equal to</td>
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<td>Less than and equal to</td>
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ABSTRACT

Background

Hypertensive disorders are major complications of pregnancy and are the commonest direct cause of maternal death in South Africa. The objective was to quantify the level of circulating cell free fetal DNA (cffDNA) and cell free total DNA (cftDNA) in Black South African women with gestational hypertension and preeclampsia.

Methods

This study is a prospective cross-sectional study involving 39 normotensive pregnant women (controls), 37 women with preeclampsia (PE) and 23 with gestational hypertension (GH), all recruited during the antenatal period from a regional health care facility, South of Durban, KwaZulu-Natal province, South Africa. Preeclampsia was classified according to the guidelines of the International Society for the Study of Hypertension in Pregnancy (ISSHP) into severe preeclampsia (SPE), mild to moderate preeclampsia (MPE), early-onset preeclampsia (EOPE) and late-onset preeclampsia (LOPE). Both cffDNA and cftDNA were quantified using real-time qPCR by exploring the difference in methylation pattern of the RASSF1A gene promoter in maternal plasma.

Results

The levels of circulating cffDNA and cftDNA were significantly higher in patients with PE compared to the levels in the controls and in patients with GH (p<0.001). There was no alteration in the level of cffDNA and cftDNA in GH compared to controls (p > 0.05). There was a significant increase in the level of cffDNA and cftDNA in SPE in contrast to the concentration in MPE (p<0.05). There was no difference in the level of cffDNA and cftDNA in EOPE compared to LOPE (p > 0.05).

Discussion/Conclusion

The use of RASSF1A gene as a universal fetal DNA marker has helped improve non-invasive prenatal diagnosis and monitoring of pregnancy complications. Our results indicate that circulating cffDNA and cftDNA are elevated in preeclampsia and consideration needs to be given for it to be used as a marker. The level of severity of PE may be a factor that influences the level of these markers in maternal plasma.
CHAPTER 1: BACKGROUND AND LITERATURE REVIEW

1.1 Background

Pregnancy-induced hypertension (PIH) such as gestational hypertension, preeclampsia, superimposed preeclampsia and eclampsia is an abnormal elevation in blood pressure diagnosed after the 20th week of pregnancy, with or without the presence of protein in the urine (Watanabe et al., 2013). The cause of hypertension in pregnancy is unknown and it’s a great health concern in both developing and advanced economies (Hutcheon et al., 2011).

The magnitude of the burden caused by hypertensive disorders of pregnancy (HDP) is felt more so by developing economies, where it accounts for most of the maternal and fetal death recorded globally (Osungbade and Ige, 2011). This is because most developing countries lack basic health care facilities, budgetary allocation to the health sector is not adequate, and lack access to timely and efficient prenatal care (Ghulmiyyah and Sibai, 2012). For a long time, it has been the priority of the United Nations to reduce maternal death, but this goal is still far from been achieved in developing countries (Say et al., 2014).

PIH such as gestational hypertension (GH) and preeclampsia (PE) are common obstetrical complications of gestation. These conditions are a leading cause of maternal morbidity and mortality (Sibai, 2011). These disorders are associated with preterm delivery, maternal and fetal mortality (Barra et al., 2012).

The development of the placenta in humans is a complex and highly regulated process involving the invasion of the uterus by cells of the cytotrophoblast, remodelling the placental vasculature and converting them into large conduits (Eastabrook et al., 2011). Spiral artery remodelling is defective in PE. It is believed that this aberration in placentation occurs in early stages of pregnancy leading to diminished blood flow to the placenta, followed by ischaemia of the placenta (Naljayan and Karumanchi, 2013); releasing placental derived particles/factors. These placenta-derived microparticles, factors and or debris are able to trigger an exaggerated inflammatory response as seen in obstetric complications like preeclampsia (Redman and Sargent, 2001). In particular, endothelial dysfunction is seen as a hallmark of PE, triggered by this inflammatory response (Tannetta and Sargent, 2013).

Other factors implicated in the development of preeclampsia include alterations in the circulating level of pro-inflammatory substances, damage to vascular endothelium and placental hypoxia. Delivery is considered a remedy as these conditions resolve with delivery of the placenta (Magee et al., 2014, Mol et al., 2016).
1.1.1 Prevalence

Globally HDP is responsible for about 62,000 to 77,000 maternal deaths yearly, which is about 18% of all maternal deaths (Abalos et al., 2013). Most of the cases of HDP are seen in low-income countries (Abalos et al., 2013). The risk of a woman dying from hypertensive complications during pregnancy is about 1 in 3800 women in the developed countries compared to the figure in Sub-Saharan Africa which is very high, viz. 1 in 39 (Abalos et al., 2013).

In South Africa, HDP continues to be the most dominant cause of maternal death (Saving Mothers Report, 2015). There was a total of 4452 maternal deaths recorded in South Africa from 2011 – 2013, and maternal deaths due to hypertensive disorders of pregnancy was 14.8 percent of this total figure at a national level. In the KwaZulu-Natal Province, hypertensive disorders of pregnancy accounted for about 8.3 percent of maternal deaths (Saving Mothers Report, 2015).

1.1.2 Risk factors of hypertension in pregnancy

Certain risk factors are known to predispose women to develop hypertension in pregnancy. These factors include previous history of hypertension, vascular disease, and renal complications, obesity, and the presence of medical conditions such as diabetes (Villar et al., 2006). Also, other known risk factors of hypertension in pregnancy include; nulliparity, an age of ≥ 40 years, a long interval between pregnancies, and multiple gestations (Lin et al., 2015, Villar et al., 2006).

Factors that predispose women to develop preeclampsia include genetic factors such as family history of the disease (Lin et al., 2015, Powe et al., 2011). The change in paternity from a previous pregnancy and the use of barrier contraception resulting in limited exposure to paternal antigens, predisposes women to preeclampsia (Powe et al., 2011).

1.1.3 Pathogenesis of hypertension in pregnancy

The mechanism of development of PIH remains elusive despite the large amount of resources dedicated to the study of these conditions. Research has shown that the underlying etiological factor responsible for the development of PIH is diminished uteroplacental blood flow resulting from impaired trophoblast invasion of the placental spiral arteries (Barra et al., 2012).

The diminished placental blood flow results in ischaemia of placenta cells followed by the destruction of these cells. The contents of these necrotic cells are released into the circulation leading to stimulation of the maternal vascular endothelium and its subsequent dysfunction. (Barra et al., 2012, Shah, 2007). Oxidative
stress resulting from dysfunction of the maternal vascular endothelium also contributes to the model of target organ injury seen in conditions like preeclampsia (Shah, 2007). More recently more defined particles have been identified such as microvesicles (Tannetta and Sergent, 2013) impacting on the endothelium.

The development of preeclampsia occurs in stages (Figure 1); viz. the pre-clinical stage and the clinical stage (Tannetta and Sargent, 2013). The first stage (the preclinical stage) of the disease, which is characterised by poor placentation, shallow invasion of cytotrophoblast cells, abnormal remodelling of the placenta spiral artery and reduced placental perfusion (Tannetta and Sargent, 2013). As a result of the abnormally remodelled spiral arteries, the placenta becomes exposed to oxidative stress, (Redman, 2011), and destruction of the syncytial architecture occurs.

Figure 1. Diagrammatic representation of the stages of pre-eclampsia. Abnormal remodelling of spiral artery which results from poor placentation causes release of placental particles or factors responsible for initiation of a systemic inflammatory response and dysfunction of the maternal vascular endothelium which manifests as the clinical signs seen in PE (Borzychowski et al., 2006).

The second stage of preeclampsia (clinical stage) develops from the destruction of the syncytial architecture, which causes the release of cellular content into maternal circulation (Granger et al., 2001, Steegers et al., 2010); a concept which has been promulgated over the years. Features of the clinical stage
of preeclampsia include increased peripheral vasoconstriction while arterial compliance is diminished (Tannetta and Sargent, 2013). Molecules released by the stressed placenta include "soluble vascular endothelial growth factor receptor-1 (VEGFR1 or the sFlt-1) and soluble endoglin (sENG)" (Redman, 2011). These molecules are known to affect the orientation of the fenestrated endothelium for which vascular endothelial growth factor (VEGF) is important for its integrity (Redman, 2011). The end result is the disorientation of the fenestrated endothelium resulting in protein leakage into the urine (Craici et al., 2013). Podocytes are pathologically affected and released into the urine of preeclamptic patients (Craici et al., 2013).

This concept of an ischemic placenta, resulting in the release of cellular debris, is currently evolving to include particles such as microvesicles, fetal DNA and exosomes. They play a significant role in initiating systemic inflammatory response and endothelial dysfunction (Tannetta and Sargent, 2013).

1.1.3.1 The role of angiogenic and antiangiogenic molecules in preeclampsia

Ischemia of the placental cells results in them secreting soluble factors in to circulation, these factors which are anti-angiogenic may cause maternal endothelial dysfunction (Tanneta and Sargent, 2013). Disruption in the balances between angiogenic and antiangiogenic factors is critical to the pathogenesis of PE. Two antiangiogenic factors, soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng) have been shown to play a great role in the pathogenesis of PE (Redman, 2011).

The anti-angiogenic molecule sFlt-1 is a splice variant of the membrane bound VEGF receptor Flt1, it is mainly secreted and released into the circulation by syncytiotrophoblast cells (Redman and Sargent 2001). Elevated levels of sFlt-1 is associated with diminished placental growth factor (PLGF) and vascular endothelial growth factor (VEGF) signalling. Soluble fms-like tyrosine kinase-1 prevents VEGF and PLGF from carrying out their normal function by binding to them in circulation and inhibiting their interaction with receptors meant for their function (Goulopoulou and Davidge, 2015).

Another peptide molecule indicted in the pathogenesis of PE is soluble endoglin (sEng), it is an antiangiogenic protein. This molecule is highly expressed on endothelial cell membranes, syncytiotrophoblast cells and invading cytotrophoblast cells (Powe et al., 2011). Soluble
endoglinng may combine with sFlt-1 and cause amplification of vascular injury (Powe et al., 2011).

1.1.4 Classification of pregnancy induced hypertension

PIH is classified into the following categories; preeclampsia, gestational hypertension, superimposed preeclampsia and eclampsia (Watanabe et al., 2013).

1.1.4.1 Preeclampsia

Preeclampsia is a HDP that occurs after the 20th week of gestation. It is characterised by a systolic blood pressure ≥ 140mmHg and or a diastolic blood pressure ≥ 90mmHg along with proteinuria (≥ 0.3g in a 24-hour urine sample) (Magee et al., 2014). It is accountable for most of the maternal and fetal deaths caused by hypertensive disorders of pregnancy in developing countries (Mol et al., 2016).

Women with preeclampsia if not given adequate medical attention will develop severe conditions such as seizures which lead to coma (Souza et al., 2013). Preeclampsia is also associated with conditions like spontaneous abortion, fetal growth restriction, and preterm delivery (see review by (Mol et al., 2016).

Preeclampsia impacts on almost all organ systems and causes devastating changes in the intrauterine milieu by modifying hormonal signalling patterns, and causes activation of unfavourable cellular signalling detrimental to the development of the fetus (Goulopoulou and Davidge, 2015).

1.1.4.2 Gestational hypertension

GH is new onset hypertension in pregnant women who were previously normotensive prior to becoming pregnant, after the 20th week of gestation (Cruz et al., 2012). This HDP is characterised by a blood pressure of ≥ 140/90 mmHg, presenting without proteinuria (Tranquilli et al., 2014).

Some authors have stated that GH, may share similar risk factors and pathologic features with PE, but is still a separate condition (Li et al., 2016, Villar et al., 2006). Others have pointed out that GH is likely to progress to PE (Romero-Arauz et al., 2014, Saudan et al., 1998, Sibai, 2003).

The dangers of the complications associated with this disorder are dependent on the maternal age of gestation at onset, as well as the maternal and fetal health at the point when the diagnosis is made (Sibai, 2011). Development of GH during pregnancy is a warning that a woman may develop essential hypertension in the future (Cruz et al., 2012).
1.1.4.3 **Superimposed preeclampsia**

This is new onset proteinuria in women with hypertension which predates pregnancy but no proteinuria preceding the 20th week of pregnancy (Watanabe *et al.*, 2013). Superimposed preeclampsia may develop from already existing chronic hypertension, and this condition is connected with grave maternal and fetal complications in pregnancy. Most cases of superimposed preeclampsia occur at term (Magee *et al.*, 2014).

Superimposed preeclampsia may also be triggered by renal complications before the 20th week of pregnancy, which is complemented by the development of new onset proteinuria and elevation of blood pressure above normal after the 20th week of pregnancy (Watanabe *et al.*, 2013).

1.1.4.4 **Eclampsia**

Severe forms of PE may result in eclampsia, this is the onset of convulsive seizures in a pregnant woman with hypertension induced by pregnancy. The Manifestation of these seizures may occur before labour, during labour or after labour (Watanabe *et al.*, 2013). The mechanism of development of eclamptic seizures still remains to be proven. However factors thought to be responsible for eclamptic seizures include cerebral vasospasm and cerebral oedema amongst other factors (Sibai, 2003).

Eclampsia accounts for the high incidence of maternal mortality in low-resource countries. Complications arising as a result of eclampsia are more severe in women who develop this disorder during the antepartum period, especially where it occurs distant to term (Ghulmiyyah and Sibai, 2012).

1.1.5 **Sub classification of Preeclampsia**

Preeclampsia (PE) can be sub classified based on severity and onset (Watanabe *et al.*, 2013). Based on severity, it is classified as follows;

I. Mild PE which is characterised by a blood pressure of ≥ 140/90 mmHg but less than 160/110 mmHg, and proteinuria of ≥ 300mg but not more than 2.0g in a 24-hour urine sample (or 2 pluses (+) on a dipstick) (Watanabe *et al.*, 2013).

II. Severe PE is characterised by a blood pressure ≥ 160/110 mmHg and urinary protein concentration > 2.0g in a 24-hour urine sample (or 3 pluses (+) on a dipstick) (Watanabe *et al.*, 2013).

Based on onset, early onset PE occurs before 34 weeks (≤ 33 weeks 6 days) of pregnancy. Late onset PE is PE at 34 or after 34 weeks post conception (Lisonkova and Joseph, 2013).
1.2 Cell free DNA and Cell free fetal DNA

Circulating cell free DNA (cfDNA) is extracellular DNA found circulating freely in plasma (Barra et al., 2015). Circulating cfDNA in human plasma was first detected by Mandel and Metais (Mandel, 1948), which was even before DNA was described as a double helix by Watson and Crick (Watson and Crick, 1953). In 1997, Lo et al discovered circulating cell free fetal DNA in maternal blood (Lo et al., 1997).

The use of cell free total DNA (cftDNA) and cell free fetal DNA (cffDNA) in molecular diagnostics has aided laboratory investigations relating to the physiologic and pathologic state during the course of pregnancy (Alberry et al., 2009). This discovery made by Lo et al., has paved a way for non-invasive prenatal diagnosis (NIPD), this fit was possible by detecting the presence of fetal Y-chromosomal segments in plasma of pregnant women (Lo et al., 1997).

The use of Cell free DNA testing, independently or in conjunction with other screening test procedures, is beginning to replace older testing methods, thus revolutionizing diagnosis for fetal abnormalities and also helping to diagnose pathological conditions in pregnant women (Cuckle et al., 2015).

1.2.1 Origin of cell free DNA and Cell free fetal DNA in maternal circulation

A greater portion of circulating DNA in maternal circulation during the course of pregnancy is of maternal origin; with the fetal fraction comprising just about 9% in the early part of pregnancy and later increases to about 20% as pregnancy progresses (Hill et al., 2012). The source of circulating cfDNA in normal individuals and also in pregnant women is not clear. However, it is known that the pool of cfDNA in circulation is from solid organs, and also from haematopoietic cells (Lui et al., 2002). In the case of the latter, this molecule is derived from the turn-over of short-lived hematopoietic cells (Hahn et al., 2014).

Postulations about the placenta being the originating source of circulating cffDNA are supported by detection of circulating cffDNA in anembryonic pregnancies, as well as the homogeneity of epigenetic markers of circulating cffDNA and the placenta tissues (Alberry et al., 2007, Chim et al., 2005). The human placenta is dynamic and constantly undergoing villous trophoblast turnover. This releases apoptotic placental material into the circulation. This physiological process is responsible for the release of cell free nucleic acids into the plasma. In pathological conditions such as preeclampsia the level is altered (Huppertz, 2004, Taglauer et al., 2014).
1.2.2 Circulating Cell free fetal DNA and Cell free total DNA (cffDNA) as indicators of placental condition

Circulating cffDNA and cftDNA are markers that can be used to establish the physiologic and pathologic states during the course of pregnancy. Previous studies have shown an increase in circulating cffDNA and cftDNA in PE and in pregnancies that experience fetal growth restriction (Alberry et al., 2009, Sekizawa et al., 2003, Sifakis et al., 2009).

Due to the continuous villous trophoblast change as pregnancy progresses, apoptotic materials including cftDNA are released into the circulation (Taglauer et al., 2014). The release of cffDNA and cftDNA is linked to the stages of placental development (Taglauer et al., 2014). Since the concentration of circulating cffDNA and cftDNA are linked to placental morphogenesis, physiological or pathological conditions that affects the placenta will also directly impact the circulating levels of cfDNA and cffDNA (Taglauer et al., 2014).

1.2.3 Quantification of circulating Cell free fetal using the Ras Association Domain Family - 1 isoform A (RASSF1A) gene.

The measurement of cffDNA routinely for NIPD in complications of pregnancy, such as preeclampsia, depends on the presence of a paternally inherited gene sequence (relating to the Y chromosome) (Alberry et al., 2009, Lazar et al., 2009, Lo et al., 1997, Sekizawa et al., 2001). Availability of a universal marker for the detection of cffDNA in the diagnosis of complications of pregnancy is of great importance (Chan et al., 2006, Salvianti et al., 2015). Fetal DNA markers of NIPD frequently used such as the Y-chromosomal sequences are not able to serve as positive controls in cases where the fetus the mother is carrying is a female, as the Y-chromosomal sequence is seen only in pregnancies with a male fetus (Chan et al., 2006).

The development of a universal marker for fetal DNA detection irrespective the sex of the fetus can be explored by the use of an epigenetic approach to distinguish circulating cfDNA of maternal origin from that of fetal origin (Chim et al., 2005). In the quest to develop a universal marker for the detection of cffDNA in circulation, Chan et al., (2006) have shown that the promoter of the RASSF1A tumor suppressor gene can be employed as a universal marker for circulating cffDNA in maternal plasma. This is due to the fact that the promoter of the RASSF1A gene of placenta origin is hypermethylated (figure 2), while that in the maternal blood is hypomethylated (Zejskova et al., 2010).

By the use of restriction enzymes such as the BstUI (figure 2) on circulating DNA extracted from the maternal plasma, the RASSFIA sequence of maternal origin is digested because it is hypomethylated,
leaving behind the hypermethylated RASSF1A sequence of fetal origin (Chan et al., 2006, White et al., 2012). The hypermethylated RASSF1A sequence of fetal origin can then be quantified using real time quantitative polymerase chain reaction (RT-qPCR) to determine the circulating level of cffDNA. Hypermethylated DNA of fetal origin is not digested because it is resistant to methylation restriction enzymes, unlike the hypomethylated DNA (White et al., 2012).

Figure 2. Diagrammatic representation of hypermethylated and hypomethylated RASSF1A gene. When RASSF1A in maternal plasma is subjected to methylation sensitive restriction enzyme such as the BstUI,
only the RASSF1A of fetal origin will remain in the sample as the RASSF1A of maternal origin will be
digested owing to the fact that it is hypomethylated (Zejskova et al., 2010).

1.2.4 Applications of circulating cell free DNA

Before the development of NIPD, conventional methods of prenatal diagnosis were based on the use of
invasive procedures (Hill et al., 2012). Clinical applications of cffDNA and cftDNA in pregnancy are based
on the fact that there is an increase in the circulating concentration in pregnancy complications (Alberry et
al., 2009). Several studies have reported increase in the circulating levels of cffDNA in complications of
pregnancy (AbdelHalim et al., 2016, Kim et al., 2015, Salvianti et al., 2015).

Asides its application in the diagnosis of pregnancy complications, it has also been of good use in the
detection of fetal gender and the diagnosis of fetal aneuploidy (Bianchi, 2004). Determination of fetal sex
can be performed using RT-qPCR to detect the sequences of inherited paternal Y chromosome in
circulation. Determination of fetal sex is critical in carriers X-linked disorders (Devaney et al., 2011). With
advances in technology and the knowledge of genetics, circulating cfDNA guarantees the use of NIPD for
detection of autosomal dominant and recessive disorders (Hill et al., 2012).

Conventionally, the detection of aneuploidy is done using circulating fetal cells from the amniotic fluid, but
with the development of NIPD using circulating cfDNA, prenatal diagnosis of aneuploidy is made easier
(Hill et al., 2012). Fetal RhD status can be confirmed using circulating cffDNA. This is of importance in
RhD-negative women with previous history of haemolytic disease of the new-born and with high anti-D
antibodies (Daniels et al., 2009)

1.3 Problem statement

The incidence of hypertensive disorders of pregnancy in South Africa is high and remains the commonest
direct cause of maternal mortality. No study has quantified the circulating levels of cell free fetal DNA
(cffDNA) and cell free total DNA (cftDNA) in Black African population, hence the need for this study
since it has been shown by previous studies that the aggressiveness of PIH differs with geographical location
and in women of African ancestry
1.4 Hypothesis

Circulating cell free fetal DNA and cell free total DNA are elevated in pregnancy induced hypertension.

1.5 Aim

To quantify cell free fetal DNA in normal pregnancy and in pregnancy induced hypertension.

1.6 Objectives

I. To quantify the circulating level of cell free fetal DNA in normal pregnancy, preeclampsia, and gestational hypertension.
II. To quantify the circulating level of cell free total DNA in normal pregnancy, preeclampsia, and gestational hypertension.

2 CHAPTER 2: MANUSCRIPT SUBMITTED TO THE INTERNATIONAL JOURNAL OF MOLECULAR SCIENCE (IJMS)

2.1 Circulating Cell Free Fetal DNA and Cell Free total DNA in Black South African Women with Gestational Hypertension and Preeclampsia.

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Abstract: The objective was to quantify the level of circulating cell free fetal DNA (cffDNA) and cell free total DNA (cftDNA) in Black South African women with gestational hypertension and preeclampsia. This was a prospective cross sectional study involving 39 controls, 37 women with preeclampsia (PE) and 23
with gestational hypertension (GH). Preeclampsia was subdivided into severe preeclampsia (SPE), mild to moderate preeclampsia (MPE), early onset preeclampsia (EOPE) and late onset preeclampsia (LOPE). Both cffDNA and cftDNA were quantified using real time qPCR by exploring the difference in methylation pattern of the RASSF1A gene promoter in maternal plasma. The levels of circulating cffDNA and cftDNA were significantly higher in patients with PE compared to the levels in the controls and in patients with GH (p<0.001). There was no alteration in the level of cffDNA and cftDNA in GH compared to controls (p > 0.05). There was a significant increase in the level of cffDNA and cftDNA in SPE in contrast to that in MPE (p<0.05). There was no difference in the level of cffDNA and cftDNA in EOPE compared to LOPE (p > 0.05).

Keywords: cell free fetal DNA; cell free total DNA; preeclampsia; gestational hypertension

1. Introduction

The hypertensive disorders of pregnancy (chronic hypertension, gestational hypertension, preeclampsia, eclampsia and the HELLP syndrome) are associated with significant perinatal and maternal morbidity and mortality [1]. Despite the large amount of resources dedicated over the years to the study of these complications of pregnancy, the exact cause still remains elusive [2].

Globally, up to 10% of pregnancies are affected by hypertensive disorders of gestation [3]. Evidence points to diminished utero-placental perfusion, leading to a hypoxic placenta, as the initiating event in preeclampsia; which leads to the secretion of factors/debris in the circulation [4]. Factors implicated in the development of preeclampsia, per se, include; alterations in the circulating level of pro-inflammatory substances, damage to vascular endothelium caused by shear stress of utero-placental blood flow and also by placental hypoxia; and systemic endothelial dysfunction. Because the exact cause or etiology of preeclampsia is not known, delivery of the baby and placenta is considered as curative [5, 6].

During pregnancy, the placenta is constantly undergoing the process of villous trophoblast turnover and morphogenesis, which leads to the release of apoptotic materials and cell free nucleic acids into maternal circulation [7]. Since the release of circulating nucleic acids is linked to placental morphogenesis, pathological conditions affecting the placenta will directly impact upon the circulating levels of cell free DNA (cfDNA) [7].
A greater percentage of cfDNA in circulation during the course of pregnancy is of maternal origin, the fetal fraction constitutes just 9% of the total circulating cfDNA in early pregnancy and about 20% as pregnancy progresses [8]. The assertion that the placenta is the source of circulating fetal DNA is supported by the presence of fetal DNA in anembryonic pregnancies, as well as the uniformity between epigenetic markers of circulating fetal DNA and the placental tissue [9, 10].

Plasma circulating placenta-derived cfDNA can be considered as a reliable marker with potential uses in the diagnosis and monitoring of the development of complications during the course of pregnancy, especially since it is released into the maternal circulation in the early stages of pregnancy [11].

To the best of our knowledge there has been no study that has quantified the circulating levels of cfDNA in a homogenous Black African population. The objective of our study therefore was to quantify circulating cell free fetal DNA (cffDNA) and cell free total DNA (cftDNA) in Black South African women with gestational hypertension and preeclampsia as the incidence of hypertensive disorders of pregnancy in South Africa is high and remains the commonest direct cause of maternal mortality [12].

2. Results

2.1 Patient demographics and their clinical characteristics

Patient demographics and their clinical characteristics are shown in table 1. There was no difference in maternal age and gestational age among the different groups of patients. There was a significant difference in the BMI of the controls compared to patients with GH (p<0.001). Also there was significant difference in BMI of patients with GH compared to patients with PE (p<0.001).

<table>
<thead>
<tr>
<th></th>
<th>Control (n=39)</th>
<th>Gestational hypertension (GH) (n=23)</th>
<th>Preeclampsia (PE) (n=37)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>27.7 ± 5.9</td>
<td>30.3 ± 4.5</td>
<td>27.1 ± 6.9</td>
</tr>
<tr>
<td><strong>BMI (kg/m^2)</strong></td>
<td>32.6 ± 7.1</td>
<td>39.8 ±6.7*</td>
<td>32.8 ± 6.8</td>
</tr>
<tr>
<td><strong>Primigravidae (number)</strong></td>
<td>12</td>
<td>4</td>
<td>14</td>
</tr>
<tr>
<td><strong>Multigravidae (number)</strong></td>
<td>27</td>
<td>19</td>
<td>23</td>
</tr>
<tr>
<td><strong>Gestational age at sampling (weeks)</strong></td>
<td>35.5 ± 4.2</td>
<td>35.3 ± 5.6</td>
<td>33.6 ± 4.4</td>
</tr>
<tr>
<td><strong>Systolic blood pressure (mmHg)</strong></td>
<td>114.7 ± 9.7</td>
<td>151.3 ± 8.3*</td>
<td>154.8 ± 14.5#</td>
</tr>
<tr>
<td><strong>Diastolic blood pressure (mmHg)</strong></td>
<td>79.8 ± 7.8</td>
<td>97.8 ± 6.9 *</td>
<td>99.7 ± 11.0 #</td>
</tr>
</tbody>
</table>

*control vs GH (p<0.05); * control vs PE (p <0.05); * GH vs PE (p <0.05)
BMI; body mass index
Values are expressed as mean ± standard deviation, and number of occurrence.
2.2 cffDNA and cftDNA quantification

Figure 1(a) shows circulating cftDNA was significantly higher in patients with PE (p < 0.001) (median 2174.0, range 904.5 – 6871.0 genomic equivalence/ml) compared to the controls (median 673.5, range 117.0 – 1754.0 genomic equivalence/ml) and patients with GH (median 682.0, range 226.5 – 1829.0 genomic equivalence/ml). There was no difference in the level of cftDNA between the controls and patients with GH (p>0.05).

Figure 1(b) shows the level of circulating cftDNA in the various sub-divisions of PE. There was a significant difference (p<0.05) in the level of circulating total cfDNA in SPE (median 3053, range 1129 - 6871 genomic equivalence/ml) compared to MPE (median 1878, range 904.5 - 5480 genomic equivalence/ml). There was no difference between EOPE (median 2518, range 1351 – 6871) and LOPE (median 1694, range 904.5 – 4751) (p>0.05).
Figure 1. Box plot of cell free total DNA (cftDNA) and cell free fetal DNA (cffDNA) in controls, GH and PE. (a) cell free total DNA in controls, GH and PE. (b) cell free total DNA in controls and the various sub-divisions of PE. (c) cell free fetal DNA in controls, GH and PE. (d) cell free fetal DNA in controls and the various sub-divisions of PE. Horizontal lines the inside box represents the median, while the whiskers represent the minimum and maximum values. GE (Genomic equivalent).
The levels of cffDNA in the various study groups are shown in figure 1(c). The level of cffDNA was significantly higher in PE (p < 0.001) (median 128.1, range 26.1 - 398.8 genomic equivalence/ml) compared to the controls (median 46.2, range 4.0 – 125.4 genomic equivalence/ml) and patients with GH (median 65.0, range 5.9 – 151.8 genomic equivalence/ml). No difference was seen in the levels cffDNA in controls compared to patients with GH (p>0.05).

Figure 1(d) shows comparison of cffDNA between the sub-divisions of PE. There was an increase in the level of cffDNA in patients with SPE (median 212.6, range 26.1 – 398.3 genomic equivalence/ml) compared to patients with MPE (median 148.9, range 52.8 – 336.0 genomic equivalence/ml) (p < 0.05). There was no difference in cffDNA between EOPE (median 148.9, range 52.8 – 336.0) and LOPE (median 125.6, range 26.1 – 398.8) (p>0.05).

3. Discussion

The development of a universal fetal DNA marker, such as the RASSF1A, which can be quantified irrespective of the sex of the fetus, has helped change the face of non-invasive prenatal diagnosis and improves the monitoring of pregnancy complications during the course of gestation [13]. Different studies have shown that circulating cffDNA regardless of the marker studied (SRY gene, DSY gene; TSPY 1 gene, RASSF1A gene and DSCR3 gene) [11, 14-18] is increased in preeclampsia and other complications of pregnancy. Though the SRY gene and the DSY gene can only be quantified in pregnancies with male fetus, studies have also shown that there exists strong correlation between the level of the SRY gene and RASSF1A [19], as well as the DSY gene and RASSF1A gene in the maternal circulation [20].

Several studies have used different techniques, some have used fluorescent labelled probes for the detection of the RASSF1A gene [13, 16], while others have used SYBR green for the detection of fluorescence [20, 21]. In this study we used a modified SYBR green real time PCR protocol published by White et al., (2012) for the detection of hypermethylated RASSF1A in maternal plasma [20]. White et al., (2012) found that interpretation of result was easier using SYBR green real time PCR compared to methods using the hydrolysis probes, and this method is easily reproducible in low resource settings [20].

From our results, we observed that the levels of circulating cffDNA and ctfDNA were significantly higher in patients with PE compared to the levels in the controls and in patients with GH. There was a 3.2-fold increase in the median level of cffDNA and ctfDNA in the patients with PE compared to the levels in the controls and GH. Our findings are in keeping with previous studies which have also reported increases
in circulating cffDNA and cftDNA in the plasma of women with preeclampsia compared to controls [11, 16]. Elevation in the level of cfDNA in PE is connected to the degree of perfusion and extent of injury to the placenta, which impacts upon the circulating levels of cffDNA and cftDNA [22-24].

There was no alteration in the level of cffDNA and cftDNA in GH compared to controls. The plasma concentration of cffDNA and cftDNA in PE was higher than that in patients with GH. Kim et al., (2015) have also reported a similar finding [16]. Some authors have stated that GH, though a hypertensive disorder of pregnancy, and shares similar risk factors and pathologic features with PE, is a separate condition [25, 26]. Noori et al., (2010) have stated that the disequilibrium in the concentration of pro and anti-angiogenic molecules and endothelial dysfunction are unique and peculiar features of PE [27], but others point out that GH is likely to progress to PE and the rate of progress is dependent on the gestational age at the time of diagnosis of GH [28-30].

In our study there was 4.5 and 2.8-fold increase in the level cftDNA in SPE and MPE when compared to the controls, and a 5 and 3.6-fold increase in the concentration of cffDNA in SPE and MPE respectively. Fetal and total cfDNA were significantly elevated in patients with SPE compared to the level in patients with MPE. It has been reported that the circulating levels of these markers are affected by the severity of the disease [14, 16]. Abdelhalim et al., (2016) has reported an increase in the level of cffDNA and cftDNA in patients with SPE compared to that in MPE, though these authors used different markers [HBB gene for estimation of cftDNA and the TSPY1 gene for quantification of cffDNA] for the quantification of cffDNA and cftDNA [14]. Kim et al., (2015) have also reported similar findings of an increase in the level of cfDNA with severity of the preeclampsia [16]. It is plausible that the finding of increased cfDNA in severe preeclampsia might help clinicians not only to differentiate between mild and severe preeclampsia but also assist in the timing of delivery. This needs further investigation.

In the EOPE and LOPE group, there was significant increase in the level of circulating cffDNA and cftDNA compared to the level in the control group, but the level did not vary between EOPE and LOPE. This finding is in keeping with the study carried out by Papantoniou et al., (2013) who noted the level of cffDNA in EOPE was not different from the level in LOPE, though the number EOPE to LOPE in their study was 2 out of 24 preeclamptic women [31].

4. Materials and Methods

4.1 Study design, setting and Patient population
Ethical approval for this study was obtained from the University of KwaZulu-Natal Biomedical Research Ethics Committee (BREC- No. 379/15). This study was done prospectively on samples obtained from patients in the antenatal ward of a regional hospital in the south of Durban, South Africa.

Pregnant women in the antenatal period without any history of obstetric complications served as controls. Gestational hypertension was defined as new onset hypertension (a blood pressure of \( \geq 140/90\) mmHg) without the presence of protein in the urine after the 20th week of gestation [5, 32]. Preeclampsia was defined as new onset hypertension, characterized by a blood pressure of \( \geq 140/90\) mmHg with the presence of protein in the urine \( \geq 300\) mg in a 24 hour urine sample (or a value of \( \geq 1+ \) on dipstick) after the 20th week of gestation [5, 32]. Blood pressure measurement as well as dipstick proteinuria values were repeated after about 4 to 6 hours later to confirm the earlier diagnosis [32].

Preeclampsia was subdivided into mild to moderate preeclampsia (MPE) defined by a blood pressure of \( \geq 140/90\) mmHg and \( \leq 159/109\) mmHg and proteinuria \( \geq 300\) mg in a 24-hour urine sample but not exceeding 2.0 g or a concentration of 1+ to 2+ on dipstick [5]. Severe preeclampsia (SPE) was defined by a blood pressure of \( \geq 160/110\) mmHg and proteinuria greater than 2.0 g in a 24 hour urine sample or \( \geq +++ \) on a dipstick [5]. Based on onset of preeclampsia, early onset preeclampsia (EOPE) was defined as PE before 34 weeks (<33 weeks 6 days) of pregnancy. Late onset preeclampsia (LOPE) was defined as PE at 34 or after 34 weeks of gestation [33].

Women having the following medical conditions were excluded from the study; chronic hypertension, cardiovascular or renal disease, diabetes mellitus, bleeding disorders and previous history of any medical conditions.

4.2 Blood collection

5 ml of blood was obtained from the patients into a sterile EDTA tube and processed within one hour of collection. Blood samples were spun at 1600g for 10 minutes. The supernatant plasma was transferred into another tube and spun for the second time at 14000g for 10 minutes. The supernatant was stored at minus (-) 80° C pending DNA extraction.

4.3 DNA extraction

Plasma cfDNA was extracted using the Roche high pure PCR template preparation kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. 200 microliter of plasma was used and the DNA was eluted in a final volume of 200 microliters.
4.4 Quantitative Real time polymerase chain reaction for quantification of cffDNA and cftDNA using the Ras association domain family 1 isoform A (RASSF1A) gene.

We used a modified Real time PCR protocol published by White et al., for the detection of hypermethylated RASSF1A in maternal plasma [20]. We used the 2X light cycler 480 SYBR green 1 master mix on the Roche light cycler 96. A concentration of 1.0µM/L of both forward and reverse primers was used. The primer sequences used were; RSF-b151F 5’-AGC CTG AGC TCA TTG AGC TG-3’, RSF-dsgrnR 5’-ACC AGC TGC CGT GTG G-3’ [13].

Fetal cffDNA was quantified using the methylated form of RASSF1A promoter after the unmethylated cffDNA was digested using methylation sensitive enzyme. 5 µl of the extracted cffDNA was digested using 10 units of Bsh1236I (Thermo Fisher scientific Inc, USA), total reaction volume was 20µl at a temperature of 37°C for 16 hours. After enzyme treatment, 5µl of the enzyme treated cffDNA underwent a qPCR reaction for the RASSF1A promoter, with a final reaction volume of 20µl. The control for the enzymatic reaction was the β-actin gene which targets the unmethylated sequence of the β-actin promoter. A fully complete enzyme digestion of the hypomethylated cffDNA was confirmed by the absence of the β-actin gene amplification signals. The primer sequence used for the β-actin gene are as follows; Actin-163F 5’-GCG CCG TTC CGA AAG TT-3’, Actin-298R 5’-CGG CGG ATC GCG AAA-3’ [13]. Total cffDNA was quantified using the undigested extracted cell free DNA. A series of different concentrations of standards was made using genomic DNA (10ng/µL) to construct the standard curve. Standards were included in all qPCR runs. Both standard standards and test samples analyzed in duplicate. Results obtained at the end of qPCR were expressed as genomic equivalence per ml, using a conversion factor of 6.6pg DNA/ml of plasma [13].

4.5 Statistical Analysis

Data analysis of the results was done using the GraphPad Prism 5.03 (Graph Pad software Inc., California). The Mann-Whitney U-test was used to compare experimental results between two groups. Quantitative variables across several groups was compared using the Kruskal–Wallis test. Categorical variables were compared using the Fisher Exact test. A p-value less than 0.05 was considered significant.

5. Conclusions

Our results indicate that circulating cffDNA and cftDNA is elevated in preeclampsia supporting its use as a potential marker in pregnancy related hypertention. Our results also indicate that that the level of severity of PE may be a factor that influences the level of these markers in maternal plasma in PE. We
propose that the use of cfDNA alone or in combination with other tests be fully standardized for the
diagnosis and monitoring of preeclampsia.

6. Strength and Limitation of the study

This is the first study to quantify circulating cffDNA and cftDNA in a homogenous Black African
population in which hypertensive disorders of pregnancy may be more prevalent than in Caucasian
population. The limitations of this study is that we did not factor in the gender of the fetus.

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3 CHAPTER 3: SYNTHESIS, CONCLUSION AND RECOMMENDATION

3.1 Synthesis

Circulating cffDNA and cftDNA are novel molecular markers, and have helped change the state prenatal diagnosis and obstetrical research (Sifakis et al., 2015). The availability of a sensitive and reproducible method for early diagnosis of pregnancy complications, would help to improve clinical management of maternal and fetal complications (Martin et al., 2014). The circulating concentrations of cffDNA and cftDNA is linked to placental development, and hence any interference in this process will impact the circulating level of these markers (Taglauer et al., 2014). The concentration of these markers can help to predict complications of pregnancy, as increase in the levels occur prior to the development of clinical symptoms of hypertensive disorders (Sifakis et al., 2015).

This study was designed to quantify circulating cffDNA and cftDNA in normal pregnancy and in pregnancy related hypertension in Black South African women, as hypertension in pregnancy remains a public health burden and a major cause of maternal mortality in South Africa (Saving mothers Report, 2015). Studies have shown that the mortality rate in black women as a result of preeclampsia is 3.1 times more than in Caucasians (MacKay et al., 2001). Currently no study has quantified the level cffDNA and cftDNA in a homogenous Black African population.

Different studies have used various techniques for the quantification of circulating cfDNA using the RASSF1A gene. Some studies have used fluorescent labelled probes for the detection of the RASSF1A gene (Chan et al., 2006, Kim et al., 2015), while others have used SYBR green for the detection of fluorescence (Moezzi et al., 2016, White et al., 2012). A modified SYBR green Real time PCR protocol published by White et al., for the detecting the presence of hypermethylated RASSF1A in plasma was used for this study (White et al., 2012). This is because White et al., (2012), found that real time qPCR data using SYBR green assay are easier to interpret and less complex compared to methods using the hydrolysis probes. These authors also showed that this technique is able to detect very low copies of the gene in plasma samples, is easily reproducible, less expensive and suitable for a low resource setting (White et al., 2012).

Extensive research has shown that circulating levels of cffDNA and cftDNA regardless of the marker studied (SRY gene, DSY gene; TSPY 1 gene, RASSF1A gene and DSCR3 gene); is elevated in preeclampsia and other pregnancy complications (AbdelHalim et al., 2016, Alberry et al., 2009, Lazar et al., 2009, Salvianti et al., 2015, Yu et al., 2013). Though the SRY gene and the DSY gene can only be quantified in pregnancies with a male fetus, a strong correlation exist between the level of the SRY gene and RASSF1A gene (Manokhina et al., 2014), as well as the DSY gene and RASSF1A gene in maternal
circulation (White et al., 2012). This study quantified circulating cfDNA and ctDNA using the RASSF1A gene as it is now accepted as a universal marker for the quantification of cfDNA regardless of the sex of the fetus.

The patient age and gestational age among the different groups of patients did not show any difference, and were matched in the present study. No difference was observed in the BMI of the PE group compared to the control group. The BMI of the GH was significantly higher compared to the BMI of the control group. In addition, there was significant difference in BMI of GH group compared to patients with PE. One of the risk factors of pregnancy-induced hypertension is obesity. Obesity and maternal over weight increases the chance of developing pregnancy induced hypertension (Rahman et al., 2015, Swank et al., 2014, Villar et al., 2006). Ramsay et al., (2002) have shown that obesity is associated with altered endothelial function in pregnancy, elevation in blood pressure, altered function of the vascular endothelium, and the upregulation in the production of inflammatory molecules (Ramsay et al., 2002).

The results obtained from this study shows a 3.2-fold increase in the concentration of cfDNA and ctDNA, which were significantly higher in patients with PE compared to the levels in the controls and in patients with GH. Our outcomes are in keeping with previous studies which have also reported increase in circulating of cfDNA and ctDNA in the plasma of women with preeclampsia compared to controls (Kim et al., 2015, Salvianti et al., 2015). Abnormal spiral artery remodelling as seen in preeclampsia leads to the development of an ischaemic placenta and subsequent destruction of placenta cells (Naljayan and Karumanchi, 2013). Destruction of the placenta cells cause the liberation of cellular contents in to the circulation, resulting in an increased level of circulating free fetal and total nucleic acids, microvessicles, and exosomes (Tannetta and Sargent, 2013). This explains the reason why these markers are elevated in pregnancy complications that affect the placenta such as PE (Taglauer et al., 2014). Circulating levels of cfDNA and ctDNA is able to serve as a mirror image of the placenta, since the concentration of these markers in circulation is affected by the rate of release of cellular debris from apoptotic placenta cells (Taglauer et al., 2014). These placental-derived factors/particles in circulation play significant roles in initiating maternal systemic inflammatory response (MSIR) and endothelial dysfunction (Tannetta and Sargent, 2013), and are thus central to the pathogenesis and clinical manifestations of PE. The development of syncytial knots and the increased infarction seen in the placenta of PE women, adds support to its role as a source of increased circulating cfDNA (Roberts and Post, 2008, VINNARS et al., 2011). Circulating levels of fetal and total cfDNA may reflect placental status, as the concentration of these markers are affected by the rate of release of cellular debris from apoptotic placental cells (Taglauer et al., 2014).
This study has shown that GH does not affect the level of circulating cffDNA and cftDNA. The level of these markers in GH was significantly lower compared to the levels in PE. This result is in keeping with results obtained by Kim et al., (2015). These authors showed the level of cffDNA and cftDNA remained low in patients with GH all through in a case control study (Kim et al., 2015). Some studies show that GH, though a hypertensive disorder of pregnancy which shares similar risk factors and pathologic features with PE, is a separate condition (Li et al., 2016, Villar et al., 2006). It has also been stated that the disequilibrium in the level of pro and anti-angiogenic molecules and endothelial dysfunction is a unique and peculiar features of PE (Noori et al., 2010). However, some authors argue that GH is likely to progress to PE and the rate of progress is reliant on the gestational age as at when the diagnosis of GH is made (Romero-Arauz et al., 2014, Saudan et al., 1998, Sibai, 2003).

Severe forms of PE are associated with more serious maternal and fetal complications. Hence identification of women with preeclampsia at risk of developing severe PE will help in the management of the patient and averting complications associated with SPE (Fong et al., 2014). Precise diagnosis and measurement of risks in cases of SPE would aid in making proper and timely obstetric decisions, which cannot be achieved by the use of conventional and routine techniques (Fong et al., 2014, Thangaratinam et al., 2011).

In our study, there was 4.5 and 2.8-fold increase in the level cftDNA in SPE and MPE when compared to the controls, and a 5.0 and 3.6-fold increase in the concentration of cffDNA in SPE and MPE respectively. Fetal and total cfDNA were significantly elevated in patients with SPE compared to the level in patients with MPE. This finding is in keeping with findings from other studies (AbdelHalim et al., 2016, Kim et al., 2015). Abdelhalim et al (2016) have reported an increase in the level of cffDNA and cftDNA in patients with SPE compared to that in MPE. Although these authors used different markers (HBB gene for estimation of cftDNA and the TSPY1 gene for quantification of cffDNA) for the quantification of fetal and total cfDNA (AbdelHalim et al., 2016). Kim et al., (2015) have also reported similar finding of an increase in the level of cfDNA with severity of the preeclampsia. Jakobsen et al., (2013) have stated that the levels of circulating cfDNA are higher in SPE than in MPE as these two conditions are different in placental pathological features, and hence should be managed as different entities. They also stated that increase cfDNA which is seen in cases of SPE results from an increase in the release of syncytiotrophoblast from ischemic lesions (Jakobsen et al., 2013).

This finding of an increase in cfDNA and cftDNA in SPE compared to MPE might help clinicians differentiate SPE from MPE, and help in accurate risk assessment in cases of SPE thus providing information needed for timing of delivery. This aspect requires further investigation.
Studies have shown that EOPE and LOPE are distinct entities with different pathologies (Von Dadelszen et al., 2003, Walker, 2000). These entities differ in pathological features and in maternal and fetal outcomes (Raymond and Peterson, 2011). Masuyama et al., (2010) have stated that angiogenic factor levels varies between EOPE and LOPE. EOPE is associated more with altered cardiovascular function resulting from placental disorder while LOPE is related more to maternal factors (Valensise et al., 2008). The finding from this current study shows a significant increase in the level of circulating cffDNA and cftDNA in EOPE and LOPE groups compared to the level in the control group, but the level did not vary between EOPE and LOPE. This finding is in keeping with the study carried out by Papantoniou et al., who noted the level of cffDNA in EOPE was not different from the level in LOPE, though the number EOPE to LOPE in their study was only 2 out of a total 24 preeclamptic women (Papantoniou et al., 2013). Though the results obtained in this study did not show any difference in the level of circulating cffDNA and cftDNA between EOPE compared to LOPE, Papantoniou et al., (2013) have shown that the circulation levels of these markers can be used to identify women who are likely to develop preeclampsia at an early stage, as the circulating concentration of fetal and total DNA in this women are elevated in the early stages of pregnancy. These authors have also stated that the increase in the level of circulating cfDNA in the early stages of pregnancy correlates to the altered placentation which is an initiating factor of PE (Papantoniou et al., 2013). This finding supports the hypothesis that cfDNA elevation in PE is due to impaired trophoblastic invasion of maternal spiral arteries leading to placental ischemia and damage, with the consequent release of apoptotic syncytiotrophoblast fragments that contain fetal DNA.

3.2 Conclusions

In conclusion, results from this study indicate that circulating cffDNA and cftDNA is elevated in preeclampsia. Consideration needs to be given to its potential use as a biomarker in the diagnosis and monitoring of complications of pregnancy. Our results also indicate that the level of severity of PE may be a factor that influences the level of these markers in maternal plasma in PE. This is promising as it can be used in making appropriate and timely decisions in patient management with SPE, and in the timing of delivery.

3.3 Recommendations

Several studies have shown that the level of circulating cffDNA and cftDNA are increased in preeclampsia. These results from previous studies show variations, as the level of increase differs with the study. It is
therefore necessary that the use of cfDNA alone or in combination with other tests be fully standardised for the diagnosis and monitoring of preeclampsia and other complications of pregnancy.
4 REFERENCES


universal fetal DNA marker that improves the reliability of noninvasive prenatal diagnosis. _Clinical chemistry_, 52, 2211-2218.


Figure 1. Standard curve using known concentration of genomic DNA to determine the concentration of circulating cffDNA and cftDNA in plasma.
APENDIX B

Research approval from the KwaZulu-Natal Department of health

Date: 16 November 2015

Dear Mr S. Eche
(University of KwaZulu Natal)
Email: echasimedi@gmail.com

Approval of research

1. The research proposal titled ‘Characterizing cell free fetal DNA in Normal pregnancy and in pregnancy related hypertension in Black South African women’ was reviewed by the KwaZulu-Natal Department of Health.

The proposal is hereby approved for research to be undertaken at Prince Mshiyeni Memorial Hospital.

NB: Please submit full ethics clearance letter before commencing with the study.

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 hrmn@kznhealth.gov.za

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

Yours Sincerely

Dr E Lutge
Chairperson, Health Research Committee
Date: 16/11/15

Fighting Disease, Fighting Poverty, Giving Hope
APENDIX C

Ethical Approval from the University of Kwazulu-Natal Biomedical Research Ethics Committee (BREC)

UNIVERSITY OF KWAZULU-NATAL

INYUNESI YAKWAZULU-NATALI

27 November 2015

Mr S Eche (215000273)
Medical Biochemistry
School Of Laboratory Medicine and Medical Sciences
eche@umk.mg@gmail.com

Dear Mr Eche

Protocol: Characterizing cell free fetal DNA in normal pregnancy and in preeclampsia.
Degree: MMedSc
BREC reference number: BE379/15

EXPEDITED APPLICATION

The Biomedical Research Ethics Committee has considered and noted your application received on 18 August 2015.

The study was provisionally approved pending appropriate responses to queries raised. Your responses dated 20 November 2015 to queries raised on 04 November 2015 have been noted and approved by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given full ethics approval.

This approval is valid for one year from 27 November 2015. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

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


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

The sub-committee’s decision will be RATIFIED by a full Committee at its meeting taking place on 08 December 2015.

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

Yours sincerely

Professor J Tsoka-Gwegweni
Chair: Biomedical Research Ethics Committee

cc supervisor: mackrai@ukzn.ac.za
cc postgrad: dudhath@ukzn.ac.za

Biomedical Research Ethics Committee
Professor J Tsoka-Gwegweni (Chair)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 4888 Facsimile: +27 (0) 31 260 4608 Email: brec@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

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100 YEARS OF ACADEMIC EXCELLENCE

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APENDIX D

Prince Mshiyeni Memorial Hospital Approval Letter to Conduct Research

TO: Mr Simeon Eche

APPROVAL LETTER TO CONDUCT RESEARCH AT PMMH

Dear researcher,

I have pleasure to inform you that PMMH has considered your application to conduct research on "CHARACTERIZING CELL FREE FETAL DNA IN NORMAL PREGNANCY AND IN PREGNANCY RELATED HYPERTENSION IN BLACK SOUTH AFRICAN WOMEN" in our institution.

Please note the following:
1. Please ensure that you adhere to all the policies, procedures, protocols and guidelines of the Department of Health with regards to this research.
2. This research will only commence once this office has received confirmation from the Provincial Health Research Committee in the KZN Department of Health.
3. Please ensure this office is informed before you commence your research.
4. The institution will not provide any resources for this research.
5. You will be expected to provide feedback on you finding to the institution.

- Full research protocol, including questionnaires and consent forms if applicable.
- Ethical approval from a recognized Ethic committee in South Africa.

Thank you.

Dr. A. Hussain
Acting Medical Manager and Intern Curator
Specialist in Family Medicine
MBBS, PG Dip in HIV/AIDS, DCH, ADV Health Management
M. Med. Fam. Med (Natal)

uml Nyango Wezempilo, Departement van Gesondheid
Fighting Disease, Fighting Poverty, Giving Hope
DATA SHEET: characterising cell free DNA in normal pregnancy and in hypertensive disorders of pregnancy in black South African women.

**Study site:** .............................................................. **Study number:** .........................................................

Study groups: Normotensive, Gestational hypertension, Mild/moderate Preeclampsia, Severe Preeclampsia.

**GENERAL HOSPITAL INFORMATION:**

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<th>Hospital number</th>
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**PATIENT DEMOGRAPHICS:**

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<th>Date of Birth</th>
<th>Rural dweller</th>
<th>Urban dweller</th>
<th>Life style</th>
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**Clinical Data**

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<td>Parity</td>
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<td>Maternal Height</td>
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<td>HIV Status</td>
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<td>If HIV positive, CD4 count</td>
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**BABY OUTCOME:**

Baby’s Birth Weight:

Baby’s gender:

Favourable /unfavourable
APENDIX F

INFORMED CONSENT FORM

Consent to Participate in Research

Dear Ms, Miss, Mrs:

I am sister Mkhize, carrying out a study with Mr Eche who is studying for a masters degree at University of Kwazulu-Natal. We will require half a tea spoon (5ml) of blood from you. We will take the blood the same time your doctors are take their blood samples for antenatal care. The taking of blood may cause you slight discomfort.

Our study may lead to finding out whether we can diagnose people with high blood pressure in early pregnancy. This may not be of help to you at the moment, but in future it may be of help to other pregnant women. Your taking part in this study is on your own free will (voluntary) and you can refuse. If you do not want to be in the study, you will not be treated differently from other patients. Our study also does not interfere with the normal treatment you get.

The results of this study will be published but no names will be used. The reason for publishing is so that other doctors and nurses can learn about our findings and because Mr Eche will get a higher qualification. Note that we may ask you a few questions about your previous pregnancy and this current pregnancy, the same questions asked at the time of your first visit to the clinic. All the information will be kept in a locked cupboard and burnt/ destroyed once the study is over.

Please do not hesitate to let me know if you would like more information.

Thank You

____________________  ____________________
Signature of Participant  Date

____________________  _____________________
Signature of Witness Date
(Ms P Mkhize)