THE ROLE OF ENDOPLASMIC RETICULUM STRESS IN SHEDDING OF SYNCYTIOTROPHOBLAST MICROPARTICLES IN PREGNANT BLACK SOUTH AFRICAN WOMEN

By

SONAL RAJ VERMA

(215081900)

Submitted in partial fulfilment for the degree of

MASTER OF MEDICAL SCIENCE

In the Department of Physiology

College of Health Sciences

University of KwaZulu-Natal

South Africa

2016

PREFACE

This study represents original work by the author and has not been submitted in any other form to another University. Where use was made of the work of others, it has been duly acknowledged in the text.

The research described in this dissertation was carried out in the Discipline of Human Physiology, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa.

Dr Sonal Raj Verma

(215081900)

Professor I Mackraj

(Supervisor)

Professor T Naicker

(Co-supervisor)

DECLARATION

I, Dr Sonal Raj Verma declare that:

(i) The research reported in this dissertation, except where otherwise indicated is my original work.

(Tomimatsu et al.) 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:

a) Their words have been rewritten but the general information attributed by them has been referenced.

b) 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: 14-03-2017

DEDICATION

Dedicated to my parents, my parents-in-laws and my dear husband

ACKNOWLEDGEMENTS

I would like to express my gratitude to the following people

- Professor Irene Mackraj for her guidance and support in the completion of this study
- Professor T. Naicker for her guidance and support both in writing the dissertation and the laboratory work (Immunohistochemistry)
- Professor Jagidesa Moodley for his continuous support and clinical input
- Denise Margolis for teaching me the steps of the immunohistochemistry assay
- Dr. Kogi Moodley, and my friends Mr. Simeon Eche, Mr. Preenan Pillay
- Miss Zinhle Mkhize for helping me with the sample collection
- My father-in -law, Dr. C.P. Shrivastava, and my mother-in-law, Mamta Shrivastava; there's never a time when you have not been there for me. Thank you for always giving me direction, and for constantly reminding me of all the possibilities. Thank you for always believing in me in my rough times.
- My husband Mr. Abhishek Shrivastava, who always stood by me. Thank you
- And to my parents for everything

TABLE OF CONTENTS

LIST OF FIGURES

Figures

LIST OF TABLES

PUBLICATION

Verma Sonal , Pillay Preenan, Naicker Thajasvarie, Moodley Jagidesa and Mackraj Irene (2016): Placental Hypoxia Inducible factor -1α and CHOP Immunohistochemical expression Relative to Maternal Circulatory Syncytiotrophoblast Microvesicles in Preeclamptic and Normotensive Pregnancies, Submitted to Placenta PLAC-S-16-00708.

LIST OF ABBREVIATIONS

PE	Preeclampsia
STBMs	Syncytiotrophoblast microparticles
ER	Endoplasmic Reticulum
BREC	Biomedical Research Ethics Committee
HIF-1 α	Hypoxia Inducible Factor-1 alpha
CHOP	C/EBP homologous protein
PLGF	Placenta growth factor
VEGF	Vascular endothelial growth factor
PLAP	Placental alkaline phosphatase
HELLP	Haemolysis, elevated liver enzymes, and low platelets
HIV	Human Immunodeficiency virus

CONFERENCE ATTENDANCE

Poster presentation entitled “PLACENTAL EXPRESSION OF HIF- 1ALPHA IN BLACK SOUTH AFRICAN PREGNANT WOMEN” at the 54th Annual Conference of Microscopic Society of Southern Africa, 5-8 December 2016, Port Elizabeth, Eastern Cape, South Africa.

ABSTRACT

Background and aim: Preeclampsia, accounts for the majority of maternal deaths emanating from hypertension in pregnancy. Although its exact aetiology is unclear, endoplasmic reticulum (ER) stress and trophoblast apoptosis are implicated. The placental microenvironment in preeclampsia is hypoxic and induces the expression of Hypoxia inducible factor-1 alpha (HIF-1 α) and CHOP (C/EBP homologous protein) which are activated due to ER stress. Hypoxia-induced oxidative and endoplasmic reticulum stress initiate a cascade of apoptotic events with the consequential shedding of microparticles which mediate the peripheral maternal syndrome of preeclampsia. The main aim of the study was to immuno-localise the expression of HIF-1 α and CHOP in placental tissues and concomitantly characterise and quantify the syncytiotrophoblast microparticles in the maternal circulation.

Materials and Methods: Plasma and placental tissue were obtained from normotensive and pre-eclamptic pregnant women. The expression of HIF-1α and CHOP was analysed using immunohistochemistry. Microvesicles in maternal circulation were isolated and their size distribution was determined using nanoparticle tracking analysis. The relative concentration of syncytiotrophoblast microvesicles (STBMs) from isolated microvesicles was determined using Placental Alkaline Phosphatase ELISA.

Results: This study demonstrated an increased immuno-expression of HIF-1 α and CHOPS in preeclampsia compared to the controls ($p < 0.05$). Additionally, a significant increase in the mean syncytiotrophoblast microparticles concentration was observed in PE, compared to the controls ($p < 0.05$). Further analysis showed a positive correlation between the immunohistochemical expression of HIF-1 α and CHOP and the STBMs concentration.

Conclusion: This study demonstrates increased placental-expression of HIF-1α and CHOP in preeclampsia compared to normotensive pregnancies which directly relate to the increase syncytiotrophoblast microvesicles concentration in maternal circulation. These findings

indicate that placental hypoxia and ER stress are contributory factors to the pathogenesis of PE and may be a key contributory factor in placental cell apoptosis and the consequent release of placental derived debris into the maternal circulation.
Key words: HIF-1α; CHOP; Syncytiotrophoblast Microvesicles; Pre- eclampsia; Endoplasmic Reticulum Stress; Hypoxia

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION AND LITERATURE REVIEW

1.1 Background

Preeclampsia (PE), a pregnancy-specific disorder, is clinically characterised by new-onset hypertension and proteinuria after 20th week of gestation.PE remains a grave obstetric disorder and a major cause of maternal and fetal morbidity and mortality (Tannetta *et al.*, 2013b). The disproportionately higher rates of PE in African women has been observed, however, the exact cause remains elusive (Rana and Karumanchi, 2017).

The hallmark of PE is abnormal placentation and an angiogenic/antiangiogenic imbalance causing a vascular dysfunction (Wenger, 2014).PE is a multisystem disease with the spectrum ranging from cardiac, cerebral, renal and haematological impairment (Rana and Karumanchi, 2017). Severe form of PE may result in the development of HELLP syndrome (haemolysis, elevated liver enzymes, and low platelets) which is a mirror image of liver abnormalities (Isler *et al*., 1999, Weiner *et al*., 2016).

1.1.1 Prevalence

The global incidence of PE is estimated to afflict 3-5% of all pregnancies (Mol *et al*., 2016). Hypertensive disorders account for 14.8% of maternal deaths in South Africa and PE accounts for the majority of maternal deaths emanating from hypertension (Cuevas et al., 2015). It is also responsible for the iatrogenic preterm delivery and is considered to be an important and common medical complication of pregnancy (Jeyabalan, 2013). It not only increases the significant health risk later in life but also an increase in health care cost.

1.1.2 Classification of Preeclampsia

Preeclampsia can be sub-categorised in various ways; classified by the onset and severity. Based on the onset of the disorder, it can be classified as early onset or late-onset PE (LOPE). Preeclampsia before 34 weeks of gestation defined as Early Onset PE (EOPE) while that which emerges after 34 weeks is defined as Late Onset PE (Watanabe *et al*., 2013).

It can also be classified based on severity as mild PE which is characterised by a blood pressure of ≥ 140/90 mmHg but < 160/110 mmHg after 20 weeks gestation, and proteinuria of ≥ 300 mg/24 hours without exceeding 2.0 g/24 hours or 3 + dipstick (Watanabe *et al*., 2013). Severe PE is defined by a blood pressure of ≥ 160/110 mmHg after 20 weeks gestation, and proteinuria of > 2.0 g/24 hours (Leeman *et al*., 2016).

1.1.3 Risk factors for Preeclampsia

Preeclampsia is commonly referred as the 'disease of theories.' Risk factors that predispose mothers to preeclampsia development include nulliparity, an age of ≥ 40 years, obesity, previous history of PE or gestational hypertension, women with previous history of renal disease, previous history of vascular disease, and a long interval between pregnancies (English *et al*., 2015). The history of hypertension before pregnancy also may lead to superimposed preeclampsia on chronic hypertension (Lin *et al*., 2015).

Genetic factors play a role, as both the maternal and paternal family history of this disease predisposes the woman to PE (Lin *et al*., 2015). Multiple gestations is also a risk factor as the risk of developing PE is increased with the number of fetuses the pregnant woman is carrying, which may be as a result of increased placental mass. Further, the change in paternity from a previous pregnancy, the use of barrier contraception as well as conception through the use of intercytoplasmic sperm injection results in limited priming to paternal antigens and as a result predisposes the woman to development of preeclampsia (Powe et al., 2011b). The other risk factors that are implicated in development of preeclampsia are ethnicity and race. The incidence of PE is found to be increased in African-American compared to Caucasians (Breathett *et al*., 2013). Moreover, a family history of preeclampsia nearly triples the risk of preeclampsia development (Jeyabalan, 2013).

1.1.4 Pathogenesis of Preeclampsia

The exact aetiology of PE is enigmatic despite years of research invested in pursuit of a'cure'. The pathology is, however, well described. During normal placentation, the

trophoblast invades the maternal placental spiral arteries, resulting in extensive remodelling of these vessels, to ensure an adequate blood supply to meet the demands of the growing fetus (Lee et al., 2012a). In PE, this process of remodelling is impaired causing hypoxic ischaemic reperfusion injury. These vascular changes and local hypoxia of surrounding tissues lead to necrosis and other pathological changes and functional derangements in various organ systems (Tannetta *et al*., 2013b). Hence, PE has been described as a complex systemic syndrome originating in the placenta.

The development of preeclampsia takes place in 3 stages: the earlier stages characterised by abnormal placentation and a later stage where symptoms manifest (Redman and Staff, 2015, Staff *et al*., 2013).

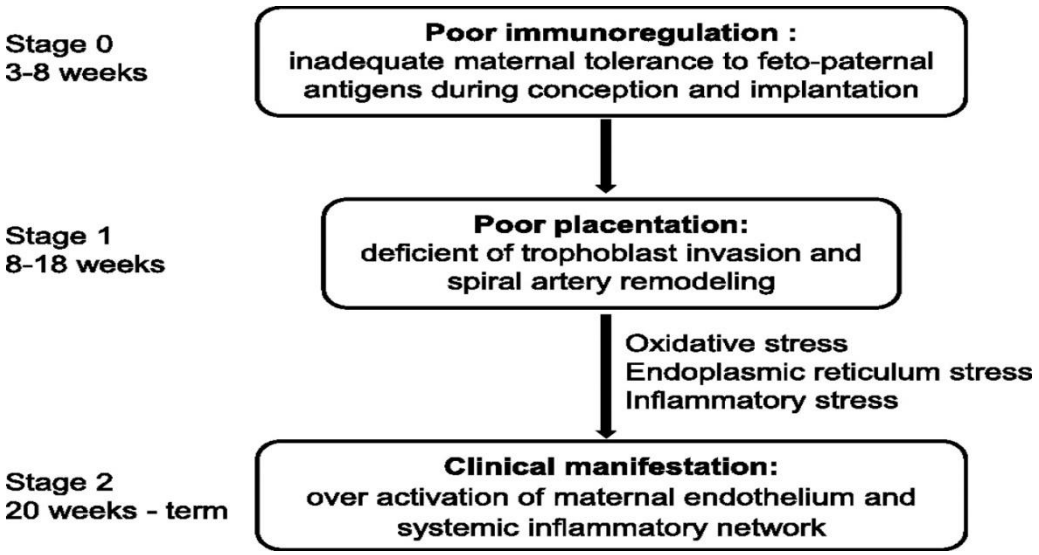


Figure 1: The three stages of preeclampsia; Adapted from (Li *et al*., 2012).

In stage 1, a clinically silent stage placental hypo perfusion develops early in pregnancy following abnormal placentation due to insufficient trophoblast invasion and the subsequent physiological change of the spiral arteries of the myometrium (until 14 to 18 weeks of gestational age) (Redman and Staff, 2015*)*. This failure of remodelling changes the constant low pressure uteroplacental perfusion to a pulsatile high pressure zone. This damages the

chorionic villi both hydro dynamically and biochemically causing an ischemic reperfusion injury (Redman and Staff, 2015). This inadequate spiral artery remodelling is a precursor of placental hypoxia, the putative culprit that initiates the whole cascade of events finally leading the maternal syndrome of preeclampsia (Tal, 2012).

The stage 2 (Figure 1) comprises of hypoxia, enhanced placental oxidative stress and endoplasmic reticulum stress (Staff *et al*., 2013). This oxidative stress releases placental factors into the maternal circulation which include exosomes, microparticles and fetal DNA (Tannetta et al., 2013b). In stage 3, this trophoblast derived debris has the potential to stimulate the systemic inflammatory response, causing endothelial dysfunction with sub sequential clinical manifestations of the disease (Redman and Sargent, 2009, Staff *et al*., 2013)

1.1.4.1 The role of angiogenic and antiangiogenic molecules in preeclampsia

The resulting ischemia of the placental cells results secreting soluble factors in to the maternal circulation, factors which are anti-angiogenic may cause maternal endothelial dysfunction (Tannetta and Sargent, 2013). Disruption in the balances between angiogenic and antiangiogenic factors is also attributed 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). 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 cytotrphoblast cells (Powe *et al*., 2011a). Soluble endoglin may combine with sFlt-1 and cause amplification of vascular injury (Powe *et al*., 2011).

1.1.5 The Placenta in Preeclampsia

The placenta plays the most significant role in the development of preeclampsia as this obstetric complication can occur in hydatidiform molar pregnancies even without a foetus.

Although the exact aetiology is unknown, it has been shown that placental factors released into the maternal circulation lead to systemic maternal inflammation and endothelial dysfunction. Gross pathological changes are seen in pre-term severe preeclampsia (Roberts and Post, 2008, Roberts and Escudero, 2012). Histological changes found in preeclampsia are believed to be because of reduced perfusion. Reduction in syncytiotrophoblast villous area has been shown in preeclampsia (Roberts and Escudero, 2012). Recent growing evidence shows that placental derived microparticles best known as syncytiotrophoblast microparticles play a major role in the development of preeclampsia (Van der Post *et al*., 2011). Factors released from the abnormal placenta within the maternal circulation stimulates the maternal systemic inflammatory response and consequently causes endothelial dysfunction (Katlama *et al*., 2013).

Placental hypoxia initiates the release of these microparticles in the maternal circulation (Rath *et al*., 2016). The key mediator of cellular hypoxia is expression of hypoxia inducible factor- 1 alpha (HIF-1 α). This protein is transiently expressed during the hypoxic condition increasing chances for cellular survival. However, persistently elevated expression can cause a deleterious effect on the syncytiotrophoblast and an angiogenic/anti-angiogenic imbalane ensues (Rath *et al*., 2016). The consistent expression of HIF-1 α under hypoxic environment makes it a suitable candidate for preeclampsia related research (Akhilesh *et al*., 2014). Oxidative stress is closely linked to endoplasmic reticulum stress.

Endoplasmic reticulum (ER) stress has recently been identified as a major regulator of cell homoestasis through its involvement in post-translational protein modifications and folding, and its capacity to activate the unfolded protein response (UPR) (Du *et al*., 2016, Moutouh *et al*., 1996).

1.2 Endoplasmic reticulum stress

Syncytiotrophoblast is equipped with high endoplasmic reticulum content in order to perform active transport of amino acids, ionic pumping, secretion of growth factors and cytokines.

These organelles are capable of adaptive responses vital to cellular homeostasis (Burton *et al*., 2016). The main function of the endoplasmic reticulum (ER) is to properly fold and process secreted and transmembrane proteins. Environmental and genetic factors that disrupt ER function can cause an accumulation of misfolded and unfolded proteins in the ER lumen, a condition termed ER stress (Burton *et al*., 2016).

Endoplasmic reticulum stress activates a cascade of signalling network called the Unfolded Protein Response (UPR). This UPR restores ER homoestasis, promoting cell survival and adaptation. However, when the adaptive mechanisms fail, the destructive pathways are initiated (Oslowski and Urano, 2011). ER stress is associated with a range of diseases, including ischemia/reperfusion injury, neurodegeneration, and diabetes making ER stress a probable instigator of pathological cell death and dysfunction (Du *et al*., 2016). As PE is also believed to be an hypoxic microenvironment, ER stress may have a role in its pathophysiology.

Placental endoplasmic reticulum stress negatively regulates the transcription of PIGF (Mizuuchi *et al*., 2016). Pregnancies complicated simultaneouly by preeclampsia and intrauterine growth restriction tend to exhibit high levels of endoplasmic reticulum stress (Yung *et al*., 2012*).* These results further support the targeting of placental ER stress as a potential new therapeutic intervention for these pregnancy complications (Mizuuchi *et al*., 2016). Higher levels of endoplasmic reticulum stress lead to activation of pro-inflammatory pathways, a feature of preeclampsia that may contribute to maternal endothelial cell activation and the aggressive feature of early preeclampsia (Burton and Yung, 2011).

1.2.1 ER stress and CHOP pathways

ER stress activates many pathways (Figure 3). It attempts to maintain intracellular homoestasis by increasing the folding of unfolded proteins, reducing the load of proteins and finally death of the cell and activation of pro-apoptotic pathways (Du *et al*., 2016). Overexpression of CHOP protein induces apoptosis, through a Bcl-2–inhibitor

mechanism. CHOP forms heterodimers with other C/EBP-family transcription factors via bZIP-domain interactions, which suppresses their binding to C/EBP sites in DNA, while promoting binding to alternative DNA sequences for target gene activation (Oyadomari and Mori, 2004). Activation of CHOP pathway leads to the initiation of apoptosis and shedding of microparticles.

1.2.2 Expression of ER stress markers in placenta

Endoplasmic reticulum (ER) stress is characterised by activation of three signalling branches: 1) PERK-pEIF2α, 2) ATF6 and 3) splicing of XBP1(U) into XBP1(S) and it is known to be implicated in pre-eclampsia (PE) as well as fetal growth restriction (FGR) (Lian et al., 2011). ER stress markers are found to be increased in the preeclamptic placenta compared to normotensive. Using real-time RT-PCR it was shown that genetic of expression of GRP78, PERK, eIF2α, ATF4, CHOP, and caspase 12 mRNA in the placentas of both the early ($p < 0.01$ each) and late ($p < 0.05$ each) onset SPE groups were significantly higher than in the control group (Jinhua Fu *et al*.,2014).

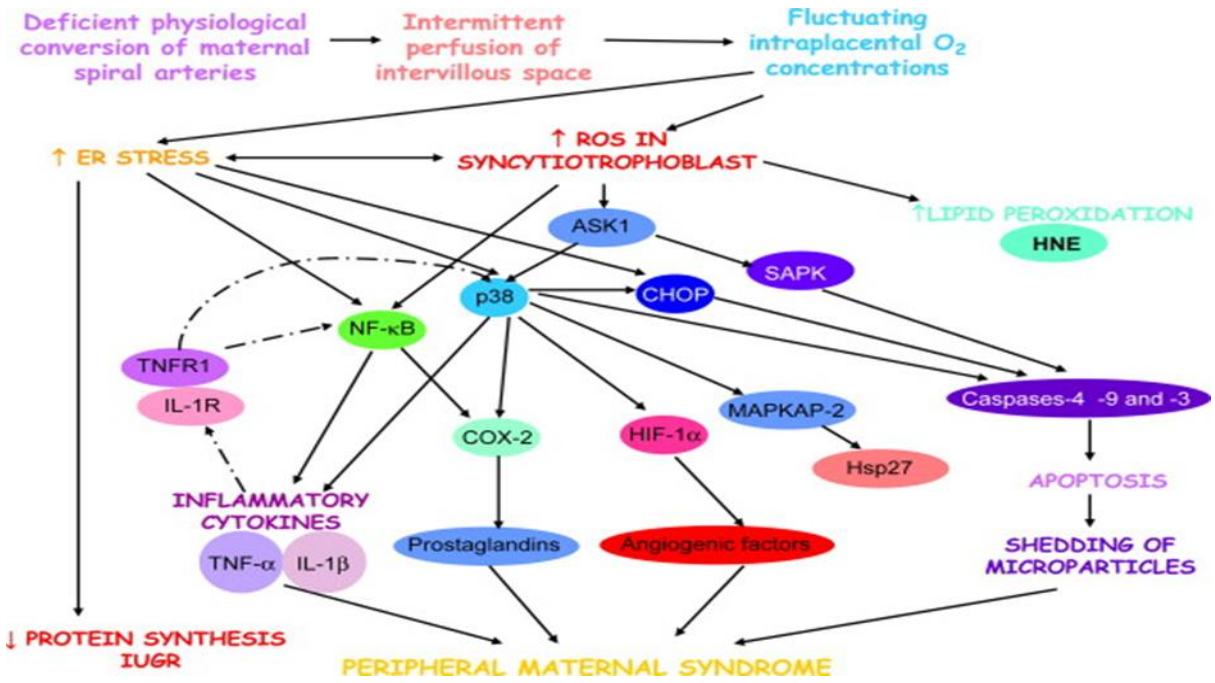


Figure 2: Schematic outline of endoplasmic reticulum stress and effect on variety of pathways leads to initiation of apoptotic pathways and finally leading to shedding of microparticles.These microparticles now mediate the peripheral symptoms of maternal syndrome. Adapted from (Cindrova-Davies, 2009).

1.2.3 Endoplasmic reticulum (ER) Stress and the shedding of microparticles

Diminished oxygen supply to the trophoblast cells results in oxidative as well as endoplasmic reticulum stress (Burton and Jauniaux, 2011). These two conditions can be initiated by inflammatory molecules. In severe cases, ER stress may not restore homeostasis as in the unfolded protein response. Such situations may lead to cellular apoptotic death (Roberts, 2014). As shown by the three stages model of PE, placental abnormalities may cause the release of placental derived debris and or microparticles into maternal blood and these particles contribute to the pathogenesis of PE by inducing generalised endothelial dysfunction, which is the hallmark of disease (Roberts and Hubel, 2009, Tomimatsu et al., 2016).

The related apoptotic effects of ER stress and necrotic effects of oxidative stress together with the resulting hypoxia cause alteration in structural changes which are secondary to oxidation. This then expedites the release of STBM into maternal circulation (Roberts, 2014). The shedding of STBMs occur in normal pregnancy but are amplified in PE, this is because there is an elevation in the level of necrotic bodies released into the circulation (Burton and Jones, 2009).

1.3 Microparticles in pregnancy

Microparticles (≥100nm) are shed from cellular structures as a result of cellular activation or necrosis (Redman and Sargent, 2008). MPs are phospholipids vesicles shed from platelets, endothelium, leucocytes and the human syncytiotrophoblast (STB). These MPs act as an identification tag for the cells from which they originate. Syncytiotrophoblast-derived microvesicles/microparticles (STBM) are larger microvesicles (0.2–2 μm) shed by the apical plasma membrane of the STB as a result of cell activation and turnover. Simultaneously with the STBM shedding, the STB produces and secretes exosomes – nanosized (30–100/150 nm) membrane-bound microvesicles that originate from the endosomal compartment. As there is a continuous activation of different cells in our body, it is natural to expect MPs even under normal physiological conditions (Jadli *et al*., 2015).

Normal pregnancy is characterised by the normal release of microparticles, however, the circulating level of these particles are impacted upon by pregnancy complications such as PE (Marques et al., 2013). Normal pregnancy is considered to be a hypercoagulable state, as there is an increase in pro-coagulant and a decrease in anticoagulant factors. There is an impact of the microparticles on placental cross talk and the intercommunication between these microparticles, thrombosis and pregnancy complications (Aharon and Brenner, 2011). Microparticles are believed to promote thrombus formation, and mediate pro-inflammatory effects thereby causing generalised endothelial dysfunction (Alijotas-Reig et al., 2013).

1.3.1 Placental derived Microparticles

The syncytiotrophoblast layer is a highly specialised multi-nucleate epithelial layer. This layer regularly forms and expands throughout the course of pregnancy by the fusion of underlying feeder layers of mononuclear villous trophoblast cells (Aharon and Brenner, 2011). During the course of gestation, this layer undergoes normal morphological changes which lead to the shedding of STBMs into the maternal circulation. These STBMs are believed to be a part of the maternal and fetal cross talk (Burton and Yung, 2011). The unbound syncytiotrophoblast released microparticles are normally detected by the second trimester in normal pregnancies with a continuous and considerable increase by the third trimester. Excess shedding of microparticles has been shown to be a feature of EOPE but not of LOPE and intrauterine growth restriction (Goswami et al., 2006).

In circulation, STBMs interact with immune cells and also the endothelium, thereby initiating the whole cascade of events leading to preeclampsia development. They also downregulate T-cell activity and may importantly contribute to fetal allograft immune escape (Redman and Sargent, 2009).

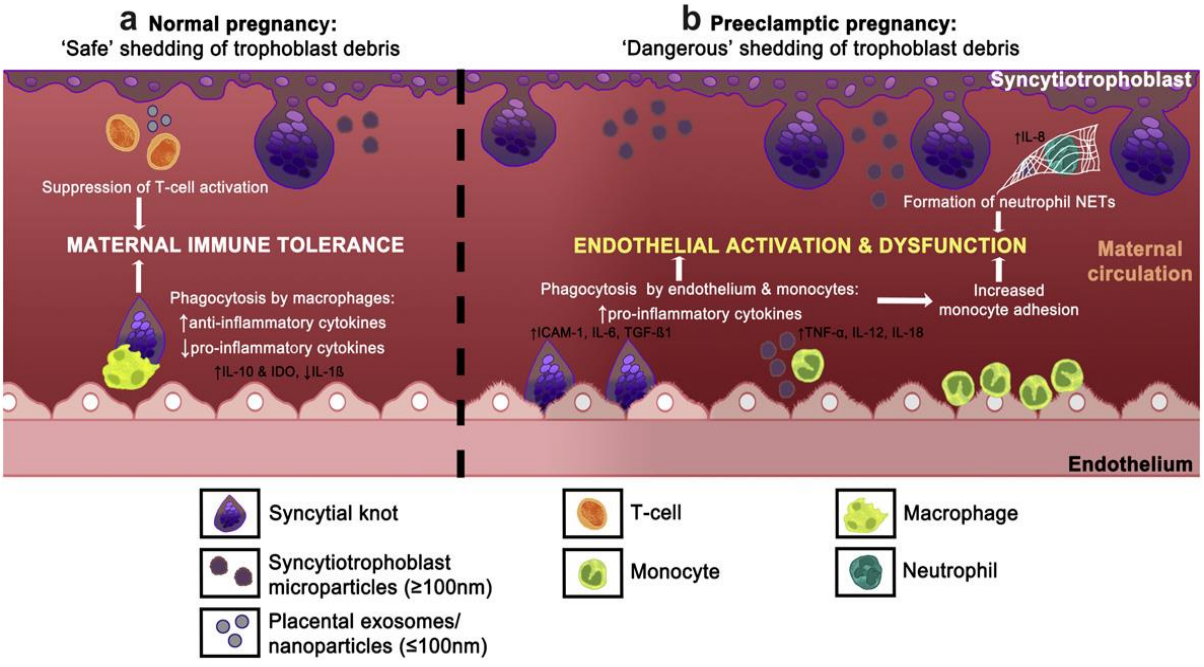


Figure 3; Trophoblastic debris in normal and complicated pregnancy; Adapted from (Panthem *et al*,2011).

A shift of safe 'shedding' of trophoblastic debris in normal pregnancy to a dangerous 'shedding' of the same debris in preeclampsia predisposes to endothelial activation and dysfunction in the maternal circulation and increased systemic inflammatory response.

It should be noted that despite considerable research the exact role of these MPs in healthy pregnancy and gestational vascular complication is still under intense discussion and inconclusive. These placental derived particles (STBM's) have been shown to be higher in uterine venous plasma (Redman *et al*., 2012). They are pro-inflammatory, procoagulant and also anti-angiogenic (Redman *et al*., 2012) .

1.3.2 Generation of STBM's

Production of these STBM is a constitutive feature of the unique hemochorial placenta. STBMs are shed during membrane remodelling.This production is preceded by several intracellular events. Firstly, at the spot or area of microvesicle formation, the intracellular calcium is increased. This calcium, derived both extra and intracellularly calcium, activates several proteolytic enzymes such as calpain. Calpain activation initiates the process of fragmentation of the cytoskeleton and starts to form blebs and release of plasma membrane bound vesicles (Freyssinet and Toti, 2010). These microparticles were first isolated *invitro* but later found as a constitutive component of plasma in normal pregnancy (Redman and Sargent, 2008)

1.3.3 Functions of STBM's

STBMs, isolated from the medium used to perfuse term placentas physiologically, have a pro-inflammatory effect (Mincheva-Nilsson and Baranov, 2014). STBMs bind to and activate blood monocytes and B cells to produce pro-inflammatory cytokines such as TNF-a, IFN-c, IL-1b, IL-6,-8,-12, and -18 (Germain *et al*., 2007, Redman *et al*., 2012, Holder *et al*., 2012).

In preclampsia, the procoagulant, pro-inflammatory and antiangiogenic properties of STBM is greatly enhanced causing widespread endothelial dysfunction and destruction of the syncytiotrophoblast (Mincheva-Nilsson and Baranov, 2014).

1.3.4 Quantification of STBM in pregnancy

Plasma STBMs were first measured with an Enzyme -Linked Immunosorbent assay (Alijotas-Reig et al.) using the syncytiotrophoblast-specific anti-placental alkaline phosphatase (PLAP) monoclonal antibody (NDOG2). NDOG2 is an anti trophoblast antibody which recognises placental alkaline phosphatase (Knight *et al*., 1998). STBMs were detected as early as the late first trimester of normal pregnancy, increased throughout normal pregnancy (Germain *et al*., 2007) and were present in significantly higher concentrations in PE *(*Knight *et al*., 1998) more so EOPE than in LOPE (Goswami D *et al*.,2006). However, although this STBM ELISA is quantitative, it gives no information about the nature of these extracellular vesicles (Tannetta et al., 2013b). The other enzyme used to detect STBM in maternal blood is Human Placental Alkaline Phosphatase.

1.4 Human Placental Alkaline Phosphatase (PLAP)

The enzyme, human placental alkaline phosphatase (PLAP), is a placenta-specific enzyme found in the trophoblast cells of a normal human mature placenta (Grgić and Bogdanović, 2009). It is part of the alkaline phosphatase (ALP) group of enzymes that hydrolyse the phosphate-containing compounds in an alkaline environment (She *et al*., 2000). This enzyme is synthesized in the placenta and is detectable in the maternal serum from the seventh week of gestation, reaching peak concentrations at term. Coryn first reported the elevation of serum PLAP during the second trimester of pregnancy in 1934 (Aliyu *et al*., 2013). The precise physiological function of this enzyme is not well-known, however, reports suggest that increased levels PLAP in the oral fluids of patients with preeclampsia (Chaparro *et al*., 2016).

1.4.1 Maternal serum PLAP

It has been shown that total serum PLAP increases from the 10-13th gestational weeks and this correlates with the developing placenta and the placental PLAP activity (Lubina Solomon, 2011). Research shows that in normal pregnancy, total PLAP increases from the 30th week of gestation reaching levels of up to two-three times the upper limit of the normal range for non-pregnant woman (Boronkai *et al*., 2005).

1.5 Rationale for the study

Controlled apoptosis plays an important role in maintaining tissue homoestasis and normal cellular growth cycles. Accumulated evidence suggests that dysregulation of apoptosis underlies the pathogenesis of several human diseases. Preeclampsia is an example of an ischaemic reperfusion injury which leads to oxidative stress. We believe that this oxidative stress leads finally to ER stress and shedding of microparticles via the activation of different signalling pathways. Amelioration of this stress can prove beneficial and must remain a research priority.

Most of the studies stated above were performed in developed countries and mostly on Caucasian women. Data available for Black South African women are sparse and no study has linked ER stress and microparticles. Furthermore, there may be racial variations in both the incidence of PE and the clinical features at presentation (Breathett *et al*., 2013). For example, the incidence of PE is much higher in South Africa and it has a much more aggressive and rapid clinical course of presentation, leading to significant mortality (Moodley, 2011-2013). Therefore the aim of this study is to quantify STBMs in South African PE and normotensive pregnant women and to investigate if ER stress in placental tissue is one of the mechanisms for the production of these microparticles.

1.6 HYPOTHESIS

I. The STBMs are raised in venous plasma of Black South African preeclamptic women compared to normal controls.

II. The ischemia reperfusion injury(I/R) in preeclampsia leads to oxidative and endoplasmic reticulum stress. ER stress activates signalling pathways which finally leads to shedding of microparticles.

III. This I/R also leads to increased expression of endoplasmic reticulum stress markers in placental tissues.

1.7 Aim of the study

To determine syncytiotrophoblast released microparticles levels and the mechanism of release in Black South African women.

1.8 Objectives of the study

I. To measure the presence of STBM's in normotensive and preeclamptic women with ELISA.

II. To measure ER stress in placental tissues with immunohistochemistry using the markers viz HIF-1α and CHOP.

III. To correlate immuno-expression of HIF- 1α and CHOP with shedding of the microparticles.

CHAPTER 2

MANUSCRIPT

MANUSCRIPT SUBMITTED TO PLACENTA

Elsevier Editorial System(tm) for Placenta

Manuscript Draft

Manuscript Number: PLAC-S-16-00708

Title: Placental Hypoxia Inducible factor -1α and CHOP Immunohistochemical- expression Relative to Maternal Circulatory Syncytiotrophoblast Microvesicles in Preeclamptic and Normotensive Pregnancies

Article Type: Full Length Article

Keywords: HIF-1α; CHOP; Syncytiotrophoblast Microvesicles; Pre- eclampsia; Endoplasmic Reticulum Stress; Hypoxia

Corresponding Author: Professor Irene Mackraj, Ph.D. Corresponding Author's Institution: University of KwaZulu-Natal,

Westville

First Author: Sonal Verma

Order of Authors: Sonal Verma; Preenan Pillay; Thajasvarie Naicker; Jagidesa Moodley; Irene Mackraj, Ph.D.

Placental Hypoxia Inducible factor -1α and CHOP Immunohistochemical expression Relative to Maternal Circulatory Syncytiotrophoblast Microvesicles in Preeclamptic and Normotensive Pregnancies

S. Verma[1], P. Pillay[1], T. Naicker[2], J. Moodley[3] and I. Mackraj[1*]

[1]Department of Physiology, University of KwaZulu-Natal, [2]Department of Optics and Imaging, University of KwaZulu-Natal, [3]Women's Health and HIV Research Group, University of KwaZulu-Natal

*Corresponding author

Telephone number: +27 729 08 5646

E-mail: mackraji@ukzn.ac.za

Abstract

Introduction and aim: Preeclampsia (PE) is thought to occur as a result of hypoxia-induced oxidative and endoplasmic reticulum stress which initiates a cascade of apoptotic events. This results in the activation of the CHOP pathway and the consequential shedding of syncytiotrophoblast microvesicles which maybe central in mediating the maternal systemic immune response. The aim of this study was to, therefore, immune-localise and morphometrically analyse CHOP and HIF-1α within the placenta of normotensive and pre-eclamptic pregnancies and concomitantly quantify syncytiotrophoblast released microvesicles in maternal circulation.

Materials and methods: Plasma and placental tissue were obtained from normotensive and pre-eclamptic pregnant women. The expression of CHOP and HIF-1α was analysed using immunohistochemistry. Circulating maternal microvesicles were isolated and their size distribution was determined using nanoparticle tracking analysis. The concentration of syncytiotrophoblast microvesicles was determined using the placental alkaline phosphatase ELISA.

Results: This study demonstrates a significant increase in Immunohistochemical expression of HIF-1 α and CHOP in preeclampsia compared to the normotensive women ($p<0.05$). A significant increase in the mean syncytiotrophoblast microvesicles concentration was observed in PE, compared to normotensives ($p<0.05$). In addition, a positive correlation between placental expression of CHOP and HIF-1α and STBMs was obtained ($p<0.05$).

Conclusion: This study demonstrates increased placental-expression of HIF-1α and CHOP in preeclampsia compared to normotensive pregnancies which correlate to the increase syncytiotrophoblast microvesicles concentration in maternal circulation. These findings indicate that placental hypoxia and ER stress are interrelated contributory factors to the pathogenesis of PE and the consequential release of placental derived debris into the maternal circulation.

Keywords: HIF-1α; CHOP; Syncytiotrophoblast Microvesicles; Pre- eclampsia; Endoplasmic Reticulum Stress; Hypoxia

Highlights

- Immuno-expression of HIF-1 α and CHOP were increased in preeclamptic pregnancies compared to normal.
- Syncytiotrophoblast microvesicles increase in preeclampsia compared to normal pregnancy.
- ER stress and Hypoxia potentiate the release of syncytiotrophoblast microvesicles in preeclampsia

Introduction

Preeclampsia (PE) is a hypertensive disorder of pregnancy characterised by new-onset hypertension and proteinuria presenting after the 20th week of gestation (Warrington et al., 2013). The global incidence of PE ranges from 2-8% (Jeyabalan, 2013). Hypertensive disorders of pregnancy are the leading cause of maternal mortality in South Africa, accounting for 14.8% of all deaths between 2011-2013 (Cuevas et al., 2015).

The underlying aetiology of PE remains elusive while the pathogenesis has been described (Ghulmiyyah and Sibai, 2012). The development of PE starts with inadequate trophoblast invasion occurring in the early stages of pregnancy. This results in improper remodelling of the myometrial spiral arteries with consequential placental hypo perfusion and an ischaemic environment (Redman and Staff, 2015).

Placental hypoxia initiates a cascade of events which lead to the maternal syndrome of PE (Tal, 2012). The hypoxic microenvironment causes oxidative stress and subsequent death of placental cells, culminating in the postulated release of trophoblast derived factors and/or debris into the maternal circulation (Tannetta et al., 2013a). These placental-derived debris such as syncytiotrophoblast microvesicles, exosomes and cell free fetal DNA stimulate the maternal systemic inflammatory response (MSIR) thus contributing to the development of PE (Redman and Sargent, 2009).

Normal pregnancy is characterised by physiological turnover of cells resulting in the release of syncytiotrophoblast microvesicles (STBMs) and other placental-derived debris into the maternal circulation (Aharon and Brenner, 2011). There is obviously an impact of the microvesicles on placental cross talk and the intercommunication between these microvesicles, thrombosis and pregnancy complications (Aharon and Brenner, 2011). In PE, there is an increase in the release of placental-derived microvesicles, which promote thrombus formation, mediate pro-inflammatory effects thereby causing endothelial dysfunction

(Katlama et al., 2013). Hypoxia and reperfusion injury in PE cause increased rate of syncytiotrophoblast apoptosis and necrosis (Burton and Yung, 2011).

One of the key factors in hypoxia is expression of hypoxia inducible factor 1 alpha (HIF-1α) (Cann and Karn, 1989). The HIF-1 α plays a crucial role in the transcription of numerous oxygen-dependent genes which encode proteins involved in angiogenesis and cell metabolism. HIF-1α is well expressed in conditions of diminished oxygen, this helps in placenta establishment early in pregnancy. However, overexpression is implicated in the development of several inflammatory conditions such as PE (Lodish et al., 2000, Cann and Karn, 1989).

The syncytiotrophoblast is equipped with a high endoplasmic reticulum (ER) content (Burton et al., 2009). In PE, oxidative and endoplasmic reticulum stress may be induced by the ischaemic reperfusion and hypoxia caused by diminished blood flow into the intervillous space, generation and accumulation of reactive oxygen species through the mitochondrial pathway as well as disturbances in homeostasis of calcium . This stress activates a cascade of signalling network called the Unfolded Protein Response (UPR). UPR restores ER homeostasis, promoting cell survival and adaptation (Oslowski and Urano, 2011). However, this initiation of UPR also leads to a homeostatic imbalance and activation of pro-apoptotic pathways such as CHOP (GADD153) (Du et al., 2016). CHOP (GADD153) is a member of the C/EBP family and bZIP transcription factors. The expression of CHOP is commonly induced by ER stress. The chop gene promoter contains binding sites for all of the major inducers of the UPR, including ATF4, ATF6, and XBP-1, and these transcription factors play causative roles in inducing chop gene transcription (Rozpędek et al., 2016). Overexpression of CHOP protein induces apoptosis, through a Bcl-2–inhibitor mechanism (Oyadomari and Mori, 2004).

While ER stress and release of microvesicles have been measured separately, we measure both events on a single sample (both placenta and blood from the same women) in an effort to

show direct link between them. This has been not done on the homogenous South African cohort.

Therefore the aim of this study is to quantify STBMs in Black South African PE and normotensive pregnant women and to assess if ER stress in placental tissue is one of the mechanisms for the production of these microvesicles.

Materials and Methods

Ethics Statement

Regulatory ethical and institutional approvals were obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BE364/15), South Africa.

Study Group and Samples

Healthy normotensive (N; n=15) pregnant and pre-eclamptic (PE; n=7) women in the third trimester of pregnancy scheduled to have elective caesarean deliveries for obstetric indications were recruited. Normotensive women were classified by a blood pressure of <140 / 90 mmHg (systolic/diastolic mmHg) and absent proteinuria as detected by a rapid urine dipstick test (Markomed®, South Africa). 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 ≥ 2+ on dipstick) after the 20th week of gestation (Magee et al., 2014, Tranquilli et al., 2014). All women had singleton pregnancies and those with evidence of any infections or medical, surgical or other obstetric complications were excluded.

Blood samples were collected [BD Vacutainer Tubes (EDTA), Becton Dickinson and Company, South Africa] and the plasma samples were stored at -80°C for analyses (N; n=7, PE; n=7). Placental tissue was dissected both from central and peripheral regions of the

placenta. The placental tissue was cut, rinsed with PBS (1X, pH 7.5) and preserved in 10% phosphate buffered formalin within 10 minutes of delivery.

Immuno-histochemical staining

Post fixation the placental tissue samples were dehydrated and paraffin embedded using the Leica automated tissue processor. Sections of 3-4 µm were cut with a Leica RM 2135 microtome and mounted on glass slides. Specimens were dewaxed in xylene and rehydrated in decreasing order of ethanol concentration. Antigen retrieval and immuno-staining was carried out using the Envision High pH Flex Mini Kit (DAKO), the monoclonal-mouse-anti-HIF-1α [1A3] antibody (Abcam, USA; ab113642, 1:1000) and monoclonal-mouse-anti-GADD153/CHOP [9C8] antibody (Novus Biologicals; NB600-1335, 1:75) served as primary antibodies. Gastric adenocarcinoma and glioblastoma multiforme tissue were used as the positive control for HIF-1 α and CHOP respectively. Appropriate buffer and negative controls (placental tissue without primary antibody) were carried out. All specimens were viewed with an Axioscope A1 microscope (Carl Zeiss, Germany). Image capturing, processing and analysis were performed using the AxoVision software (Carl Zeiss, Germany 4.8.3). At least four fields of view per slide were randomly selected and captured at an initial objective magnification of 20X.

Isolation of microvesicles from maternal circulation

Microvesicles were isolated according to a modified method as described by Dragovic *et al.,* (2015) on seven normotensive and seven preeclamptic pregnancies. Plasma (1ml) was diluted with an equal volume of phosphate buffered saline (PBS; pH 7.4) and centrifuged at 1500g at 4°C for 20 min. The supernatant was collected and centrifuged at 10 000g at 4°C for 30 min. The pellet was re-suspended in 2 ml PBS (pH 7.4) and thereafter centrifuged at 10 000g at 4°C for 30 min. This step was repeated to reduce contamination by soluble proteins.

Microvesicle protein concentration was determined using the RC DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA).

Size distribution of microvesicles using nanoparticle tracking analysis and Syncytiotrophoblast microvesicles quantification

Quantification and size distribution of microvesicles were determined using the NS500 as previously described by our group (Nano Sight NTA 3.0 Nanoparticle Tracking and Analysis Release, Version Build 0069) (Pillay et al., 2016). The relative concentration of STBMs was determined by the quantification of human placental alkaline phosphatase (PLAP) in the total microvesicle fraction using a commercial ELISA kit (Elabscience, E-EL-H1976, WuHan, P.R.C). Briefly, isolated microvesicles (25 µg) were allowed to bind to the primary PLAP specific antibody coated plates by incubation at 37°C for 90 min. Plates were washed and 50 µl of HRP-conjugate was added to each well and incubated at 37°C for 20 min. Plates were washed and incubated with 50 µl of substrate A and 50 µl of substrate B at 37°C for 15 min. The incubation was terminated using 50 µl of stop solution at RT for 2 min under agitation. Absorbance was measured at 450 nm and microvesicle PLAP was expressed as pg/ml plasma. The quantification of PLAP in the microvesicle fraction indicates the relative concentration of placental-derived microvesicles (STBMs) in maternal circulation.

Statistical Analysis

Statistical significance was defined as $p<0.05$. Statistical significance between the two groups was assessed using unpaired t-test. Data is presented as the mean ± SEM. Pearson's correlation analysis was done to determine the relationship between syncytiotrophoblast microvesicles in maternal circulation and placental protein expression. Statistical analyses were preformed using commercially available packages (Prism 6, GraphPad Inc, La Jolla, CA 92037 USA).

Results

Patient Clinical Data

Patient demographics are presented in table 1. The table shows that there were no significant differences in clinical parameters between the N and PE groups ($p>0.05$) except for increased blood pressure levels in the PE group ($p<0.05$). The mean birth weight in the N group was significantly higher than that in the PE group (3.4 ± 0.3 *vs* 2.7 ± 0.5 kg, $p<0.05$).

Table 1 The Clinical Characteristics of both study groups

Variables	Normotensive (N) (n=15)	Preeclampsia (PE) (n=7)
Age (years)	30.0 ± 5.1	25.1 ± 7.1
BMI (kg/m^2)	31.6 ± 6.4	26.7 ± 5.9
Nulliparous (number)	1	2
Multiparous (number and percentage)	14	5
Gestational age at sampling/ delivery (weeks)	36.2 ± 1.3	35.8 ± 1.5
Systolic blood pressure (mmHg)	108.8 ± 6.7	148.1 ± 11.2*
Diastolic blood pressure (mmHg)	71.8 ± 6.4	96.4 ± 8.8*
Baby weight (kg)	3.4 ± 0.3*	2.7 ± 0.5
Baby gender (Male)	7	3
Baby gender (Female)	8	4

All values are expressed as mean ± SEM. All pregnancies were singleton without any intrauterine infection or any other medical condition. PE versus N *$p<0.05$ in blood pressure. BMI N vs PE *$p<0.05$.

Expression of CHOP and HIF-1α in preeclamptic and normotensive placenta

Immunohistochemistry showed strong reactivity for HIF-1 α and CHOP in preeclamptic placentas than normotensive controls (Fig.1). More specifically, the nuclear immune-expression of CHOP and cytoplasmic HIF-1α was enhanced in PE in comparison to normotensives (Fig.1 A, B, D & E). The majority of the positive staining was found in the

syncytiotrophoblast. In addition, the analysis of the mean percentage area of CHOP and HIF-1α immuno-staining indicated that there was a significant increase in CHOP and HIF-1α immune-expression in PE in comparison to N (13.1 ± 4.1 % vs 6.5 ± 4.3 % and 14.1 ± 6.8 % vs 9.24 ± 2.4 %, respectively, $p < 0.0001$; Fig.1 C & F).

Quantification and characterisation of microvesicles in maternal circulation

The size distribution profiles (Fig. 2A) indicate that the microvesicles ranged in size from 50 - 450 nm. A statistically significant difference in the mean microvesicle particle size between preeclamptic and normotensive groups was obtained (127.6 ± 7.93 nm vs 137.4 ± 5.08 nm, $p < 0.005$; Fig. 2B). A significant increase in microvesicle concentration was obtained in PE in comparison to N pregnancies ($1.38 \pm 0.34 \times 10^{11}$ vs $5.06 \pm 0.61 \times 10^{10}$ particles/ml plasma, $p < 0.05$; Fig. 3).

The relative concentration of syncytiotrophoblast (STB) microvesicles was obtained 574.3 ± 55.99 pg/ml plasma, in PE compared to vs 218.8 ± 29.35 pg/ml plasma, in normotensive pregnancies ($p<0.005$; Fig. 4)

Relationship between Syncytiotrophoblast Microvesicles in maternal circulation and HIF-1α & CHOP Expression

Figure 5 shows the relationship between the relative concentration of syncytiotrophoblast microvesicles and placental protein (HIF-1α & CHOP) expression. This relationship was determined by the correlation between the $PLAP^+$ microvesicles (pg/ml plasma) and protein expression (HIF-1α & CHOP, % Area). A positive correlation between the relative concentration of STBMs and placental HIF-1α & CHOP protein expression in PE and N groups were obtained (Pearson's $r > 0.5$, $p < 0.05$; Fig. 5).

Discussion

While ER stress and an increase in microvesicle concentration have been reported in complicated pregnancies like PE, no study has examined the combined relationship of these factors. Our findings demonstrate a significant increase in the immuno-expression of CHOP and HIF-1α in PE placental tissue in comparison to the normotensive group ($p<0.05$), which is in keeping with previous studies (Augusto Korkes et al., 2017). In combination with these findings, nanoparticle tracking analysis of the isolated microvesicles in maternal circulation showed a significant decrease in mean microvesicle particle size and increased particle concentration in PE in comparison to normotensive women ($p<0.05$). The size distribution profile ranged from 50-450nm which is characteristics of microvesicles. Further quantification of the relative concentration of STBMs from the isolated microvesicles by PLAP ELISA indicated that there was a significant increase in STBMs in PE in comparison to normotensive woman ($p<0.05$). In addition, we report a positive correlation between placental CHOP & HIF-1α expression and STBMs in maternal circulation. These findings support the theory whereby the oxidatively stressed placenta results in the expression of ER stress proteins which enhances the production of placental derived STBMs.

Hypoxia is essential in early stages of gestation for supporting a successful pregnancy, however, prolonged hypoxia is observed in PE and other inflammatory conditions (Cann and Karn, 1989). It is therefore probable that the increased expression of placental HIF-1 α in PE could be as a result of the imbalance in oxygen homeostasis due to diminished placental perfusion caused by improper spiral artery remodelling in early pregnancy, a key feature of PE (Caniggia et al., 2000). Increased HIF-1 α mRNA expression in preeclamptic placentae have also been observed by others (Du et al., 2016). In addition, severe and prolonged imbalances in oxygen homeostasis could result in antiangiogenic/angiogenic imbalance induced by HIF-1α, which acts in combination with many other factors and potentiates the clinical manifestation of PE (Greijer and van der Wall, 2004).

The syncytiotrophoblast performs many important functions such as regulation of oxygen delivery and synthesis of a variety of proteins and possess abundant free ribosomes and endoplasmic reticulum (ER) (Mincheva-Nilsson and Baranov, 2014). Oxidative stress in syncytiotrophoblast predisposes it to ER stress. CHOP is a known marker of ER stress, and is up regulated in PE (Moutouh et al., 1996, Du et al., 2016). Endoplasmic Reticulum stress-induced apoptosis is primarily mediated through ATF4-mediated transcriptional induction of the C/EBP homologous protein CHOP (Redman, 2011). The increased expression of syncytiotrophoblast nuclear CHOP observed in PE in comparison to normotensive placentae suggests that ER stress maybe a major contributing factor to the increased apoptosis in PE syncytiotrophoblast. This is supported by the findings of this study whereby the increased expression of STBMs in PE positively correlates to placental CHOP & HIF-1α expression. In addition, this relationship suggests that that both the ER stress and hypoxia response pathways could potentiate the release of STBMs in maternal circulation.

STBMs are key factors involved in mediating the maternal systemic inflammatory response (MSIR) (Göhner et al., 2012). It is therefore plausible that the increased release of STBMs into maternal circulation leads to the exaggeration of the MSIR resulting in PE. In this study we observed that the isolated vesicles in PE constituted of an elevated concentration of vesicles with a particle size of 60-120nm in comparison to normotensives, this size profile is a key characteristic of microvesicles. This observation is in keeping with our previous study which showed that placental-derived microvesicles and exosomes were increased in early and late onset preeclampsia (Pillay et al., 2016). This suggests that the cascade of cellular events induced by the enhanced expression of placental CHOP and HIF-1α not only results in the increased release of STBMs but also the specific biogenesis of vesicles such as exosomes. These signalling vesicles also may contain key DNA, RNA and protein factors which potentiate the MSIR (Pillay et al., 2016). It is therefore necessary that future studies incorporate the isolation and characterisation of the sub-classes of STBMs in conjunction with

determining their biological relationship with ER stress and hypoxia, these studies are currently ongoing within our research group.

Limitations

There were limitations to this study. Oxidative stress and ER stress start early in pregnancy, however, it was not possible to do an early gestational study. Sample size was a huge limitation as chances of encountering HIV-ve pregnant woman with preeclampsia who delivered babies by caesarean section are low.

Conclusion

Our findings show that the placental immuno-expression of CHOP and HIF-1α are elevated in PE and could be key placental factors involved in the pathogenesis of PE. Additionally, the correlation between placental protein expression and STBMs indicate that ER stress and hypoxia may potentiate the release of STBMs into the maternal circulation. In combination, these novel findings form the basis for future research whereby we will isolate and characterise STBM constituents for further biological function in ER stress and hypoxia.

Acknowledgements

Dr Kogi Moodley, Mr. Simeon Eche, Denise Margolis for their assistance and Miss Zinhle Mkhize for the collection of samples. This project was funded by the National Research Foundation of South Africa (Grant Number: 91544).

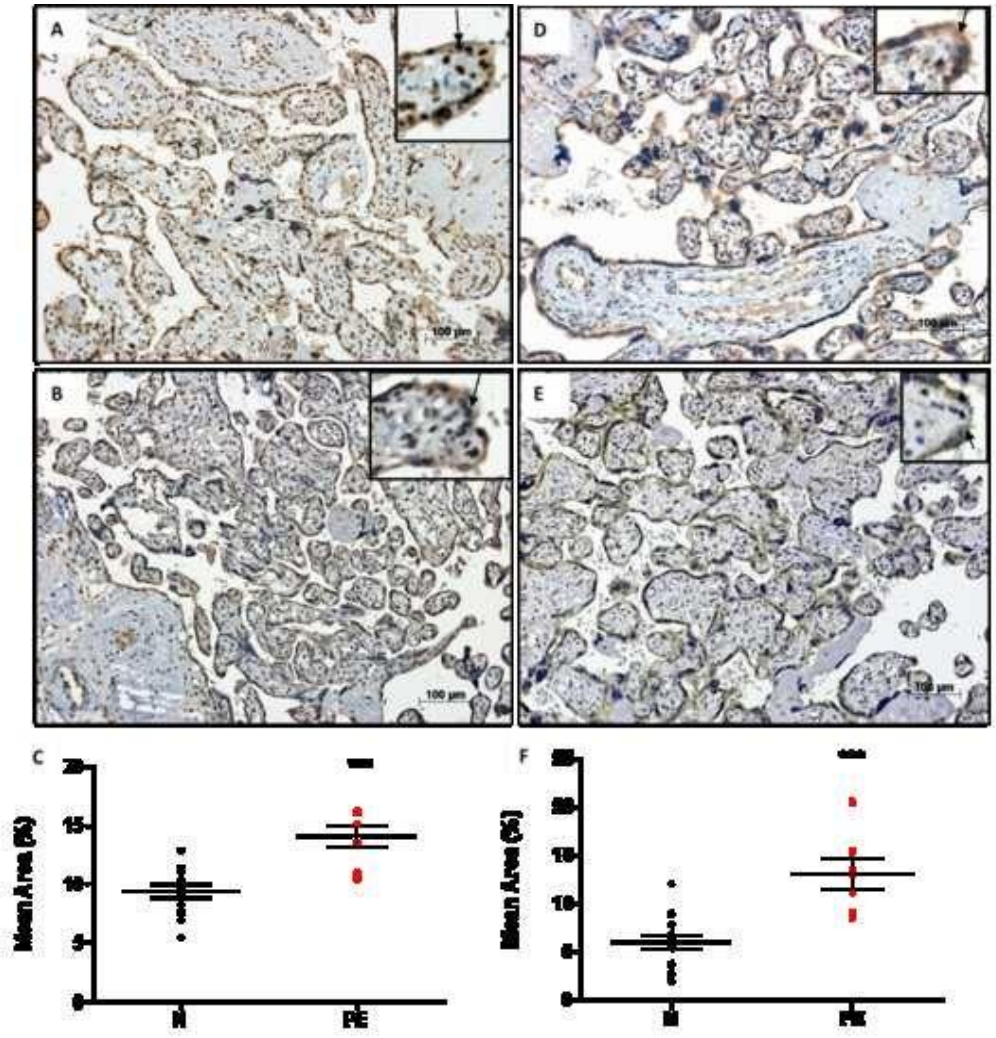
References

1. Warrington JP, George EM, Palei AC, Spradley FT, Granger JP: **Recent advances in the understanding of the pathophysiology of preeclampsia**. *Hypertension* 2013, **62**(4):666-673.
2. Jeyabalan A: **Epidemiology of preeclampsia: impact of obesity**. *Nutrition reviews* 2013, **71**(suppl 1):S18-S25.
3. Moodley J: **Saving Mothers Report**. 2011-2013.

4. Ghulmiyyah L, Sibai B: **Maternal Mortality From Preeclampsia/Eclampsia**. *Seminars in Perinatology* 2012, **36**(1):56-59.
5. Redman CWG, Staff AC: **Preeclampsia, biomarkers, syncytiotrophoblast stress, and placental capacity**. *American Journal of Obstetrics and Gynecology* 2015, **213**(4, Supplement):S9.e1-S9.e4.
6. Tal R: **The role of hypoxia and hypoxia-inducible factor-1alpha in preeclampsia pathogenesis**. *Biology of reproduction* 2012, **87**(6):134.
7. Tannetta D, Collett G, Vatish M, Redman C, Sargent I: **Syncytiotrophoblast extracellular vesicles – Circulating biopsies reflecting placental health**. *Placenta* 2016,1-5.
8. Redman CWG, Sargent IL: **Placental Stress and Preeclampsia: A Revised View**. *Placenta* 2009, **30, Supplement**:38-42.
9. Aharon A, Brenner B: **Microparticles and pregnancy complications**. *Thrombosis research* 2011, **127**:S67-S71.
10. Tannetta D, Sargent I: **Placental disease and the maternal syndrome of preeclampsia: missing links?** *Current hypertension reports* 2013, **15**(6):590-599.
11. Burton GJ, Yung H-W: **Endoplasmic reticulum stress in the pathogenesis of early-onset preeclampsia**. *Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health* 2011, **1**(1):72-78.
12. Rath G, Aggarwal R, Jawanjal P, Tripathi R, Batra A: **HIF-1 Alpha and Placental Growth Factor in Pregnancies Complicated With Preeclampsia: A Qualitative and Quantitative Analysis**. *Journal of clinical laboratory analysis* 2014.
13. Rajakumar A, Brandon H, Daftary A, Ness R, Conrad K: **Evidence for the functional activity of hypoxia-inducible transcription factors overexpressed in preeclamptic placentae**. *Placenta* 2004, **25**(10):763-769.
14. Burton GJ, Yung HW, Cindrova-Davies T, Charnock-Jones DS: **Placental endoplasmic reticulum stress and oxidative stress in the pathophysiology of unexplained intrauterine growth restriction and early onset preeclampsia**. *Placenta* 2009, **30 Suppl A**:S43-48.
15. Marques FK, Campos FM, Sousa LP, Teixeira-Carvalho A, Dusse LM, Gomes KB: **Association of microparticles and preeclampsia**. *Molecular biology reports* 2013, **40**(7):4553-4559.
16. Oslowski CM, Urano F: **Measuring ER stress and the unfolded protein response using mammalian tissue culture system**. *Methods in enzymology* 2011, **490**:71.
17. Fu J, Zhao L, Wang L, Zhu X: **Expression of markers of endoplasmic reticulum stress-induced apoptosis in the placenta of women with early and late onset severe preeclampsia**. *Taiwanese Journal of Obstetrics and Gynecology* 2015, **54**(1):19-23.
18. Du L, He F, Kuang L, Tang W, Li Y, Chen D: **eNOS/iNOS and endoplasmic reticulum stress-induced apoptosis in the placentas of patients with preeclampsia**. *Journal of human hypertension* 2016.
19. Rozpędek W, Pytel D, Mucha B, Leszczyńska H, Diehl J, Majsterek I: **The role of the PERK/eIF2α/ATF4/CHOP signaling pathway in tumor progression during Endoplasmic Reticulum stress**. *Current molecular medicine* 2016.
20. Oyadomari S, Mori M: **Roles of CHOP/GADD153 in endoplasmic reticulum stress**. *Cell death and differentiation* 2004, **11**(4):381-389.
21. Magee LA, Pels A, Helewa M, Rey E, von Dadelszen P: **Diagnosis, evaluation, and management of the hypertensive disorders of pregnancy**. *Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health* 2014, **4**(2):105-145.
22. Tranquilli AL, Dekker G, Magee L, Roberts J, Sibai BM, Steyn W, Zeeman GG, Brown MA: **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* 2014, **4**(2):97-104.

23. Pillay P, Maharaj N, Moodley J, Mackraj I: **Placental exosomes and preeclampsia: Maternal circulating levels in normal pregnancies and, early and late onset pre-eclamptic pregnancies**. *Placenta* 2016, **46**:18-25.
24. Caniggia I, Mostachfi H, Winter J, Gassmann M, Lye SJ, Kuliszewski M, Post M: **Hypoxia-inducible factor-1 mediates the biological effects of oxygen on human trophoblast differentiation through TGFbeta(3)**. *J Clin Invest* 2000, **105**(5):577-587.
25. Greijer AE, van der Wall E: **The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis**. *J Clin Pathol* 2004, **57**(10):1009-1014.
26. Mincheva-Nilsson L, Baranov V: **Placenta-Derived Exosomes and Syncytiotrophoblast Microparticles and their Role in Human Reproduction: Immune Modulation for Pregnancy Success**. *American Journal of Reproductive Immunology* 2014, **72**(5):440-457.
27. Rath G, Aggarwal R, Jawanjal P, Tripathi R, Batra A: **HIF-1 Alpha and Placental Growth Factor in Pregnancies Complicated With Preeclampsia: A Qualitative and Quantitative Analysis**. *J Clin Lab Anal* 2016, **30**(1):75-83.

Figures



.

Fig. 1. Placental Immune Histochemical Expression of CHOP and HIF-1α in Preeclamptic (n=7) and Normal Pregnancies (n=15). (A) CHOP expression in PE and (B) normotensive pregnancies. (D) HIF-1α expression in PE and (E) Normotensive pregnancies. Mean area (%) of (C) CHOP and (F) HIF-1α in PE and N. In A and B arrows indicate the increased nuclear expression of CHOP in the terminal villi. In D and E arrows indicate the increased cytoplasmic expression of HIF-1α in the terminal villi. In C indicates CHOP expression and F HIF-1α expression, ***p < 0.0001.

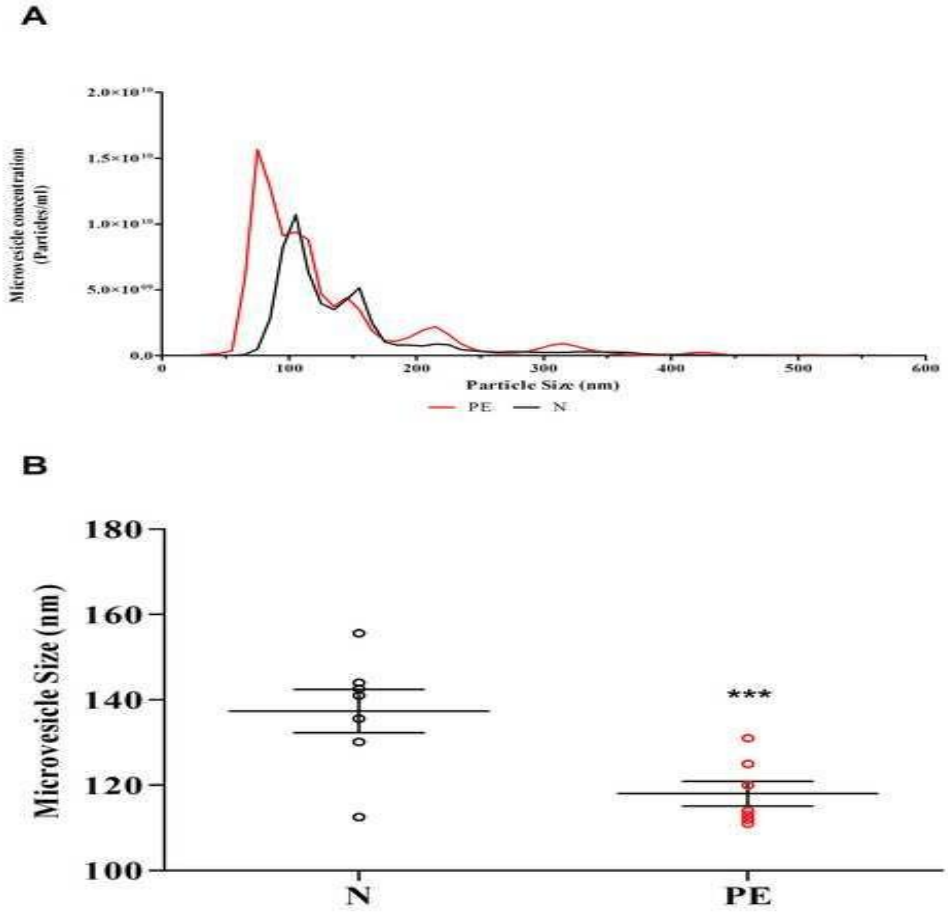


Fig. 2. Characterisation of Microvesicles in Preeclamptic (PE; n=7) and Normotensive (N; n=7) Maternal Circulation. (A) Microvesicles size distribution profile (nm). (B) Mean particle size (nm).

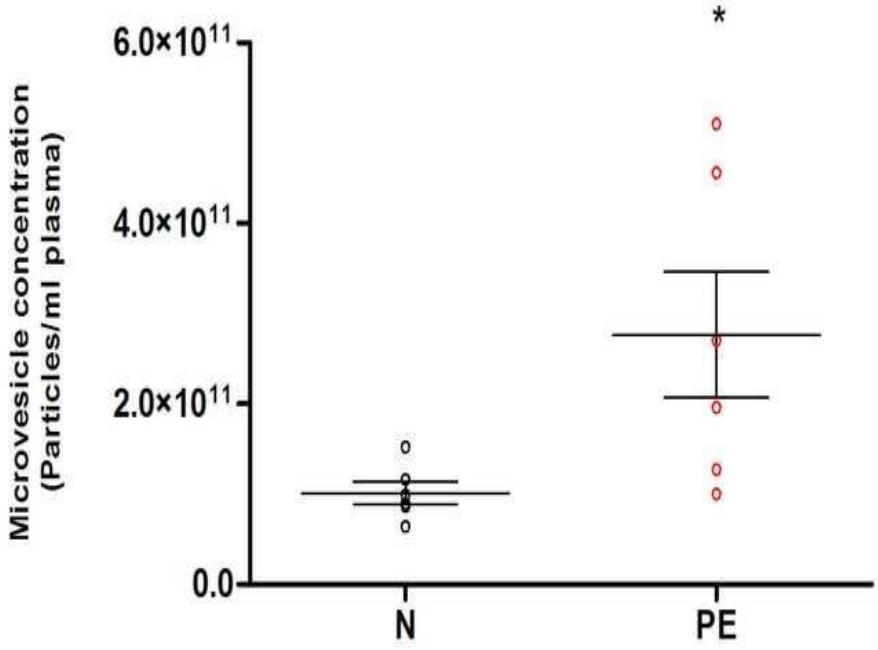


Fig.3.Microvesicle concentration in maternal circulation of normotensive (N; n=7) and preeclamptic (PE; n=7) pregnant woman. The data is expressed as aligned dot plot and values are mean ± SEM.*$p < 0.05$ PE vs N.

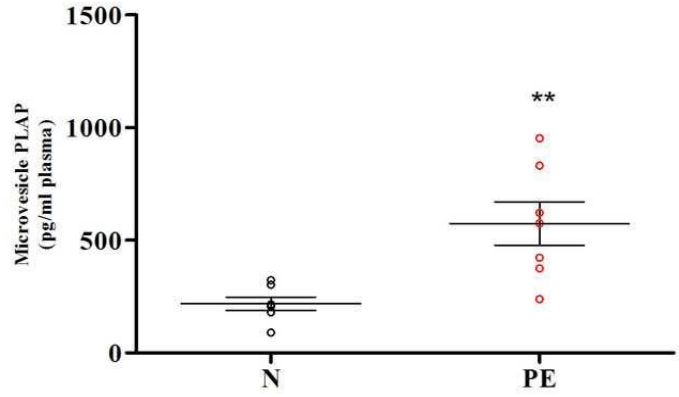


Fig. 4. Syncytiotrophoblast microvesicles in preeclampsia (PE; n=7) and normotensive (N; n=7) pregnancies. The data is expressed as aligned dot plot and values are mean ±SEM.

N PE

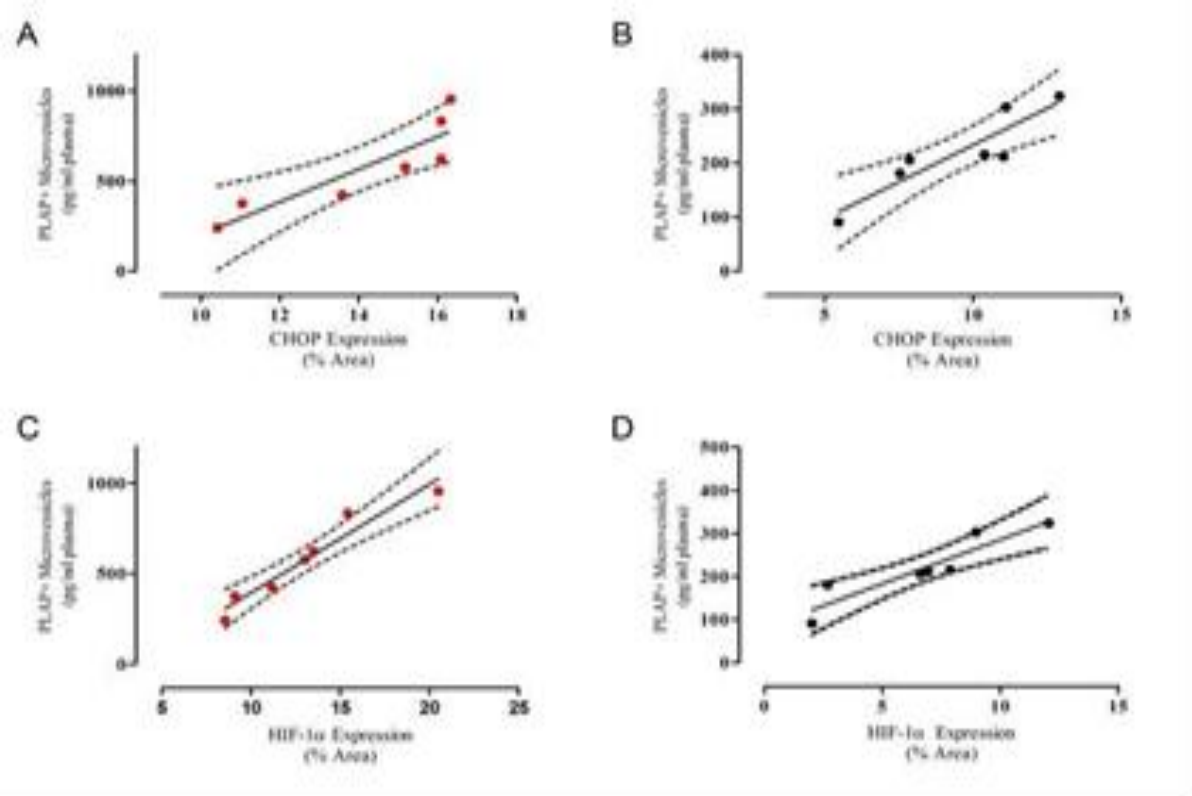


Figure 5: Relationship between the relative concentration of $PLAP^+$ microvesicles in maternal circulation with immunohistochemical expression HIF-1α and CHOP. (A) PLAP+ microvesicles and CHOP expression (% Area) in PE (r = 0.8891; p = 0.0074). (B) PLAP+ microvesicles and CHOP expression (% Area) in N (r = 0.9065 p = 0.0049). (C) $PLAP^+$ microvesicles and HIF-1α expression (% Area) in PE (r = 0.9668, p = 0.0004) and (D) $PLAP^+$ microvesicles and HIF-1α expression (% Area) in N (r = 0.9218, p = 0.0031).

CHAPTER 3

SYNTHESIS, CONCLUSION AND RECOMMENDATION

SYNTHESIS, CONCLUSION AND RECOMMENDATION

Synthesis

The main aim of this study was to evaluate if endoplasmic reticulum (ER) stress was one of the mechanisms for the production of syncytiotrophoblast microparticles in the maternal plasma. Immuno-localization of one of the hypoxic marker i.e. HIF-1 α and one of the endoplasmic reticulum stress marker i.e. CHOP was performed using immunohistochemistry in the placenta. Concomitantly, microvesicles size distribution and quantification was done using Nanoparticle Tracking Analysis and ELISA (PLAP).

The syncytiotrophoblast layer forms a very critical maternal foetal interface which performs many important functions which impact on the success of pregnancies. Disturbances in functions have deleterious effects on the development of placenta (Tannetta *et al*., 2016). Normal pregnancy is characterised by physiological turnover of the syncytiotrophoblast layer with consequential shedding of microvesicles and, hence, the cells are continuously active. Cellular apoptosis plays a crucial role in maintaining tissue homeostasis, but the dysregulation of apoptotic process is involved in the cause of many human diseases (McIlwain *et al*., 2013).

In pregnancy disorders like PE, placental hypoperfusion develops due to lack of the physiological transformation of spiral arteries within the myometrium (Moutouh et al., 1996). The resulting hypoxic placenta increases transcription of oxygen dependent genes such as factors such as HIF-1 α which helps in establishment of the placenta in early stages of gestation. Overexpression of HIF-1 α is implicated in the pathogenesis of inflammatory conditions like PE (Lodish et al., 2000, Redman, 2011). Hypoxia, oxidative stress and endoplasmic reticulum (ER) stress are interrelated contributory factors to the aetiopathogenesis of PE. Recent findings implicate ER stress in the pathophysiology of PE (Myatt, 2010, Burton and Yung, 2011, Moutouh et al., 1996). Failure of homeostatic pathways to restore and maintain metabolic equilibrium, will induce oxidative, mitochondrial

and ER stress which together induce apoptosis via the activation of mitochondrial dependent and independent apoptotic pathways which include the caspase and CHOP pathways (Burton *et al*., 2016). We and others hypothesized that the syncytiotrophoblast under ER stress, increases shedding of microvesicles in the maternal circulation. Importantly, recent studies indicate that these microvesicles may activate different signalling pathways and may cause an exaggerated inflammatory response and an increase cellular apoptosis associated with PE (Cindrova-Davies, 2009, Tannetta *et al*., 2016). It is this key point that illustrates the clinical manifestation in pre-eclamptic mothers, necessitating intense investigation in their biogenesis and as potential early biomarkers.

Activation of apoptotic pathways present a major problem for the syncytiotrophoblast layer because of the absence of cell boundaries which endangers this area as "apoptosis" sweeps easily through the entire epithelium causing failure in the maintenance of pregnancy (Burton *et al*., 2016). The syncytiotrophoblast layer is highly active and performs many important functions so are susceptible to apoptosis and necrosis which is elevated in pregnancy complications like PE and fetal growth restriction (Lee et al., 2012b), due to increased stress levels.

The present study was designed to evaluate ER stress in placental tissues by immuno-localisation of Hif-1α and CHOP, and to correlate these parameters to the concentration of circulating syncytiotrophoblast microparticles in normal pregnancy and preeclamptic pregnancy. The study population was a cohort of Black South African women as hypertension in pregnancy remains a major public health concern and one of the main cause of maternal mortality in South Africa (Moodley, 2011-2013). While increased expression of ER stress markers and HIF-1 α and increased microparticles in complicated pregnancies have been reported by other researchers (Fu *et al*., 2015, Du *et al*., 2016, Rath *et al*., 2016),to the best of our knowledge, no work has been performed to show a link between within a single patient sample.

In this study, results showed a significant difference in placental immunohistochemical studies, morphometric analysis, and microvesicles size and concentration between preeclamptic and normotensive pregnant women. This supports earlier findings that the cause of PE is of placental origin and hence, the understanding of the pathophysiology of PE requires placental examination (Göhner et al., 2012).

In the present study, maternal and gestational age as well as the BMI was similar between the normotensive pregnant and the PE groups. Birth weight was significantly lower in the preeclamptic compared to the normotensive pregnant group. A low birth weight in early onset but not late onset PE is as a result of the diminished blood flow and nutrient supply to the developing foetus during the course of pregnancy (Xiong et al., 2002).

In the present study, the mean area % of HIF-1α was significantly elevated in the preeclamptic compared to normotensive. This finding is in line with similar findings that also showed an increased immunoexpression of HIF-1α in PE compared to controls (Göhner et al., 2012, Akhilesh et al., 2014). In hypoxic environments, HIF-1α plays crucial roles in vascularization and embryo survival, thus helping in the early establishment of pregnancy (Rath *et al*., 2016), however, chronic elevated expression of HIF-1 α leads to dysregulation of angiogenesis and angiogenic/antiangiogenic imbalance (Göhner et al., 2012). A limitation of our finding is that we used term placenta, whereas, vascular remodelling occurs in the first trimester. The other limitation to our study was the collection preeclamptic placenta delivered by elective caesarean section. Studies have shown that in preeclamptic placentae, there is an up-regulation of HIF-1α mRNA, as well as an elevated expression of HIF-1α implicating it in the pathogenesis of this hypertensive disorders of pregnancy (Rajakumar *et al*., 2004, Iwagaki *et al*., 2004, Caniggia and Winter, 2002).

Another key finding in this study is the significantly elevated CHOP immunohistochemical expression in preeclamptic placentae compared to normal. CHOP is marker of ER stress, and the CHOP mRNA and protein expression level has been shown to be up regulated in women

with PE compared to that in women with normal pregnancies (Fu *et al*., 2015, Du *et al*., 2016). Mccullough *et al*., (2001) showed that an increased expression of CHOP causes ER stress as Bcl2 is downregulated (McCullough *et al*., 2001) and the rate of apoptosis is increased. This downregulation of Bcl2 suggests that that the exaggerated ER stress seen in PE may be a major contributory factor to the increased placental cell death consequent increase in the release of cellular debris into the maternal circulation.

Results obtained from the present study show a statistically significant difference in mean microvesicles particle size between the PE group and the controls. We reported the size distribution profiles ranging between 50 - 450nm were present in the patient's samples which are characteristic features of microvesicles. A statistically significant difference in the mean microvesicles concentration in preeclamptic pregnancies compared to normal pregnancy was also observed ($p<0.005$). The relative concentration of the isolated syncytiotrophoblast (STB) microvesicles using the placental alkaline phosphatase ELISA indicates that an increase in STB microvesicles is present in PE group compared to the controls ($p<0.05$). Our findings are in keeping with similar studies that have found an increase in the STB microvessicles level in PE (Salomon et al., 2013, Sabapatha et al., 2006, Pillay et al., 2016). Importantly, we also observed a positive correlation between the placental immunohistochemical expressions of HIF-1 α and CHOP with microparticles in the maternal circulation. This key finding might provide a link between stressed placenta and increased production of microvesicles and also paves a way to novel pharmaceutical interventions.

We have previously shown in our laboratories that the Placental Alkaline Phosphatase (PLAP) marker used for the quantification of placental-derived exosomes (a sub classification of microvesicles within the size range of 20-130nm) is elevated in PE (Pillay *et al*., 2016). This was attributed to placental hypoxia, increased apoptosis and increased release of STBMS, exosomes and fetal DNA into maternal circulation (Redman and Sargent, 2009). Furthermore, STBMS carry the danger molecules HSP70 and HMGB1 which facilitates their pro-inflammatory effect, whereas their pro-coagulant activity results from the expression of

tissue factor (Mincheva-Nilsson and Baranov, 2014, Göhner *et al*., 2012). They have also been shown to be immunoregulatory, as they suppress NK and T cell responses *in vitro* due to their expression of MICA/B and Fas ligand (Christianne *et al*., 2015). This has implications in rejection of the placenta. Clearly STBMS are major role players implicated in both placentation and foetal development.

Conclusion

This study shows that the immunoexpression of HIF-1α and CHOP, markers of hypoxia and ER stress were significantly elevated in PE compared to normotensive pregnancies. We also observed a statistically significant difference in the mean microvesicles concentration in PE compared to normal pregnancy. A correlation between these changes was observed. This study showed that placental hypoxia and ER stress are implicated to the pathogenesis of PE. This increased placental hypoxia and oxidative stress results in the release of placenta derived factors or debris into maternal circulation which has the capacity to stimulate an exaggerated inflammatory response and cause generalised endothelial dysfunction which is hallmark of preeclampsia.

Recommendations:

These syncytiotrophoblast microparticles are capable of cellular signalling and carry a complex cargo of lipids, mRNA, proteins and are of critical importance in maternal, fetal and placental cross-talk during pregnancy. It is therefore important to isolate and fully characterise sub -classes of STBMs in an effort to determine their relationship with hypoxia and ER stress. In addition, the evaluation of placenta would be performed by electron microscopy and electron immunostaining. For future studies a larger cohort is recommended for extrapolation of finding for the general population. Amelioration of ER stress through pharmaceutical interventions can be a breakthrough in the management of preeclampsia.

CHAPTER 4

REFERENCES

REFERENCES

AHARON, A. & BRENNER, B. 2011. Microparticles and pregnancy complications. *Thrombosis research,* 127**,** S67-S71.

AKHILESH, M., MAHALINGAM, V., NALLIAH, S., ALI, R. M., GANESALINGAM, M. & HALEAGRAHARA, N. 2014. Participation of hypoxia-inducible factor-1α in the pathogenesis of preeclampsia-related placental ischemia and its potential as a marker for preeclampsia. *Biomarkers and Genomic Medicine,* 6**,** 121-125.

ALIJOTAS-REIG, J., PALACIO-GARCIA, C., LLURBA, E. & VILARDELL-TARRES, M. 2013. Cell-derived microparticles and vascular pregnancy complications: a systematic and comprehensive review. *Fertility and sterility,* 99**,** 441-449.

ALIYU, I. S., RANDAWA, A. J., ISAH, H. S. & AFONJA, O. A. 2013. Pattern of serum total alkaline phosphatase activity in different stages of normal third trimester pregnancy in Zaria, Northern Nigeria. *Annals of Nigerian Medicine,* 7**,** 28.

AUGUSTO KORKES, H., OLIVEIRA, L. D., SASS, N., SALAHUDDIN, S., KARUMANCHI, S. A. & RAJAKUMAR, A. 2017. Relationship between hypoxia and downstream pathogenic pathways in preeclampsia. *Hypertension in Pregnancy***,** 1-6.

BORONKAI, A., THAN, N., MAGENHEIM, R., BELLYEI, S., SZIGETI, A., DERES, P., HARGITAI, B., SUMEGI, B., PAPP, Z. & RIGO, J. 2005. Extremely high maternal alkaline phosphatase serum concentration with syncytiotrophoblastic origin. *Journal of clinical pathology,* 58**,** 72-76.

BREATHETT, K., MUHLESTEIN, D., FORAKER, R. & GULATI, M. 2013. Abstract P192: The Incidence of Pre-Eclampsia Remains Higher in African-American Women Compared to Caucasian Women: Trends from the National Hospital Discharge Survey 1979-2006. *Circulation,* 127**,** AP192.

BURTON, G., YUNG, H. & MURRAY, A. 2016. Mitochondrial–Endoplasmic reticulum interactions in the trophoblast: Stress and senescence. *Placenta*.

BURTON, G. J. & JAUNIAUX, E. 2011. Oxidative stress. *Best Practice & Research Clinical Obstetrics & Gynaecology,* 25**,** 287-299.

BURTON, G. J. & JONES, C. J. 2009. Syncytial knots, sprouts, apoptosis, and trophoblast deportation from the human placenta. *Taiwanese Journal of Obstetrics and Gynecology,* 48**,** 28-37.

BURTON, G. J. & YUNG, H.-W. 2011. Endoplasmic reticulum stress in the pathogenesis of early-onset pre-eclampsia. *Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health,* 1**,** 72-78.

BURTON, G. J., YUNG, H. W., CINDROVA-DAVIES, T. & CHARNOCK-JONES, D. S. 2009. Placental endoplasmic reticulum stress and oxidative stress in the pathophysiology of unexplained intrauterine growth restriction and early onset preeclampsia. *Placenta,* 30 Suppl A**,** S43-8.

CANIGGIA, I., MOSTACHFI, H., WINTER, J., GASSMANN, M., LYE, S. J., KULISZEWSKI, M. & POST, M. 2000. Hypoxia-inducible factor-1 mediates the biological effects of oxygen on human trophoblast differentiation through TGFbeta(3). *J Clin Invest,* 105**,** 577-87.

CANIGGIA, I. & WINTER, J. 2002. Adriana and Luisa Castellucci Award Lecture 2001 Hypoxia Inducible Factor-1: Oxygen regulation of trophoblast differentiation in normal and pre-eclamptic pregnancies—a review. *Placenta,* 23**,** S47-S57.

CANN, A. J. & KARN, J. 1989. Molecular biology of HIV: new insights into the virus life-cycle. *Aids,* 3**,** S19-34.

CHAPARRO, A., GAEDECHENS, D., RAMÍREZ, V., ZUÑIGA, E., KUSANOVIC, J. P., INOSTROZA, C., VARAS-GODOY, M., SILVA, K., SALOMON, C. & RICE, G. 2016. Placental biomarkers and angiogenic factors in oral fluids of patients with preeclampsia. *Prenatal diagnosis,* 36**,** 476-482.

CHRISTIANNE, A., BOER, K., STURK, A. & SARGENT, I. The functions of microparticles in pre-eclampsia. Seminars in Thrombosis and Hemostasis, 2015. 146-152.

CINDROVA-DAVIES, T. 2009. Gabor Than Award Lecture 2008: Pre-eclampsia – From Placental Oxidative Stress to Maternal Endothelial Dysfunction. *Placenta,* 30, Supplement**,** 55-65.

CUEVAS, J. M., GELLER, R., GARIJO, R., LOPEZ-ALDEGUER, J. & SANJUAN, R. 2015. Extremely High Mutation Rate of HIV-1 In Vivo. *PLoS Biol,* 13**,** e1002251.

DU, L., HE, F., KUANG, L., TANG, W., LI, Y. & CHEN, D. 2016. eNOS/iNOS and endoplasmic reticulum stress-induced apoptosis in the placentas of patients with preeclampsia. *Journal of human hypertension*.

ENGLISH, F. A., KENNY, L. C. & MCCARTHY, F. P. 2015. Risk factors and effective management of preeclampsia. *Integrated Blood Pressure Control,* 8**,** 7-12.

FREYSSINET, J.-M. & TOTI, F. 2010. Formation of procoagulant microparticles and properties. *Thrombosis research,* 125**,** S46-S48.

FU, J., ZHAO, L., WANG, L. & ZHU, X. 2015. Expression of markers of endoplasmic reticulum stress-induced apoptosis in the placenta of women with early and late onset severe pre-eclampsia. *Taiwanese Journal of Obstetrics and Gynecology,* 54**,** 19-23.

GERMAIN, S. J., SACKS, G. P., SOORANA, S. R., SARGENT, I. L. & REDMAN, C. W. 2007. Systemic inflammatory priming in normal pregnancy and preeclampsia: the role of circulating syncytiotrophoblast microparticles. *The Journal of Immunology,* 178**,** 5949-5956.

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

GÖHNER, C., BONNKE, C., BRÜCKMANN, A., SCHLEUSSNER, E., MARKERT, U., SOSSDORF, M. & FITZGERALD, J. 2012. Pro-coagulant capacity of syncytiotrophoblastic microparticles. *Journal of Reproductive Immunology,* 94**,** 121.

GOSWAMI, D., TANNETTA, D., MAGEE, L., FUCHISAWA, A., REDMAN, C., SARGENT, I. & VON DADELSZEN, P. 2006. Excess syncytiotrophoblast microparticle shedding is a feature of early-onset pre-eclampsia, but not normotensive intrauterine growth restriction. *Placenta,* 27**,** 56-61.

GREIJER, A. E. & VAN DER WALL, E. 2004. The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis. *J Clin Pathol,* 57**,** 1009-14.

GRGIĆ, G. & BOGDANOVIĆ, G. 2009. Placental alkaline phosphatase in the prediction of preterm delivery. *Acta Medica Academica,* 38**,** 16-20.

HOLDER, B. S., TOWER, C. L., JONES, C. J., APLIN, J. D. & ABRAHAMS, V. M. 2012. Heightened pro-inflammatory effect of preeclamptic placental microvesicles on peripheral blood immune cells in humans. *Biology of reproduction,* 86**,** 103.

ISLER, C. M., RINEHART, B. K., TERRONE, D. A., MARTIN, R. W., MAGANN, E. F. & MARTIN, J. N. 1999. Maternal mortality associated with HELLP (hemolysis, elevated liver enzymes, and low platelets) syndrome. *American journal of obstetrics and gynecology,* 181**,** 924-928.

IWAGAKI, S., YOKOYAMA, Y., TANG, L., TAKAHASHI, Y., NAKAGAWA, Y. & TAMAYA, T. 2004. Augmentation of leptin and hypoxia-inducible factor 1α mRNAs in the pre-eclamptic placenta. *Gynecological endocrinology,* 18**,** 263-268.

JADLI, A., SHARMA, N., DAMANIA, K., SATOSKAR, P., BANSAL, V., GHOSH, K. & SHETTY, S. 2015. Promising prognostic markers of Preeclampsia: New avenues in waiting. *Thrombosis research,* 136**,** 189-195.

JEYABALAN, A. 2013. Epidemiology of preeclampsia: impact of obesity. *Nutrition reviews,* 71**,** S18-S25.

KATLAMA, C., DEEKS, S. G., AUTRAN, B., MARTINEZ-PICADO, J., VAN LUNZEN, J., ROUZIOUX, C., MILLER, M., VELLA, S., SCHMITZ, J. E., AHLERS, J., RICHMAN, D. D. & SEKALY, R. P.

2013. Barriers to a cure for HIV: new ways to target and eradicate HIV-1 reservoirs. *The Lancet,* 381**,** 2109-2117.

KNIGHT, M., REDMAN, C. W., LINTON, E. A. & SARGENT, I. L. 1998. Shedding of syncytiotrophoblast microvilli into the maternal circulation in pre-eclamptic pregnancies. *BJOG: An International Journal of Obstetrics & Gynaecology,* 105**,** 632-640.

LEE, S. M., ROMERO, R., LEE, Y. J., PARK, I. S., PARK, C.-W. & YOON, B. H. 2012a. Systemic inflammatory stimulation by microparticles derived from hypoxic trophoblast as a model for inflammatory response in preeclampsia. *American Journal of Obstetrics and Gynecology,* 207**,** 337.e1-337.e8.

LEE, S. M., ROMERO, R., LEE, Y. J., PARK, I. S., PARK, C.-W. & YOON, B. H. 2012b. Systemic inflammatory stimulation by microparticles derived from hypoxic trophoblast as a model for inflammatory response in preeclampsia. *American journal of obstetrics and gynecology,* 207**,** 337. e1-337. e8.

LEEMAN, L., DRESANG, L. T. & FONTAINE, P. 2016. Hypertensive Disorders of Pregnancy. *American family physician,* 93.

LI, J., LAMARCA, B. & RECKELHOFF, J. F. 2012. A model of preeclampsia in rats: the reduced uterine perfusion pressure (RUPP) model. *American Journal of Physiology-Heart and Circulatory Physiology,* 303**,** H1-H8.

LIAN, I. A., LØSET, M., MUNDAL, S. B., FENSTAD, M. H., JOHNSON, M. P., EIDE, I. P., BJØRGE, L., FREED, K. A., MOSES, E. K. & AUSTGULEN, R. 2011. Increased endoplasmic reticulum stress in decidual tissue from pregnancies complicated by fetal growth restriction with and without pre-eclampsia. *Placenta,* 32**,** 823-829.

LIN, S., LEONARD, D., CO, M. A., 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.

LODISH, H., BERK, A., ZIPURSKY, S. L., MATSUDAIRA, P., BALTIMORE, D. & DARNELL, J. 2000. Mutations: types and causes. *Molecular Cell Biology,* 4.

LUBINA SOLOMON, A. 2011. *The role of placental alkaline phosphatase in the regulation of insulin-like growth factor binding protein-1 in pregnancy complicated by diabetes.* University of Manchester.

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.

MARQUES, F. K., CAMPOS, F. M. F., SOUSA, L. P., TEIXEIRA-CARVALHO, A., DUSSE, L. M. S. & GOMES, K. B. 2013. Association of microparticles and preeclampsia. *Molecular Biology Reports,* 40**,** 4553-4559.

MCCULLOUGH, K. D., MARTINDALE, J. L., KLOTZ, L.-O., AW, T.-Y. & HOLBROOK, N. J. 2001. Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. *Molecular and cellular biology,* 21**,** 1249-1259.

MCILWAIN, D. R., BERGER, T. & MAK, T. W. 2013. Caspase functions in cell death and disease. *Cold Spring Harbor perspectives in biology,* 5**,** a008656.

MINCHEVA-NILSSON, L. & BARANOV, V. 2014. Placenta-Derived Exosomes and Syncytiotrophoblast Microparticles and their Role in Human Reproduction: Immune Modulation for Pregnancy Success. *American Journal of Reproductive Immunology,* 72**,** 440-457.

MIZUUCHI, M., CINDROVA-DAVIES, T., OLOVSSON, M., CHARNOCK-JONES, D. S., BURTON, G. J. & YUNG, H. W. 2016. Placental endoplasmic reticulum stress negatively regulates transcription of placental growth factor via ATF4 and ATF6beta: implications for the pathophysiology of human pregnancy complications. *J Pathol,* 238**,** 550-61.

MOL, B. W. J., ROBERTS, C. T., THANGARATINAM, S., MAGEE, L. A., DE GROOT, C. J. M. & HOFMEYR, G. J. 2016. Pre-eclampsia. *The Lancet,* 387**,** 999-1011.

MOODLEY, J. 2011-2013. Saving Mothers Report.

MOUTOUH, L., CORBEIL, J. & RICHMAN, D. D. 1996. Recombination leads to the rapid emergence of HIV-1 dually resistant mutants under selective drug pressure. *Proceedings of the National Academy of Sciences,* 93**,** 6106-6111.

MYATT, L. 2010. Review: reactive oxygen and nitrogen species and functional adaptation of the placenta. *Placenta,* 31**,** S66-S69.

OSLOWSKI, C. M. & URANO, F. 2011. Measuring ER stress and the unfolded protein response using mammalian tissue culture system. *Methods in enzymology,* 490**,** 71.

OYADOMARI, S. & MORI, M. 2004. Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death Differ,* 11**,** 381-9.

PILLAY, P., MAHARAJ, N., MOODLEY, J. & MACKRAJ, I. 2016. Placental exosomes and pre-eclampsia: Maternal circulating levels in normal pregnancies and, early and late onset pre-eclamptic pregnancies. *Placenta,* 46**,** 18-25.

POWE, C. E., LEVINE, R. J. & KARUMANCHI, S. A. 2011a. Preeclampsia, a disease of the maternal endothelium. *Circulation,* 123**,** 2856-2869.

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

RAJAKUMAR, A., BRANDON, H., DAFTARY, A., NESS, R. & CONRAD, K. 2004. Evidence for the functional activity of hypoxia-inducible transcription factors overexpressed in preeclamptic placentae. *Placenta,* 25**,** 763-769.

RANA, S. & KARUMANCHI, S. A. 2017. 172 - Pathophysiology of Preeclampsia A2 - Polin, Richard A. *In:* ABMAN, S. H., ROWITCH, D. H., BENITZ, W. E. & FOX, W. W. (eds.) *Fetal and Neonatal Physiology (Fifth Edition).* Elsevier.

RATH, G., AGGARWAL, R., JAWANJAL, P., TRIPATHI, R. & BATRA, A. 2016. HIF-1 Alpha and Placental Growth Factor in Pregnancies Complicated With Preeclampsia: A Qualitative and Quantitative Analysis. *J Clin Lab Anal,* 30**,** 75-83.

REDMAN, C. 2011. Preeclampsia: a multi-stress disorder. *La Revue de médecine interne,* 32**,** 41-44.

REDMAN, C., TANNETTA, D., DRAGOVIC, R., GARDINER, C., SOUTHCOMBE, J., COLLETT, G. & SARGENT, I. 2012. Review: Does size matter? Placental debris and the pathophysiology of pre-eclampsia. *Placenta,* 33**,** S48-S54.

REDMAN, C. W. G. & SARGENT, I. L. 2008. Circulating Microparticles in Normal Pregnancy and Pre-Eclampsia. *Placenta,* 29**,** 73-77.

REDMAN, C. W. G. & SARGENT, I. L. 2009. Placental Stress and Pre-eclampsia: A Revised View. *Placenta,* 30, Supplement**,** 38-42.

REDMAN, C. W. G. & STAFF, A. C. 2015. Preeclampsia, biomarkers, syncytiotrophoblast stress, and placental capacity. *American Journal of Obstetrics and Gynecology,* 213**,** S9.e1-S9.e4.

ROBERTS, D. J. & POST, M. D. 2008. The placenta in pre-eclampsia and intrauterine growth restriction. *Journal of clinical pathology,* 61**,** 1254-1260.

ROBERTS, J. M. Pathophysiology of ischemic placental disease. Seminars in perinatology, 2014. Elsevier, 139-145.

ROBERTS, J. M. & ESCUDERO, C. 2012. The placenta in preeclampsia. *Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health,* 2**,** 72-83.

ROBERTS, J. M. & HUBEL, C. A. 2009. The two stage model of preeclampsia: variations on the theme. *Placenta,* 30**,** 32-37.

ROZPĘDEK, W., PYTEL, D., MUCHA, B., LESZCZYŃSKA, H., DIEHL, J. & MAJSTEREK, I. 2016. The role of the PERK/eIF2α/ATF4/CHOP signaling pathway in tumor progression during Endoplasmic Reticulum stress. *Current molecular medicine*.

SABAPATHA, A., GERCEL-TAYLOR, C. & TAYLOR, D. D. 2006. Specific Isolation of Placenta-Derived Exosomes from the Circulation of Pregnant Women and Their Immunoregulatory Consequences1. *American Journal of Reproductive Immunology,* 56**,** 345-355.

SALOMON, C., RYAN, J., SOBREVIA, L., KOBAYASHI, M., ASHMAN, K., MITCHELL, M. & RICE, G. E. 2013. Exosomal signaling during hypoxia mediates microvascular endothelial cell migration and vasculogenesis. *PloS one,* 8**,** e68451.

SHE, Q.-B., MUKHERJEE, J. J., HUANG, J.-S., CRILLY, K. S. & KISS, Z. 2000. Growth factor-like effects of placental alkaline phosphatase in human fetus and mouse embryo fibroblasts. *FEBS letters,* 469**,** 163-167.

STAFF, A. C., BENTON, S. J., VON DADELSZEN, P., ROBERTS, J. M., TAYLOR, R. N., POWERS, R. W., CHARNOCK-JONES, D. S. & REDMAN, C. W. 2013. Redefining preeclampsia using placenta-derived biomarkers. *Hypertension,* 61**,** 932-942.

TAL, R. 2012. The role of hypoxia and hypoxia-inducible factor-1alpha in preeclampsia pathogenesis. *Biology of reproduction,* 87**,** 134.

TANNETTA, D., COLLETT, G., VATISH, M., REDMAN, C. & SARGENT, I. 2013a. Syncytiotrophoblast extracellular vesicles – Circulating biopsies reflecting placental health. *Placenta*.

TANNETTA, D., MASLIUKAITE, I., VATISH, M., REDMAN, C. & SARGENT, I. 2016. Update of syncytiotrophoblast derived extracellular vesicles in normal pregnancy and preeclampsia. *Journal of Reproductive Immunology*.

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

TANNETTA, D. S., DRAGOVIC, R. A., GARDINER, C., REDMAN, C. W. & SARGENT, I. L. 2013b. Characterisation of syncytiotrophoblast vesicles in normal pregnancy and pre-eclampsia: expression of Flt-1 and endoglin. *PloS one,* 8**,** e56754.

TOMIMATSU, T., MIMURA, K., ENDO, M., KUMASAWA, K. & KIMURA, T. 2016. Pathophysiology of preeclampsia: an angiogenic imbalance and long-lasting systemic vascular dysfunction. *Hypertension Research*.

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.

VAN DER POST, J., LOK, C., BOER, K., STURK, A., SARGENT, I. L. & NIEUWLAND, R. The functions of microparticles in pre-eclampsia. Seminars in thrombosis and hemostasis, 2011. 146-152.

WARRINGTON, J. P., GEORGE, E. M., PALEI, A. C., SPRADLEY, F. T. & GRANGER, J. P. 2013. Recent advances in the understanding of the pathophysiology of preeclampsia. *Hypertension,* 62**,** 666-673.

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.

WEINER, E., SCHREIBER, L., GRINSTEIN, E., FELDSTEIN, O., RYMER-HASKEL, N., BAR, J. & KOVO, M. 2016. The placental component and obstetric outcome in severe preeclampsia with and without HELLP syndrome. *Placenta,* 47**,** 99-104.

WENGER, N. K. 2014. Recognizing pregnancy-associated cardiovascular risk factors. *The American journal of cardiology,* 113**,** 406-409.

XIONG, X., DEMIANCZUK, N. N., SAUNDERS, L. D., WANG, F.-L. & FRASER, W. D. 2002. Impact of preeclampsia and gestational hypertension on birth weight by gestational age. *American journal of epidemiology,* 155**,** 203-209.

YUNG, H. W., HEMBERGER, M., WATSON, E. D., SENNER, C. E., JONES, C. P., KAUFMAN, R. J., CHARNOCK-JONES, D. S. & BURTON, G. J. 2012. Endoplasmic reticulum stress disrupts placental morphogenesis: implications for human intrauterine growth restriction. *J Pathol,* 228**,** 554-64.

CHAPTER 5

Abstract presented at the 54th Annual Microscopic Society of Southern Africa

PLACENTAL EXPRESSION OF HIF-1 ALPHA IN PREGNANT SOUTH AFRICAN WOMEN

S. Verma[1], T. Naicker[2], J. Moodley[3] and I. Mackraj[1]

[1]Department of Physiology, University of KwaZulu-Natal, [2]Department of Optics and Imaging, University of KwaZulu-Natal, [3]Women's Health and HIV Research Unit, University of KwaZulu-Natal

Hypertensive disorders (chronic hyper-tension, gestational hypertension, preeclampsia, eclampsia and the HELLP syndrome) is considered a major complication of pregnancy and is the commonest direct cause of maternal mortality in South Africa (Saving Mother's Report; 2011-2013). Preeclampsia, accounts for majority of maternal deaths emanating from hypertension. It is also responsible for the iatrogenic preterm delivery and is associated with perinatal and maternal morbidity and mortality. Although its exact pathophysiology is unclear, endoplasmic reticulum stress and trophoblast apoptosis are implicated[1].

The placental microenvironment in preeclampsia is hypoxic and induces the expression of Hypoxia inducible factor-1 alpha (HIF-1 alpha). HIF-1 alpha is transiently expressed enabling adaptation to the hypoxic environment[2]. This promotes the production of anti-angiogenic molecules with resultant endothelial dysfunction[3]. Hypoxia-induced oxidative and endoplasmic reticulum stress initiate a cascade of apoptotic events with consequential shedding of microparticles. These microparticles mediate the peripheral maternal syndrome of preeclampsia[4]. The aim of this study is to immunolocalise and morphometrically analyse HIF-1 alpha within the placenta of normotensive and pre-eclamptic pregnancies.

This study was approved by the Biomedical Research Ethics Committee (BREC no: 364/15), University of KwaZulu-Natal. All patients gave written informed consent. Placentae were obtained after caesarean section at a regional hospital, Durban, KwaZulu-Natal, South Africa. The study population consisted of normotensive pregnant (n=14) and preeclampsia (n=7) women. The placenta was collected within 10 minutes of delivery and immediately fixed in 10% phosphate-buffered formalin. Immunohistochemistry was performed using anti HIF-1

alpha antibody (1:1000; Abcam113642) and Envision Flex Mini High pH Link kit (Dako). All specimens were viewed with an Axioscope A1 microscope (Carl Zeiss, Germany). Image capturing, processing and analysis were performed using the AxoVision software (Carl Zeiss, Germany 4.8.3). At least two fields of view per slide were randomly selected and captured at an initial objective magnification of 20X.

Within the placental villi (both exchange and conducting), HIF-1 alpha immunostaining occurred within the syncytiotrophoblast and cytotrphoblast cell population (Fig. 1A). Additionally, the immuno-expression was also noted in endothelial cells lining arteries, veins and capillaries across all villi types. HIF-1 alpha was also immune-localised in the amnion (Fig. 1 B), fibroblast- like cells of the chorion and the extra- villous trophoblast cells of the decidua. Preliminary qualitative evaluation of placental villi showed an upregulation of the immuno-expression of HIF-1 alpha in pre-eclamptic versus normotensive pregnancies.

This study demonstrates an increased immumo-expression of HIF-1 alpha in preeclampsia compared to normotensive pregnancies. This elevation contributes to the angiogenic/anti-angiogenic imbalance that characterises preeclampsia. Further morphometric image analysis of HIF-1 alpha immuno-expression is currently under investigation.

References:

1. Redman, C. and Sargent, I. (2009) Placenta. 30**,** 38-42.

2. Noha Abdellatif Ibrahim et al. (2014) Cell Biology. 6, 72-80.

3. Tal, R. (2012) Biol Reprod. 87**,** 134.

4. Akhilesh, M., *et al*. (2014) Bio Geno Med. 6**,** 121-125.

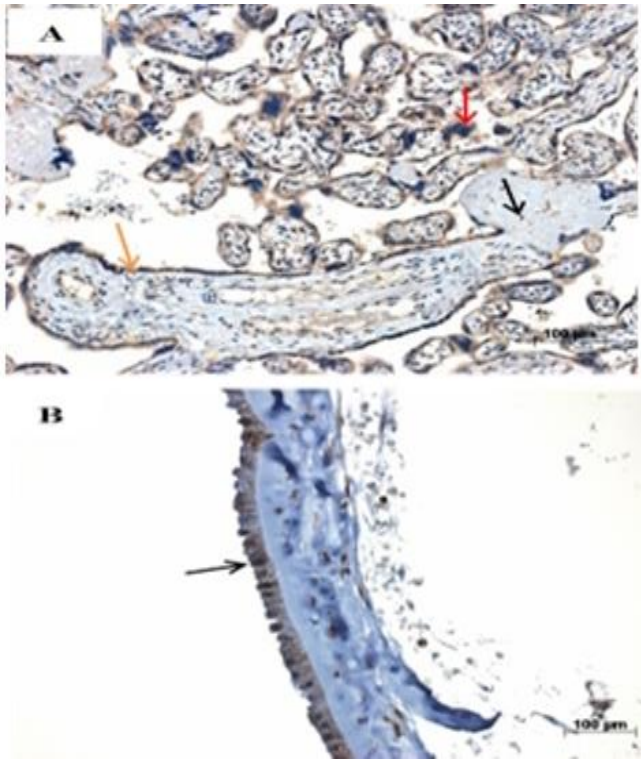


Figure 1. Syncytial knot (Red arrow), Fibrinoid (Black arrow), positive syncytiotrophoblast (Brown arrow) (A) and positive amnion (Black arrow) (B) in HIF-1 Alpha stained pre-eclamptic placenta. Scale: 100 µm.

Corresponding author: rite2sonal@yahoo.com

APPENDICES

APPENDIX A

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

27 November 2015

Dr SR Verma (215081900)
Discipline of Physiology
School of Laboratory Medicine and Medical Sciences
naina.minal.slb@gmail.com

Protocol: A study to detect levels of syncytiotrophoblast released microparticles and the mechanism of release in preeclampsia (early and late) compared to normal pregnancy in black South African women.
Degree: MMedSc
BREC reference number: BE364/15 *(sub-study of BE036/12)*

EXPEDITED APPLICATION

The Biomedical Research Ethics Committee has considered and noted your application received on 07 August 2015.

The study was provisionally approved pending appropriate responses to queries raised. Your responses dated 18 November 2015 to queries raised on 03 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: mackraj@ukzn.ac.za
cc postgrad: dudhra@ukzn.ac.za

APPENDIX 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: 313/15
KZ_2015RP41_691

Date: 18 November 2015

Dear Dr S. Verma
(University of KwaZulu Natal)
Email: naina.minal.slb@gmail.com

Approval of research

1. The research proposal titled **'Detection of syncytiotrophoblast released microparticles mechanism of release in preeclampsia compared to normotensive pregnancy 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 before commencing with data collection.

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

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

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

Yours Sincerely

[redacted]

Dr E Lutge
Chairperson, Health Research Committee
Date: 24/11/15

Fighting Disease, Fighting Poverty, Giving Hope