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

Simeon Eche

BMLS (Hons) (UNICAL-NIG)

Submitted in partial fulfilment of the requirements for the degree of Master of Medical Science

in the

Discipline of Medical Biochemistry and Chemical Pathology

School of Laboratory Medicine and Medical Sciences

College of Health Sciences

University of KwaZulu-Natal

2016

Supervisor

Professor Irene Mackraj, PhD.

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.

Simeon Eche _____ 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.

Where their exact words have been used their writing had been placed inside quotation marks and referenced.

(v) Where I have reproduced a publication of which I am an author, co-author, I have indicated in detail which part of the publication was written by myself alone and have fully referenced such publications.

(vi) This dissertation does not contain text, graphics, or tables copied and pasted from the internet, unless specifically acknowledged and the source being detailed in the dissertation and the reference sections.

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.

My sincere and heartfelt appreciation goes to my supervisor, Professor Irene Mackraj for her advice and encouragement that contributed to the success of this work.

Special thanks to my co-supervisor Professor Jagidesa Moodley, for his constructive criticism and guidance throughout this work.

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

I want to say a big thank you to Dr. Kogi Moodley, Sister Zinhle Mkhize, Mr. Nerolen Soobryan and all members of the placenta research group for their contributions to the success of this research project.

CONTENTS

LIST OF FIGURES

Figures in Manuscript

LIST OF TABLES

Table in Manuscript

PUBLICATION

The following manuscript has been submitted to the International Journal of Molecular Science (IJMS). Manuscript number: IJMS-142616

Simeon Eche, Irene Mackraj, Jagidesa Moodley. Circulating Cell Free Fetal DNA and Cell Free Total DNA in Black South African Women with Gestational Hypertension and Preeclampsia.

ABBREVIATIONS AND SYMBOLS

ANOVA	Analysis of Variance
BMI	Body Mass Index
BREC	Biomedical Research Ethics Committee
Bsh 1236I (BSTUI)	Restriction enzyme
cfDNA	Cell Free DNA
cffDNA	Cell Free Fetal DNA
cftDNA	Cell Free Total DNA
DSCR3	Down Syndrome Critical Region 3
EDTA	Ethylene Diamine Tetra-acetic Acid
EOPE	Early Onset Preeclampsia
GE	Genomic Equivalence
GH	Gestational Hypertension
HDP	Hypertensive Disorder of Pregnancy
HELLP	Hemolysis Elevated Liver Enzyme and Low Platelet
LOPE	Late Onset Preeclampsia
Ml	Mililiter
MPE	Mild to Moderate Preeclampsia
NIPD	Non-Invasive Prenatal Diagnosis
PIH	Pregnancy Induced Hypertension
PE	Preeclampsia

RASSF1A	Ras Association Domain Family - 1 isoform A
RhD	Rhesus D
RT-PCR	Real Time Polymerase Chain Reaction
sENG	Soluble Endoglin
sFlt-1	Soluble Fms-like Tyrosine Kinase-1
SPE	Severe Preeclampsia
SRY	Sex-determining Region Y
TSPY	Testis Specific Protein-Y
T-test	A type of statistical test
µl	Microliter
VEGF	Vascular Endothelial Growth Factor
VEGF-R1	Vascular Endothelial Growth Factor Receptor-1
≥	Greater and equal to
≤	Less than and equal to
>	Greater than
<	Less than

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.

1 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 $\geq$ 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 a microvescicles (Tannneta 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.

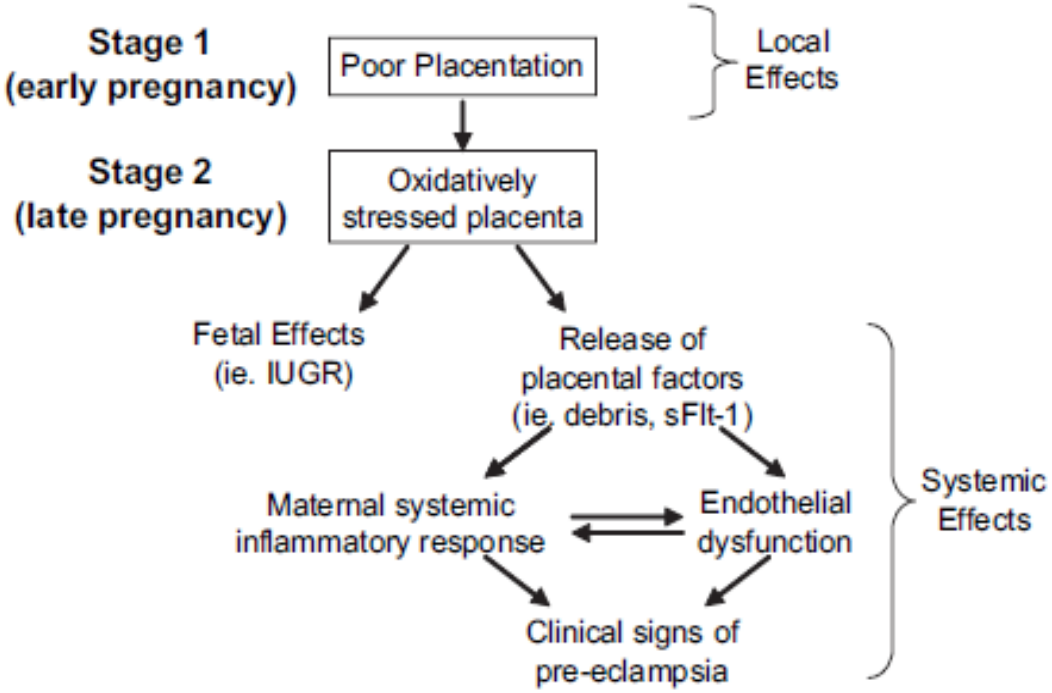


Figure 1. Diagrammatic representation of the stages of pre-eclampsia. Abnormal remodelling of spiral artery which results from poor placentation causes release of placental particles or factors responsible for initiation of a systemic inflammatory response and dysfunction of the maternal vascular endothelium 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 cftDNA and cffDNA 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 *RASSF1A* 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).

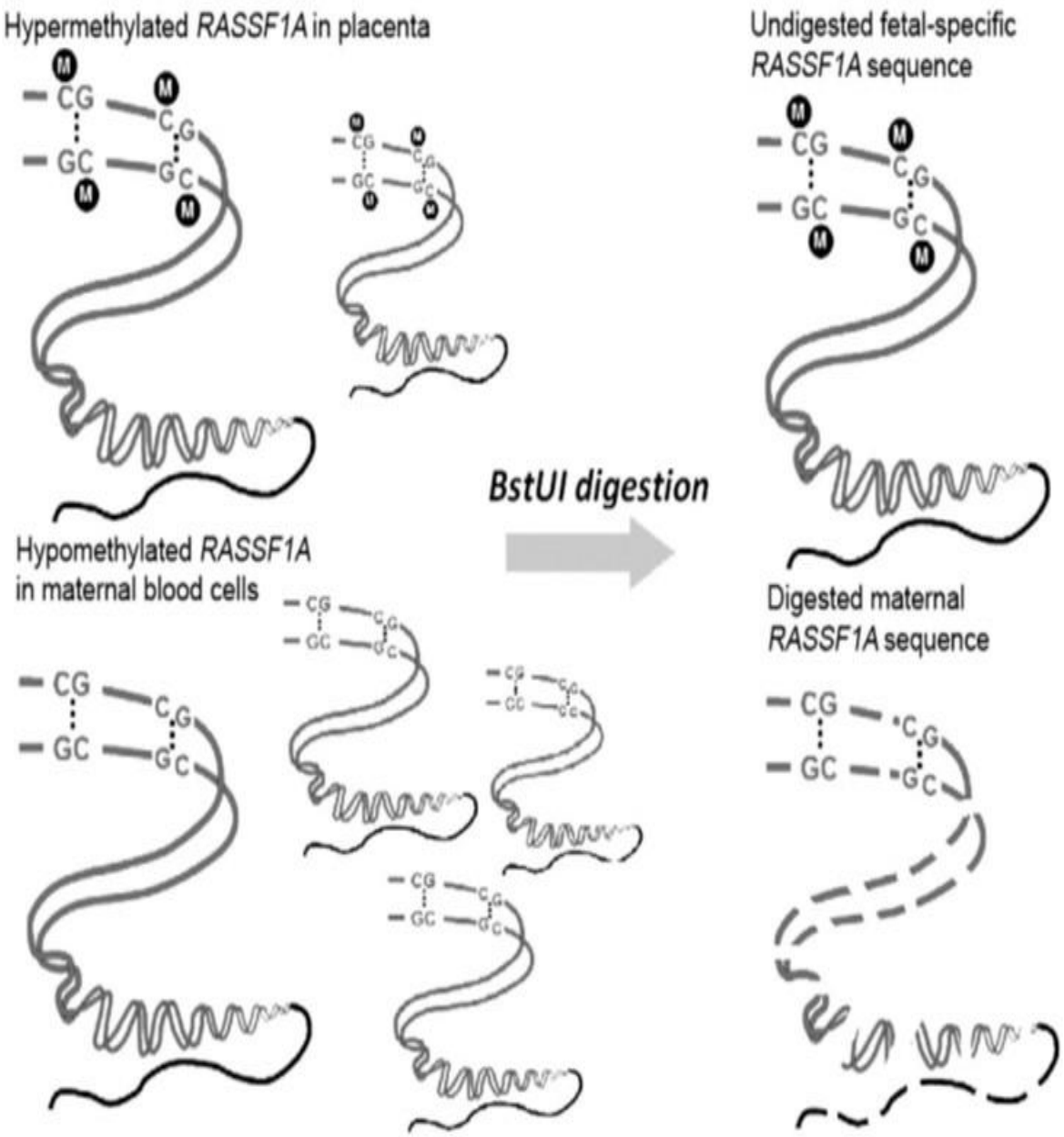


Figure 2. Diagrammatic representation of hypermethylated and hypomethylated RASSF1A gene. When RASSF1A in maternal plasma is subjected to methylation sensitive restriction enzyme such as the BstUI,

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.

Simeon Eche[1], Irene Mackraj[2*], Jagidesa Moodley [3]

[1]Department of Medical Biochemistry, University of KwaZulu-Natal, Durban, South Africa; echesimeon@gmail.com

[2]Department of Human Physiology, University of KwaZulu-Natal, Durban, South Africa; mackraji@ukzn.ac.za.

[3]Department of Obstetrics and Gynecology and Women's Health and HIV Research Group, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa; jmog@ukzn.c.za

*Correspondence: Tel; 031-260 7770; mackraji@ukzn.ac.za

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 1. Patient demographics and clinical characteristics.

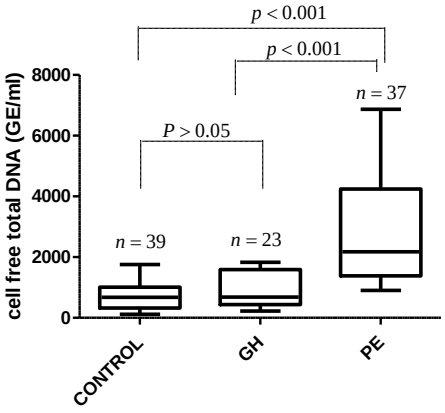
	Control (n=39)	**Gestational hypertension (GH) (n=23)**	**Preeclampsia (PE) (n=37)**
Age (years)	27.7 ± 5.9	30.3 ± 4.5	27.1 ± 6.9
BMI (kg/m^2)	32.6 ± 7.1	39.8 ±6.7*$^{\mu}$	32.8 ± 6.8
Primigravidae (number)	12	4	14
Multigravidae (number)	27	19	23
Gestational age at sampling (weeks)	35.5 ± 4.2	35.3 ±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

*control vs GH ($p<0.05$); # control vs PE ($p<0.05$); $^{\mu}$ GH vs PE ($p<0.05$)
BMI; body mass index
Values are expressed as mean ± standard deviation, and number of occurrence.

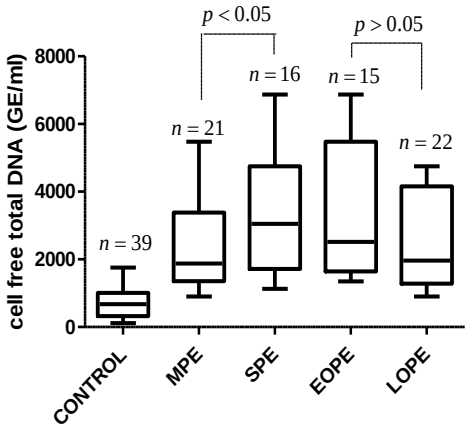
2.2 cffDNA and cftDNA quantification

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 ($p>0.05$).

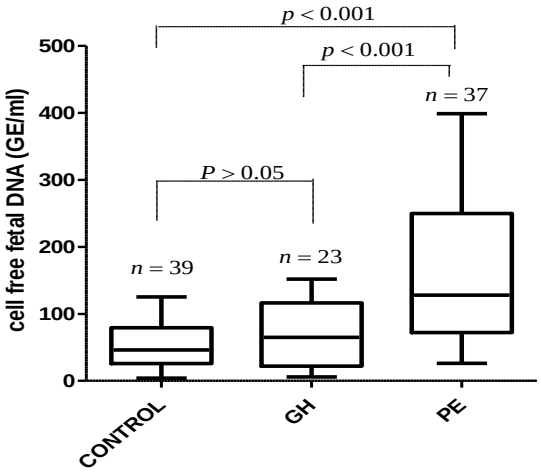
Figure 1(b) shows the level of circulating cftDNA in the various sub-divisions of PE. There was a significant difference ($p<0.05$) in the level of circulating total cfDNA in SPE (median 3053, range 1129 - 6871 genomic equivalence/ml) compared to MPE (median 1878, range 904.5 - 5480 genomic equivalence/ml). There was no difference between EOPE (median 2518, range 1351 – 6871) and LOPE (median 1694, range 904.5 – 4751) ($p>0.05$).



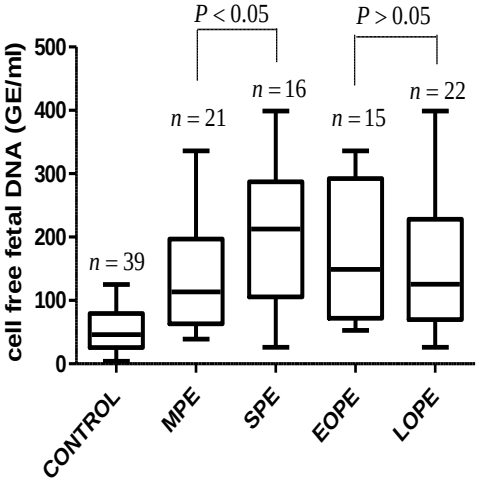
(**a**)



(**b**)



(C)



(d)

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 cftDNA 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 findings [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 ≥ 140/90mmHg) 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 ≥ 140/90mmHg with the presence of protein in the urine ≥ 300mg in a 24 hour urine sample (or a value of ≥ 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 ≥ 140/90mmHg and ≤ 159/109 mmHg and proteinuria ≥ 300mg 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 ≥160/110 mmHg and proteinuria greater than 2.0 g in a 24 hour urine sample or ≥ +++ 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^{o} 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-dsgnR 5'-ACC AGC TGC CGT GTG G-3' [13].

Fetal cfDNA was quantified using the methylated form of RASSF1A promoter after the unmethylated cfDNA was digested using methylation sensitive enzyme. 5 µl of the extracted cfDNA was digested using 10 units of Bsh1236I (Thermo Fisher scientific Inc, USA), total reaction volume was 20µl at a temperature of 37^0C for 16 hours. After enzyme treatment, 5µl of the enzyme treated cfDNA 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 GGC AAA-3' [13]. Total cfDNA 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.

Acknowledgments

We would like to thank the National Research Foundation of South Africa (NRF) for funding this project. We also acknowledge Dr R. Maharaj, Dr K. Moodley, Ms Zinhle Mkhize and Mr Nerolen Soobryan for their assistance.

References

1. Barra S, do Carmo Cachulo M, Providência R, Leitão-Marques A. Hypertension in pregnancy: The current state of the art. Rev Port Cardiol (English Ed). 2012;31(6):425-32.
2. Hutcheon JA, Lisonkova S, Joseph K. Epidemiology of pre-eclampsia and the other hypertensive disorders of pregnancy. Best Pract Res Clin Obstet Gynaecol. 2011;25(4):391-403.
3. Magee LA, Pels A, Helewa M, Rey E, von Dadelszen P. The hypertensive disorders of pregnancy (29.3). Best Pract & Res Clin Obstet Gynaecol. 2015;29(5):643-57.
4. Granger JP, Alexander BT, Bennett WA, Khalil RA. Pathophysiology of pregnancy-induced hypertension. Am J Hypertens. 2001;14(S3):178S-85S.
5. Magee LA, Pels A, Helewa M, Rey E, von Dadelszen P. Diagnosis, evaluation, and management of the hypertensive disorders of pregnancy. Preg Hypertens: An International Journal of Women's Cardiovascular Health. 2014;4(2):105-45.
6. Mol BW, Roberts CT, Thangaratinam S, Magee LA, de Groot CJ, Hofmeyr GJ. Pre-eclampsia. The Lancet. 2016;387(10022):999-1011.
7. Taglauer E, Wilkins-Haug L, Bianchi D. Review: cell-free fetal DNA in the maternal circulation as an indication of placental health and disease. Placenta. 2014;35:S64-S8.
8. Hill M, Barrett AN, White H, Chitty LS. Uses of cell free fetal DNA in maternal circulation. Best Pract Res Clin Obstet Gynaecol. 2012;26(5):639-54.
9. Alberry M, Maddocks D, Jones M, Abdel Hadi M, Abdel-Fattah S, Avent N, *et al*. Free fetal DNA in maternal plasma in anembryonic pregnancies: confirmation that the origin is the trophoblast. Prenatal Diag. 2007;27(5):415-8.

10. Chim SS, Tong YK, Chiu RW, Lau TK, Leung TN, Chan LY, *et al*. Detection of the placental epigenetic signature of the maspin gene in maternal plasma. Proc Nat Acad Sci USA. 2005;102(41):14753-8.
11. Salvianti F, Inversetti A, Smid M, Valsecchi L, Candiani M, Pazzagli M, *et al*. Prospective evaluation of RASSF1A cell-free DNA as a biomarker of pre-eclampsia. Placenta. 2015;36(9):996-1001.
12. Saving Mothers 2011-2013. A short report of the National Committee on Confidential Enquiries into Maternal Deaths. Pattinson R ed. Nat Dept of Halth. Pretoria. 2015.
13. Chan KA, Ding C, Gerovassili A, Yeung SW, Chiu RW, Leung TN, *et al*. Hypermethylated RASSF1A in maternal plasma: a universal fetal DNA marker that improves the reliability of noninvasive prenatal diagnosis. Clin Chem. 2006;52(12):2211-8.
14. AbdelHalim RM, Ramadan DI, Zeyada R, Nasr AS, Mandour IA. Circulating Maternal Total Cell-Free DNA, Cell-Free Fetal DNA and Soluble Endoglin Levels in Preeclampsia: Predictors of Adverse Fetal Outcome? A Cohort Study. Mol Diagn Ther. 2016;20(2):135-49.
15. Alberry MS, Maddocks DG, Hadi MA, Metawi H, Hunt LP, Abdel-Fattah SA, *et al*. Quantification of cell free fetal DNA in maternal plasma in normal pregnancies and in pregnancies with placental dysfunction. Am J Obstet Gynecol. 2009;200(1):98.e1-.e6.
16. Kim HJ, Kim SY, Lim JH, Kwak DW, Park SY, Ryu HM. Quantification and application of potential epigenetic markers in maternal plasma of pregnancies with hypertensive disorders. Int J Mol Sci. 2015;16(12):29875-88.
17. Lazar L, Rigó J, Nagy B, Balogh K, Makó V, Cervenak L, *et al*. Relationship of circulating cell-free DNA levels to cell-free fetal DNA levels, clinical characteristics and laboratory parameters in preeclampsia. BMC Med Genet. 2009;10(1):120.
18. Yu H, Shen Y, Ge Q, He Y, Qiao D, Ren M, *et al*. Quantification of maternal serum cell-free fetal DNA in early-onset preeclampsia. Int J Mol Sci. 2013;14(4):7571-82.
19. Manokhina I, Singh TK, Peñaherrera MS, Robinson WP. Quantification of cell-free DNA in normal and complicated pregnancies: overcoming biological and technical issues. PloS one. 2014;9(7):e101500.
20. White HE, Dent CL, Hall VJ, Crolla JA, Chitty LS. Evaluation of a novel assay for detection of the fetal marker RASSF1A: facilitating improved diagnostic reliability of noninvasive prenatal diagnosis. PloS one. 2012;7(9):e45073.
21. Moezzi L, Keshavarz Z, Ranjbaran R, Aboualizadeh F, Behzad-Behbahani A, Abdullahi M, *et al*. Fetal RHD Genotyping Using Real-Time Polymerase Chain Reaction Analysis of Cell-Free Fetal DNA in Pregnancy of RhD Negative Women in South of Iran. Int J Fertili Steril. 2016;10(1):62.

22. Reddy A, Zhong X, Rusterholz C, Hahn S, Holzgreve W, Redman C, *et al*. The effect of labour and placental separation on the shedding of syncytiotrophoblast microparticles, cell-free DNA and mRNA in normal pregnancy and pre-eclampsia. Placenta. 2008;29(11):942-9.
23. Sekizawa A, Jimbo M, Saito H, Iwasaki M, Matsuoka R, Okai T, *et al*. Cell-free fetal DNA in the plasma of pregnant women with severe fetal growth restriction. Am Journal Obstet Gynecol. 2003;188(2):480-4.
24. Sifakis S, Zaravinos A, Maiz N, Spandidos DA, Nicolaides KH. First-trimester maternal plasma cell-free fetal DNA and preeclampsia. Am J Obstet Gynecol. 2009;201(5):472. e1-. e7.
25. Li X, Tan H, Huang X, Zhou S, Hu S, Wang X, *et al*. Similarities and differences between the risk factors for gestational hypertension and preeclampsia: A population based cohort study in south China. Pregn Hypertens: An International Journal of Women's Cardiovascular Health. 2016;6(1):66-71.
26. Villar J, Carroli G, Wojdyla D, Abalos E, Giordano D, Ba'aqeel H, *et al*. Preeclampsia, gestational hypertension and intrauterine growth restriction, related or independent conditions? Am J Obstet Gynecol. 2006;194(4):921-31.
27. Noori M, Donald AE, Angelakopoulou A, Hingorani AD, Williams DJ. Prospective study of placental angiogenic factors and maternal vascular function before and after preeclampsia and gestational hypertension. Circulation. 2010;122(5):478-87.
28. Romero-Arauz J, Ortiz-Díaz C, Leaños-Miranda A, Martínez-Rodríguez O. [Progression of gestational hypertension to preeclampsia]. Ginecol Obstet Mex. 2014;82(4):229-35.
29. Saudan P, Brown MA, Buddle ML, Jones M. Does gestational hypertension become pre-eclampsia? BJOG: An International Journal of Obstetrics & Gynaecology. 1998;105(11):1177-84.
30. Sibai BM. Diagnosis and management of gestational hypertension and preeclampsia. Obstet Gynecol. 2003;102(1):181-92.
31. Papantoniou N, Bagiokos V, Agiannitopoulos K, Kolialexi A, Destouni A, Tounta G, *et al*. RASSF1A in maternal plasma as a molecular marker of preeclampsia. Prenatal Diag. 2013;33(7):682-7.
32. Tranquilli AL, Dekker G, Magee L, Roberts J, Sibai BM, Steyn W, *et al*. The classification, diagnosis and management of the hypertensive disorders of pregnancy: A revised statement from the ISSHP. Preg Hypertens: An International Journal of Women's Cardiovascular Health. 2014;4(2):97-104.
33. Lisonkova S, Joseph K. Incidence of preeclampsia: risk factors and outcomes associated with early-versus late-onset disease. Am J Obstet Gynecol. 2013;209(6):544. e1-. e12.

3 CHAPTER 3: SYNTHESIS, CONCLUSION AND RECOMENDATION

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 cffDNA and cftDNA using the RASSF1A gene as it is now accepted as a universal marker for the quantification of cffDNA 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 cffDNA and ctfDNA, 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 cffDNA and cftDNA 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 cffDNA and cftDNA 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 cffDNA 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 cffDNA 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

Abalos, E., Cuesta, C., Grosso, A. L., Chou, D. & Say, L. 2013. Global and regional estimates of preeclampsia and eclampsia: a systematic review. *European Journal of Obstetrics & Gynecology and Reproductive Biology,* 170**,** 1-7.

Abdelhalim, R. M., Ramadan, D. I., Zeyada, R., Nasr, A. S. & Mandour, I. A. 2016. Circulating Maternal Total Cell-Free DNA, Cell-Free Fetal DNA and Soluble Endoglin Levels in Preeclampsia: Predictors of Adverse Fetal Outcome? A Cohort Study. *Molecular diagnosis & therapy,* 20**,** 135-149.

Alberry, M., Maddocks, M., Jones, M., Abdel Hadi, M., Abdel-Fattah, S., Avent, N. & Soothill, P. 2007. Free fetal DNA in maternal plasma in anembryonic pregnancies: confirmation that the origin is the trophoblast. *Prenatal diagnosis,* 27**,** 415-418.

Alberry, M. S., Maddocks, D. G., Hadi, M. A., Metawi, H., Hunt, L. P., Abdel-Fattah, S. A., Avent, N. D. & Soothill, P. W. 2009. Quantification of cell free fetal DNA in maternal plasma in normal pregnancies and in pregnancies with placental dysfunction. *American Journal of Obstetrics and Gynecology,* 200**,** 98.e1-98.e6.

Barra, G. B., Santa Rita, T. H., De Almeida Vasques, J., Chianca, C. F., Nery, L. F. A. & Costa, S. S. S. 2015. EDTA-mediated inhibition of DNases protects circulating cell-free DNA from ex vivo degradation in blood samples. *Clinical biochemistry*.48, 976-981

Barra, S., Do Carmo Cachulo, M., Providência, R. & Leitão-Marques, A. 2012. Hypertension in pregnancy: The current state of the art. *Revista Portuguesa de Cardiologia (English Edition),* 31**,** 425-432.

BianchI, D. 2004. Circulating fetal DNA: its origin and diagnostic potential—a review. *Placenta,* 25**,** S93-S101.

Borzychowski, A. M., Sargent, I. L. & Redman, C. W. G. 2006. Inflammation and pre-eclampsia. *Seminars in Fetal and Neonatal Medicine,* 11**,** 309-316.

Chan, K. A., Ding, C., Gerovassili, A., Yeung, S. W., Chiu, R. W., Leung, T. N., Lau, T. K., Chim, S. S., Chung, G. T. & Nicolaides, K. H. 2006. Hypermethylated RASSF1A in maternal plasma: a

universal fetal DNA marker that improves the reliability of noninvasive prenatal diagnosis. *Clinical chemistry,* 52**,** 2211-2218.

Chim, S. S., Tong, Y. K., Chiu, R. W., Lau, T. K., Leung, T. N., Chan, L. Y., Oudejans, C. B., Ding, C. & Lo, Y. D. 2005. Detection of the placental epigenetic signature of the maspin gene in maternal plasma. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 14753-14758.

Craici, I. M., Wagner, S. J., Bailey, K. R., Fitz-Gibbon, P. D., Wood-Wentz, C. M., Turner, S. T., Hayman, S. R., White, W. M., Brost, B. C. & Rose, C. H. 2013. Podocyturia Predates Proteinuria and Clinical Features of Preeclampsia Longitudinal Prospective Study. *Hypertension,* 61**,** 1289-1296.

Cruz, M. O., Gao, W. & Hibbard, J. U. 2012. What is the optimal time for delivery in women with gestational hypertension? *American Journal of Obstetrics and Gynecology,* 207**,** 214.e1-214.e6.

Cuckle, H., Benn, P. & Pergament, E. 2015. Cell-free DNA screening for fetal aneuploidy as a clinical service. *Clinical Biochemistry*. 48, 932-941.

Daniels, G., Finning, K., Martin, P. & Massey, E. 2009. Noninvasive prenatal diagnosis of fetal blood group phenotypes: current practice and future prospects. *Prenatal diagnosis,* 29**,** 101-107.

Devaney, s. a., Palomaki, G. E., Scott, J. A. & Bianchi, D. W. 2011. Noninvasive fetal sex determination using cell-free fetal DNA: a systematic review and meta-analysis. *Jama,* 306**,** 627-636.

Eastabrook, G., Brown, M. & Sargent, I. 2011. The origins and end-organ consequence of pre-eclampsia. *Best Practice & Research Clinical Obstetrics & Gynaecology,* 25**,** 435-447.

Fong, F. M., Sahemey, M. K., Hamedi, G., Eyitayo, R., Yates, D., Kuan, V., Thangaratinam, S. & Walton, R. T. 2014. Maternal genotype and severe preeclampsia: a HuGE review. *American Journal of Epidemiology,* 180**,** 335-345.

Ghulmiyyah, L. & Sibai, B. 2012. Maternal Mortality From Preeclampsia/Eclampsia. *Seminars in Perinatology,* 36**,** 56-59.

Goulopoulou, S. & Davidge, S. T. 2015. Molecular mechanisms of maternal vascular dysfunction in preeclampsia. *Trends in molecular medicine,* 21**,** 88-97.

Granger, J. P., Alexander, B. T., Bennett, W. A. & Khalil, R. A. 2001. Pathophysiology of pregnancy-induced hypertension. *American Journal of Hypertension,* 14**,** 178S-185S.

Hahn, S., Giaglis, S., Buser, A., Hoesli, I., Lapaire, O. & Hasler, P. 2014. Cell-free nucleic acids in (maternal) blood: any relevance to (reproductive) immunologists? *Journal of reproductive immunology,* 104**,** 26-31.

Hahn, S., Rusterholz, C., Hösli, I. & Lapaire, O. 2011. Cell-free nucleic acids as potential markers for preeclampsia. *Placenta,* 32**,** S17-S20.

Hill, M., Barrett, A. N., White, H. & Chitty, L. S. 2012. Uses of cell free fetal DNA in maternal circulation. *Best Practice & Research Clinical Obstetrics & Gynaecology,* 26**,** 639-654.

Huppertz, B. 2004. Apoptosis in the trophoblast—role of apoptosis in placental morphogenesis. *Journal of the Society for Gynecologic Investigation,* 11**,** 353-362.

Hutcheon, J. A., Lisonkova, S. & Joseph, K. 2011. Epidemiology of pre-eclampsia and the other hypertensive disorders of pregnancy. *Best practice & research Clinical obstetrics & gynaecology,* 25**,** 391-403.

Jakobsen, T. R., Clausen, F. B., Rode, l., Dziegiel, M. H. & Tabor, A. 2013. Identifying mild and severe preeclampsia in asymptomatic pregnant women by levels of cell-free fetal DNA. *Transfusion,* 53**,** 1956-1964.

Kim, H. J., Kim, S. Y., Lim, J. H., Kwak, D. W., Park, S. Y. & Ryu, H. M. 2015. Quantification and application of potential epigenetic markers in maternal plasma of pregnancies with hypertensive disorders. *International journal of molecular sciences,* 16**,** 29875-29888.

Lazar, L., Rigó, J., Nagy, B., Balogh, K., Makó, V., Cervenak, L., Mézes, M., Prohászka, Z. & Molvarec, A. 2009. Relationship of circulating cell-free DNA levels to cell-free fetal DNA levels, clinical characteristics and laboratory parameters in preeclampsia. *BMC medical genetics,* 10**,** 120.

Li, X., Tan, H., Huang, X., Zhou, S., Hu, S., Wang, X., Xu, X., Liu, Q. & Wen, S. W. 2016. Similarities and differences between the risk factors for gestational hypertension and preeclampsia: A population based cohort study in south China. *Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health,* 6**,** 66-71.

Lin, S., Leonard, D., Co, M. A. M., Mukhopadhyay, D., Giri, B., Perger, L., Beeram, M. R., Kuehl, T. J. & Uddin, M. N. 2015. Pre-eclampsia has an adverse impact on maternal and fetal health. *Translational Research,* 165**,** 449-463.

Lisonkova, S. & Joseph, K. 2013. Incidence of preeclampsia: risk factors and outcomes associated with early-versus late-onset disease. *American journal of obstetrics and gynecology,* 209**,** 544. e1-544. e12.

Lo, Y. M. D., Corbetta, N., Chamberlain, P. F., Rai, V., Sargent, I. L., Redman, C. W. G. & WAINSCOAT, J. S. 1997. Presence of fetal DNA in maternal plasma and serum. *The Lancet,* 350**,** 485-487.

Lui, Y. Y., Chik, K.-W., Chiu, R. W., Ho, C.-Y., Lam, C. W. & Lo, Y. D. 2002. Predominant hematopoietic origin of cell-free DNA in plasma and serum after sex-mismatched bone marrow transplantation. *Clinical chemistry,* 48**,** 421-427.

Mackay, A. P., Berg, C. J. & Atrash, H. K. 2001. Pregnancy-related mortality from preeclampsia and eclampsia. *Obstetrics & Gynecology,* 97**,** 533-538.

Magee, L. A., Pels, A., Helewa, M., Rey, E. & Von Dadelszen, P. 2014. Diagnosis, evaluation, and management of the hypertensive disorders of pregnancy. *Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health,* 4**,** 105-145.

Magee, L. A., Pels, A., Helewa, M., Rey, E. & Von Dadelszen, P. 2015. The hypertensive disorders of pregnancy (29.3). *Best Practice & Research Clinical Obstetrics & Gynaecology,* 29**,** 643-657.

Mandel, P. M., P 1948. Les acides nucleiques du plasma sanguin chez l'Homme. *C R Acad Sci Paris,* 142**,** 241-243.

Manokhina, I., Singh, T. K., Peñaherrera, M. S. & Robinson, W. P. 2014. Quantification of cell-free DNA in normal and complicated pregnancies: overcoming biological and technical issues. *PloS one,* 9**,** e101500.

Martin, A., Krishna, I., Martina, B. & Samuel, A. 2014. Can the quantity of cell-free fetal DNA predict preeclampsia: a systematic review. *Prenatal diagnosis,* 34**,** 685-691.

Masuyama, H., Segawa, T., Sumida, Y., Masumoto, A., Inoue, S., Akahori, Y. & Hiramatsu, Y. 2010. Different profiles of circulating angiogenic factors and adipocytokines between early-and late-onset pre-eclampsia. *BJOG: An International Journal of Obstetrics & Gynaecology,* 117**,** 314-320.

Moezzi, L., Keshavarz, Z., Ranjbaran, R., Aboualizadeh, F., Behzad-Behbahani, A., Abdullahi, M., Ramezani, A., Samsami, A. & Sharifzadeh, S. 2016. Fetal RHD Genotyping Using Real-Time Polymerase Chain Reaction Analysis of Cell-Free Fetal DNA in Pregnancy of RhD Negative Women in South of Iran. *International journal of fertility & sterility,* 10**,** 62.

Mol, B. W., Roberts, C. T., Thangaratinam, S., Magee, L. A., De Groot, C. J. & Hofmeyr, G. J. 2016. Pre-eclampsia. *The Lancet,* 387**,** 999-1011.

Moodley, J. 2011. Maternal deaths associated with hypertension in South Africa: lessons to learn from the Saving Mothers report, 2005–2007. *Cardiovascular journal of Africa,* 22**,** 31.

Naljayan, M. V. & Karumanchi, S. A. 2013. New developments in the pathogenesis of preeclampsia. *Advances in chronic kidney disease,* 20**,** 265-270.

Noori, M., Donald, A. E., Angelakopoulou, A., Hingorani, A. D. & Williams, D. J. 2010. Prospective study of placental angiogenic factors and maternal vascular function before and after preeclampsia and gestational hypertension. *Circulation,* 122**,** 478-487.

Osungbade, K. O. & Ige, O. K. 2011. Public health perspectives of preeclampsia in developing countries: implication for health system strengthening. *Journal of pregnancy,* 2011.

Papantoniou, N., Bagiokos, V., Agiannitopoulos, K., Kolialexi, A., Destouni, A., Tounta, G., Kanavakis, E., Antsaklis, A. & Mavrou, A. 2013. RASSF1A in maternal plasma as a molecular marker of preeclampsia. *Prenatal diagnosis,* 33**,** 682-687.

Powe, C. E., Levine, R. J. & Karumanchi, S. A. 2011. Preeclampsia, a disease of the maternal endothelium the role of antiangiogenic factors and implications for later cardiovascular disease. *Circulation,* 123**,** 2856-2869.

Rahman, M., Abe, S., Kanda, M., Narita, S., Rahman, M., Bilano, V., Ota, E., Gilmour, S. & Shibuya, K. 2015. Maternal body mass index and risk of birth and maternal health outcomes in low-and middle-income countries: a systematic review and meta-analysis. *Obesity Reviews,* 16**,** 758-770.

Ramsay, J. E., Ferrell, W. R., Crawford, L., Wallace, A. M., Greer, I. A. & Sattar, N. 2002. Maternal obesity is associated with dysregulation of metabolic, vascular, and inflammatory pathways. *The Journal of Clinical Endocrinology & Metabolism,* 87**,** 4231-4237.

Raymond, D. & Peterson, E. 2011. A critical review of early-onset and late-onset preeclampsia. *Obstetrical & gynecological survey,* 66**,** 497-506.

Reddy, A., Zhong, X., Rusterholz, C., Hahn, S., Holzgreve, W., Redman, C. & Sargent, I. 2008. The effect of labour and placental separation on the shedding of syncytiotrophoblast microparticles, cell-free DNA and mRNA in normal pregnancy and pre-eclampsia. *Placenta,* 29**,** 942-949.

Redman, C. W. G. 2011. Preeclampsia: A multi-stress disorder. *La Revue de Médecine Interne,* 32, Supplement 1**,** S41-S44.

Redman, C. W. G. & Sargent, I. L. 2001. The pathogenesis of pre-eclampsia. *Gynécologie Obstétrique & Fertilité,* 29**,** 518-522.

Romero-Arauz, J., Ortiz-Díaz, C., Leaños-Miranda, A. & Martínez-Rodríguez, O. 2014. [Progression of gestational hypertension to preeclampsia]. *Ginecologia y obstetricia de Mexico,* 82**,** 229-235.

Salvianti, F., Inversetti, A., Smid, M., Valsecchi, L., Candiani, M., Pazzagli, M., Cremonesi, L., Ferrari, M., Pinzani, P. & Galbiati, S. 2015. Prospective evaluation of RASSF1A cell-free DNA as a biomarker of pre-eclampsia. *Placenta,* 36**,** 996-1001.

Saudan, P., Brown, M. A., Buddle, M. L. & Jones, M. 1998. Does gestational hypertension become pre-eclampsia? *BJOG: An International Journal of Obstetrics & Gynaecology,* 105**,** 1177-1184.

Saving Mothers 2011-2013. A short report of the National Committee on Confidential Enquiries into Maternal Deaths. Pattinson R ed. Nat Dept of Halth. Pretoria. 2015.

Say, L., Chou, D., Gemmill, A., Tunçalp, Ö., Moller, A.-B., Daniels, J., Gülmezoglu, A. M., Temmerman, M. & Alkema, L. 2014. Global causes of maternal death: a WHO systematic analysis. *The Lancet Global Health,* 2**,** e323-e333.

Sekizawa, A., Jimbo, M., Saito, H., Iwasaki, M., Matsuoka, R., Okai, T. & Farina, A. 2003. Cell-free fetal DNA in the plasma of pregnant women with severe fetal growth restriction. *American Journal of Obstetrics and Gynecology,* 188**,** 480-484.

Sekizawa, A., Kondo, T., Iwasaki, M., Watanabe, A., Jimbo, M., Saito, H. & Okai, T. 2001. Accuracy of fetal gender determination by analysis of DNA in maternal plasma. *Clinical chemistry,* 47**,** 1856-1858.

Shah, D. M. 2007. Preeclampsia: new insights. *Current opinion in nephrology and hypertension,* 16**,** 213-220.

Sibai, B. M. 2003. Diagnosis and management of gestational hypertension and preeclampsia. *Obstetrics & Gynecology,* 102**,** 181-192.

Sibai, B. M. 2011. Management of Late Preterm and Early-Term Pregnancies Complicated by Mild Gestational Hypertension/Pre-Eclampsia. *Seminars in Perinatology,* 35**,** 292-296.

Sifakis, S., Koukou, Z. & Spandidos, D. A. 2015. Cell-free fetal DNA and pregnancy-related complications (Review). *Molecular medicine reports,* 11**,** 2367-2372.

Sifakis, S., Zaravinos, A., Maiz, N., Spandidos, D. A. & Nicolaides, K. H. 2009. First-trimester maternal plasma cell-free fetal DNA and preeclampsia. *American journal of obstetrics and gynecology,* 201**,** 472. e1-472. e7.

Souza, J. P., Gülmezoglu, A. M., Vogel, J., Carroli, G., Lumbiganon, P., Qureshi, Z., Costa, M. J., Fawole, B., Mugerwa, Y. & Nafiou, I. 2013. Moving beyond essential interventions for reduction of maternal mortality (the WHO Multicountry Survey on Maternal and Newborn Health): a cross-sectional study. *The Lancet,* 381**,** 1747-1755.

Steegers, E. A. P., Von dadelszen, P., Duvekot, J. J. & Pijnenborg, R. 2010. Pre-eclampsia. *The Lancet,* 376**,** 631-644.

Swank, M., Caughey, A., Farinelli, C., Main, E., Melsop, K., Gilbert, W. & Chung, J. 2014. The impact of change in pregnancy body mass index on the development of gestational hypertensive disorders. *Journal of Perinatology,* 34.

Taglauer, E., Wilkins-Haug, L. & Bianchi, D. 2014. Review: cell-free fetal DNA in the maternal circulation as an indication of placental health and disease. *Placenta,* 35**,** S64-S68.

Tannetta, D. & Sargent, I. 2013. Placental disease and the maternal syndrome of preeclampsia: missing links? *Current hypertension reports,* 15**,** 590-599.

Thangaratinam, S., Koopmans, C. M., Iyengar, S., Zamora, J., Ismail, K. M., Mol, B. W. & Khan, K. S. 2011. Accuracy of liver function tests for predicting adverse maternal and fetal outcomes in women with preeclampsia: a systematic review. *Acta obstetricia et gynecologica Scandinavica,* 90**,** 574-585.

Tranquilli, A. L., Dekker, G., Magee, L., Roberts, J., Sibai, B. M., Steyn, W., Zeeman, G. G. & Brown, M. A. 2014. The classification, diagnosis and management of the hypertensive disorders of pregnancy: A revised statement from the ISSHP. *Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health,* 4**,** 97-104.

Valensise, H., Vasapollo, B., Gagliardi, G. & Novelli, G. P. 2008. Early and late preeclampsia two different maternal hemodynamic states in the latent phase of the disease. *Hypertension,* 52**,** 873-880.

Villar, J., Carroli, G., Wojdyla, D., Abalos, E., Giordano, D., Ba'Aqeel, H., Farnot, U., Bergsjø, P., Bakketeig, L., Lumbiganon, P., Campodónico, L., Al-Mazrou, Y., Lindheimer, M. & Kramer, M. 2006. Preeclampsia, gestational hypertension and intrauterine growth restriction, related or independent conditions? *American Journal of Obstetrics and Gynecology,* 194**,** 921-931.

Von Dadelszen, P., Magee, L. A. & Roberts, J. M. 2003. Subclassification of preeclampsia. *Hypertension in pregnancy,* 22**,** 143-148.

Walker, J. J. 2000. Pre-eclampsia. *The Lancet,* 356**,** 1260-1265.

Watanabe, K., Naruse, K., Tanaka, K., Metoki, H. & Suzuki, Y. 2013. Outline of definition and classification of "pregnancy induced hypertension (PIH)". *Hypertension Research in Pregnancy,* 1**,** 3-4.

Watson, J. D. & Crick, F. H. The structure of DNA. Cold Spring Harbor Symposia on Quantitative Biology, 1953. Cold Spring Harbor Laboratory Press, 123-131.

White, H. E., Dent, C. L., Hall, V. J., Crolla, J. A. & Chitty, L. S. 2012. Evaluation of a novel assay for detection of the fetal marker RASSF1A: facilitating improved diagnostic reliability of noninvasive prenatal diagnosis. *PloS one,* 7**,** e45073.

Yu, H., Shen, Y., Ge, Q., He, Y., Qiao, D., Ren, M. & Zhang, J. 2013. Quantification of maternal serum cell-free fetal DNA in early-onset preeclampsia. *International journal of molecular sciences,* 14**,** 7571-7582.

Zejskova, L., Jancuskova, T., Kotlabova, K., Doucha, J. & Hromadnikova, I. 2010. Feasibility of fetal-derived hypermethylated RASSF1A sequence quantification in maternal plasma—Next step toward reliable non-invasive prenatal diagnostics. *Experimental and molecular pathology,* 89**,** 241-247.

APENDIX A

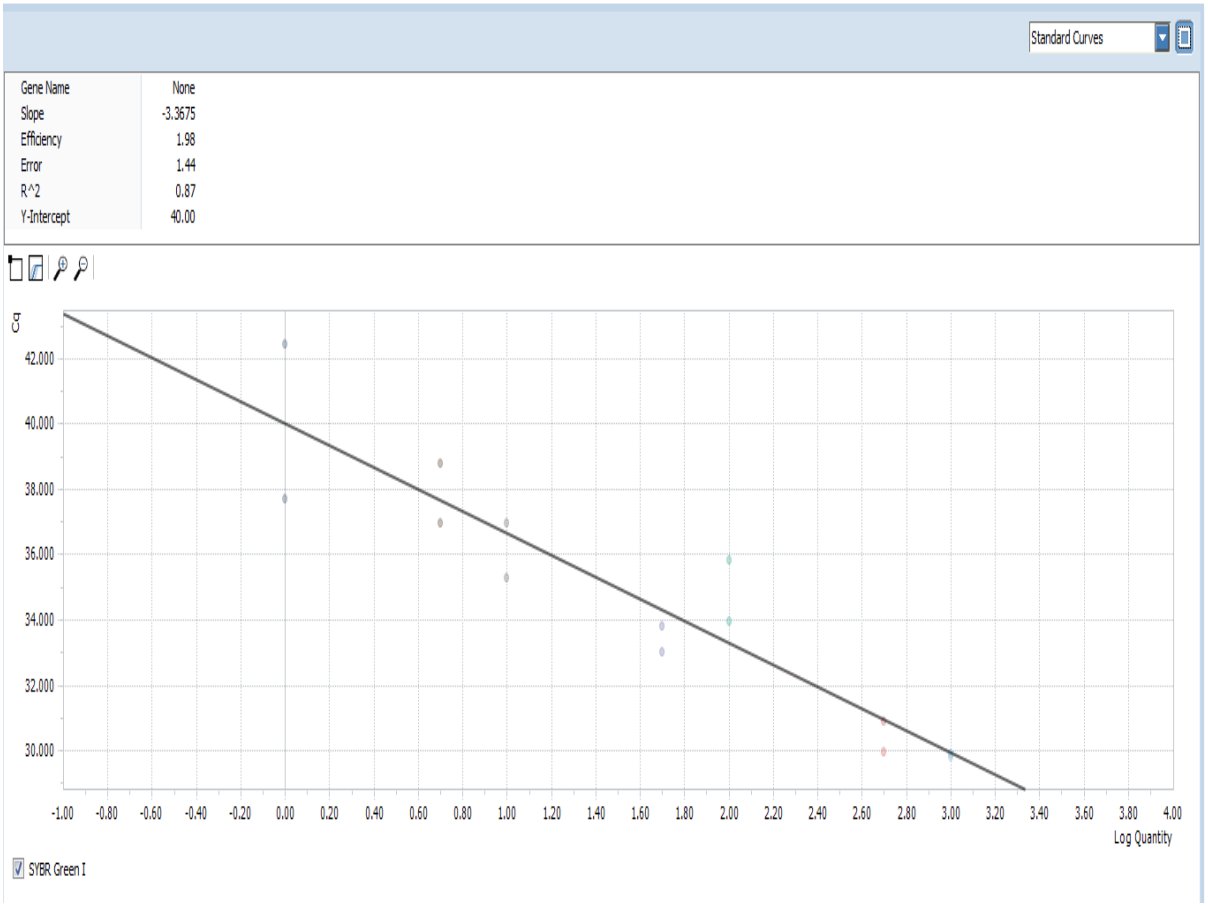


Figure 1. Standard curve using known concentration of genomic DNA to determine the concentration of circulating cffDNA and cftDNA in plasma.

APENDIX B

Research approval from the Kwazulu-Natal Department of health

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

Physical Address: 330 Langalibalele Street, Pietermaritburg
Postal Address: Private Bag X9051
Tel: 033 395 2805/ 3189/ 3123 Fax: 033 394 3782
Email: hrkm@kznhealth.gov.za
www.kznhealth.gov.za

DIRECTORATE:
Health Research & Knowledge Management

Reference: 269/15
KZ_2015RP30_889

Date: 18 November 2015

Dear Mr S. Eche
(University of KwaZulu Natal)
Email: echesimem@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 hrkm@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: 20/11/15

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
INYUVESI YAKWAZULU-NATALI

27 November 2015

Mr S Eche (215000273)
Medical Biochemistry
School Of Laboratory Medicine and Medical Sciences
echesimem@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.

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

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

The sub-committee's decision will be **RATIFIED** by a full Committee at its 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: mackraji@ukzn.ac.za
cc postgrad: dudhrajhp@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 2486 **Facsimile:** +27 (0) 31 260 4609 **Email:** brec@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

APENDIX D

Prince Mshiyeni Memorial Hospital Approval Letter to Conduct Research

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

PRINCE MSHIYENI MEMORIAL HOSPITAL
MEDICAL MANAGER OFFICE
DR. A.Hussain
Private Bag X07; Mobeni 4060
Mangosuthu Highway
Tel.:031-907 8317/ 8304
Fax.:031- 906 1044
Email.:Aktarhussain@kznhealth.gov.za
www.kznhealth.gov.za

Enquiry: Dr A.HUSSAIN
Ref No:30 /RESH/2105
Date: 16/10/2015

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, PGDip in HIV/AIDS.DCH .ADV Health Management
M.Med.Fam.Med (Natal)

APENDIX E

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:

Hospital number	Admission number

PATIENT DEMOGRAPHICS:

Date of Birth	Rural dweller	Urban dweller	Life style

Clinical Data

Gravidity	
Parity	
Gestational age	
Maternal Height	
Maternal weight	
Systolic blood pressure	
Diastolic blood pressure	
Proteinuria	
HIV Status	
If HIV positive, CD4 count	

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)