THE ROLE OF c-JUN N-TERMINAL KINASE (JNK) AND TUMOUR PROTEIN (p53) IN HIV ASSOCIATED PRE-ECLAMPSIA

By

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Submitted in partial fulfilment of the requirements for the degree of

MASTER OF MEDICAL SCIENCE

in the

Discipline of Optics and Imaging

College of Health Sciences

University of KwaZulu-Natal

Durban

2017

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 Optics & Imaging Centre, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa.

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DECLARATION

I, Yazira Pillay declare that:

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

(ii) This dissertation has not been submitted for any degree or examination at any other university.

(iii) This dissertation does not contain other person's data, pictures, graphs or other information,

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

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(v) 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: _____18-12-2017_____

DEDICATION

To my parents and brother, Tariq.

Thank you for your endless love, support and encouragement.

I love you. Dad, Mom and Tariq.

FUNDING

- Funding for this project was received from the College of Health Science (Masters Scholarship), University of KwaZulu-Natal.
- The project was additionally supported by the publication fund of Prof T Naicker.

ACKNOWLEDGEMENTS

I wish to express my sincere thanks and gratitude to:

- Prof T Naicker, for her continuous guidance, invaluable advice and help. You have not only been an excellent supervisor, but an incredible role model.
- Dr Margaret Alese for her laboratory assistance.
- Dr Frederick Odun-Ayo for his laboratory assistance.
- Miss S Thakoordeen, Miss P Nydoo, and Miss L Nandlal for all their assistance throughout the year.
- The Optics and Imaging Centre.
- To my entire family for their kindness, love and support.

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LIST OF ABBREVIATIONS

Human Immunodeficiency Virus……. HIV

Highly active antiretroviral treatment…….. HAART

Pre-eclampsia…… PE

Haemolysis, elevated liver enzymes, and low platelets……... HELLP

Human leukocyte antigen…… HLA

Tumour necrosis factor…… TNF

Reactive oxygen species…….. ROS

Apoptosis protease activating factor-1…… APAF-1

Inhibitor of apoptosis proteins……. IAPs

Syncytial nuclear aggregates…… SNAs

Murine double minute 2…… Mdm2

Bcl-2 homology domains…….. BH

Mitochondrial outer membrane permeabilization……. MOMP

Responsive element…… RE

Mitogen activated protein kinases……. MAPKs

c-Jun N-terminal protein kinase…… JNK

Extracellular signal-regulated kinase…… ERK

Activator protein-1…… AP-1

Primary apoptosis signalling regulated kinase 1……... ASK1

Hypoxia-reoxygenation……. H/R

Syncytiotrophoblast microvillous membrane particles……..... STBMs

Streptavidin-phycoerythrin conjugate……... SAPE

Median fluorescent intensity…… MFI

Standard error mean…….. SEM

Early-onset pre-eclampsia…… EOPE

Late-onset pre-eclampsia……... LOPE

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ABSTRACT

Background: In pre-eclampsia, immune maladaptation to the foetal allograft results in impaired trophoblast invasion and defective spiral arterial remodelling with consequential placental oxidative stress. Placental apoptosis can be initiated by various stimuli including hypoxia and oxidative stress and is notably exaggerated in pre-eclampsia. This elevated apoptosis prevents normal replenishment of the syncytiotrophoblast, promotes syncytial degeneration and releases vasoactive or inflammatory factors into the maternal circulation thereby provoking the endothelial dysfunction seen in pre-eclampsia. Since the p53 antigen and JNK are important mediators of apoptosis we determined their expression in HIV associated pre-eclampsia.

Method: Blood samples were collected from normotensive pregnant and pre-eclamptic HIV infected and negative women. Buffy coat was extracted and a Bio-plex multiplex assay to quantify expression of phosphorylated-p53 and JNK.

Results: Based on pregnancy type, a significant difference in the expression of p53 was noted between the pre-eclamptic vs normotensive pregnant group regardless of HIV status ($p=0.0162$). Irrespective of pregnancy type, there was also a significant difference found between the HIV positive and HIV negative groups ($p=0.0469$). Furthermore, there was a significant difference in the expression of p53 between the HIV negative pre-eclamptic vs the HIV negative normotensive group and HIV infected normotensive *vs* the HIV negative pre-eclamptic group. Despite having no statistical significance ($p=0.8701$), the expression of JNK was found to be increased in the pre-eclamptic compared to the normotensive pregnant group. Similarly, based on HIV status, regardless of pregnancy type no statistical significance was found ($p=0.2227$), however, there was a decrease in the expression of JNK in the HIV positive compared to the HIV negative group.

Conclusion: These experiments demonstrate a significant increase in the expression of p53 with an upwards trend in the expression of JNK in pre-eclampsia, confirming their influence on trophoblast cell invasion in pre-eclampsia development. This increase in expression of p53 and JNK in pre-eclampsia, holds potential value as a risk indicator of pre-eclamptic development. In contrast, a significant down regulation of p53 with a downwards trend of JNK expression was noted in the HIV positive group, possibly due to immune reconstitution following HAART.

CHAPTER ONE

1.0 BACKGROUND AND LITERATURE REVIEW

1.1 Maternal Mortality

Globally, the rate of maternal mortality is high. Every day, there are about 830 maternal deaths due to pregnancy or childbirth related complications worldwide, with 99% of all maternal deaths occurring in developing countries (Alkema *et al*., 2016). In 2015, it was estimated that approximately 303, 000 women died during and following pregnancy and childbirth, most of these deaths were preventable (Alkema *et al*., 2016).

In South Africa, during 2011-2013 there were 4452 maternal deaths. The top three causes of maternal death were: non-pregnancy related infections (mainly HIV infected pregnant women), obstetric haemorrhage and hypertension which accounted for 34.7%, 15.8% and 14.8% maternal deaths respectively. Majority (67%) of the cases of maternal deaths emanating from hypertension was preventable (South African Department of Health, 2013).

1.2 HIV

Human Immunodeficiency Virus (HIV) infection remains one of the leading health concerns in the world. In 2015, roughly 2.1 million new infections were reported worldwide, with a recent tally of 36.7 million people living with HIV infection (UNAIDS, 2016). In South Africa, the overall HIV prevalence rate is estimated to be 12.7%. For adults of reproductive age (15–49 years), an estimated 18.9% of the population is HIV infected (Statistics South Africa, 2016).

HIV is a retrovirus that can integrate its DNA into the host genome (Craigie and Bushman, 2012). The primary receptor for HIV-1 is CD4+. The CD4+ cell is expressed on the surface of T lymphocytes, monocytes, and macrophages (Février *et al*., 2011). HIV can be transmitted through infected body fluids that come into contact with mucosal tissue, blood or broken skin (Deeks *et al*., 2015). HIV causes progressive loss of CD4+ T cells, which results in a decline of cell-mediated immunity (Moir *et al*., 2011, McCune, 2001). This renders the individual vulnerable to a range of opportunistic infections (Holmes *et al*., 2006).

Management of HIV infection includes the use of multiple antiretroviral drugs. Highly active antiretroviral treatment (HAART), is a powerful therapy which suppresses HIV replication and allows partial restoration of $CD4^+$ T cell populations. (Schmidt-Westhausen *et al*., 2000, Ramirez-Amador *et al*., 2003, Février *et al*., 2011).

1.2.1 HIV and women

Currently, one-fifth of South African women in their reproductive ages are HIV infected (Statistics South Africa, 2016). Women are more likely to face increased tissue injury and due to their anatomy have a larger mucosal surface area, resulting in longer exposure times to pathogens and infectious fluid during sexual intercourse. Thus, the women have a higher physiological risk of infection (Ackermann and Klerk, 2002).

During pregnancy, the high levels of oestrogen and progesterone may alter the genital mucosa structure, alternatively it can cause immunological changes including increased mucosal lymphoid aggregates or the up-regulation in the expression of co-receptors that are involved with HIV infection (Chersich and Rees, 2008). HIV infection and hypertensive disorders of pregnancies, especially pre-eclampsia are important conditions in developing countries (Hall *et al*., 2014).

1.3 Pre-Eclampsia

Pre-eclampsia (PE) is a condition specific to pregnancy that occurs after the $20t^h$ week of gestation (Silasi *et al*., 2010) and is characterized by new-onset hypertension (high blood pressure), with proteinuria. PE is defined as a blood pressure reading of ≥ 140/90 mmHg on two or more occasions after the 20^{th} week of pregnancy with significant proteinuria >300mg/dl present in a 24-hour period (Davey and MacGillivray, 1988). It is estimated that 3-5% of all pregnancies are affected worldwide by PE, making it the most common pregnancy complication (Wang *et al*., 2009), and a leading cause of perinatal and maternal morbidity and mortality (Levy, 2005).

Some of the risk factors of PE include; primigravidae, a maternal and paternal family history of PE, nulliparity as well as pre-existing metabolic disorders such as diabetes, chronic hypertension, obesity and kidney disease (Silasi *et al*., 2010).

1.3.1 Complications of pre-eclampsia

Maternal complications of PE include; acute renal failure, placental abruption, seizures, pulmonary oedema and the HELLP (haemolysis, elevated liver enzymes, and low platelets) syndrome (Wang *et al*., 2009). When vascular dysfunction of the brain occurs, eclampsia can develop which is characterized by seizures (Coppage and Sibai, 2005). Complications which affect the developing foetus include; prematurity (Zhang *et al*., 2007), intrauterine foetal growth restriction and an increased risk of perinatal death (Wang *et al*., 2009). Increased maternal and foetal morbidity and mortality are largely due to a lack of predictive biomarkers and effective pharmaceutical interventions (Tannetta and Sargent, 2013). There is no treatment for PE currently, except early delivery of the placenta and the baby, however this increases the risk of neonatal morbidity and mortality (Williamson *et al*., 2017).

1.3.2 The placenta

The placenta is fundamental to the pathogenesis of PE, as PE only occurs when the placenta is present, even when there is no foetus, as in a hydatidiform molar pregnancy. The maternal symptoms of PE usually resolve when the placenta is delivered (Naljayan, 2013). The placenta is a fetally-derived multifaceted organ which connects the developing foetus to the uterine wall providing nutrition, removal of waste products, performing endocrine functions and affording foetal protection from the maternal immune system (Gude *et al*., 2004). Altered placental development and function can have repercussions on the foetus affecting its ability to cope with the intrauterine micro-environment (Gude *et al*., 2004).

1.3.3 Placentation

During implantation, the secretion of hormones regulate growth factors, cytokines and adhesion molecules, thereby altering the endometrial surface and opening the implantation window. Following

attachment, the trophoblast layer of the blastocyst proliferates rapidly and differentiates (Gude *et al*., 2004).

Cytotrophoblast cells differentiate into; the multinucleate syncytiotrophoblast and the invasive extra-villous trophoblast cell populations. Syncytiotrophoblasts cover the surface of the placenta and allow foetal-maternal exchange, while also secreting hormones into the maternal circulation necessary for the maintenance and immunological adaptation of pregnancy (Bansal *et al*., 2012, Gude *et al*., 2004). It has been proposed that fusion between the cytotrophoblasts and the syncytiotrophoblast is a means of replenishment of the syncytiotrophoblast (Sharp *et al*., 2010). The extravillous trophoblasts invade the uterine spiral arteries of the decidua and myometrium in a set timed sequence, replacing the endothelial layer of the maternal spiral arteries and transforming them from small, high-resistance vessels to high-calibre capacitance, low resistance vessels that can sustain the growth of the foetus (Meekins *et al*., 1994) (Figure 1).

In normal placental development, intramural cytotrophoblasts differentiate from an epithelial phenotype to an endothelial phenotype by pseudovasculogenesis (Zhou *et al*., 1997). In PE, failure to adopt an invasive endothelial phenotype in cytotrophoblasts is observed (Zhou *et al*., 1997).

1.3.4 Pathogenesis of pre-eclampsia

The exact pathophysiology of PE remains unknown. In PE, the physiological conversion of the spiral arteries is incomplete and is limited to the decidua (Meekins *et al*., 1994). Failure of trophoblast invasion results in reduced uteroplacental perfusion which leads to the placenta becoming ischemic (Levy, 2005).

Retention of vasoreactivity of the spiral arteries, could result in intermittent perfusion of the intervillous space, fluctuating oxygen tension, and ischemia-reperfusion injury of the villi (Hung *et al*., 2001). The re-established blood flow seen after placental reperfusion injury releases cytokines as well as other inflammatory factors and damaging levels of reactive oxygen species (Sánchez-Aranguren *et al*., 2014).

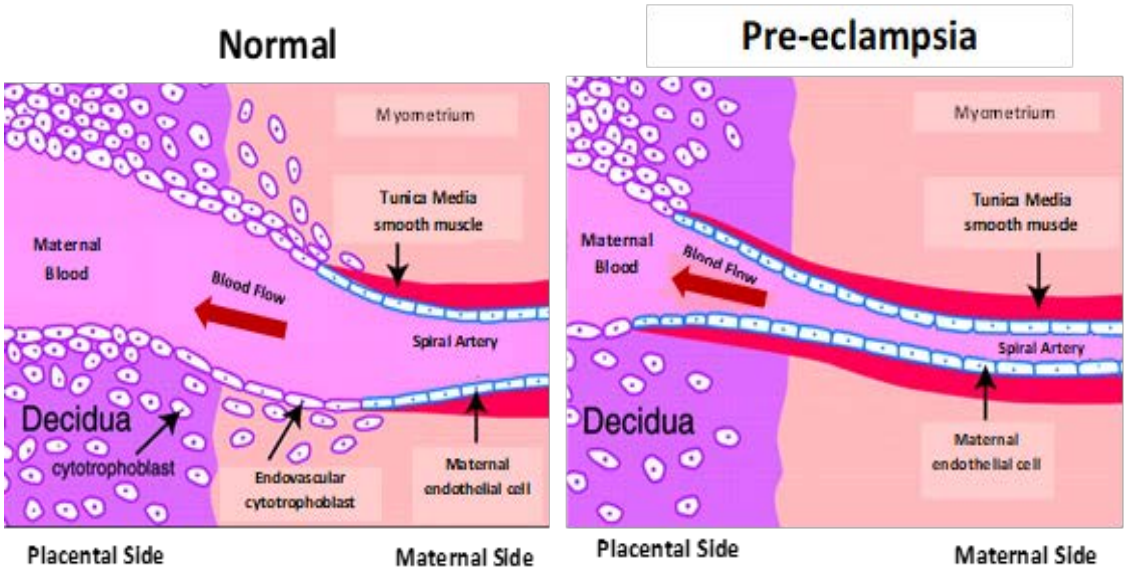

Figure 1. Normal vs Abnormal Placentation. In normal placentation (Left) the extravillous cytotrophoblasts invade the uterine spiral arteries of the decidua and myometrium, replacing the endothelial layer of the maternal spiral arteries, transforming them from small, high-resistance vessels to high-calibre capacitance vessels that can supply the necessary placental perfusion to sustain the growth of the foetus. In PE (Right) remodelling of the spiral arteries is inadequate and limited to the superficial decidua, with the myometrial segments remaining narrow. Figure adapted from (Lam *et al*., 2005).

1.3.5 The role of the immune system in pre-eclampsia

A common theory to explain the disease mechanism of PE is immune maladaptation to the foetal allograft (Dekker and Sibai, 1998). An exacerbated maternal inflammatory response directed against foreign foetal antigens can result in impaired trophoblast invasion and defective spiral arterial remodelling (Matthiesen *et al*., 2005).

Contact between paternal antigens in the semen and the female genital tract through sexual intercourse allows for immune tolerance. However, when there is a new partner the risk of PE increases due to insufficient duration of exposure to sperm (Verwoerd *et al*., 2002, Hall, 2007b).

The fetus and placenta is regarded as a semi-allograft to the mother's immune system and therefore can produce an immune rejection response (Aagaard-Tillery *et al*., 2006). During early stages of pregnancy, there is an increased influx of macrophages and lymphocytes into the decidua at the maternal-foetal

interface (Bulmer and Johnson, 1985). A common theory is that human leukocyte antigen (HLA) G has an immunologically permissive function of the antigenic mismatch between mother and foetus, and this protects the developing foetus from these cells (Le Bouteiller *et al*., 2003). Defective HLA-G expression occurs in the extravillous cytotrophoblast of pre-eclamptic placentas. Serum levels of soluble HLA-G1 isoform involved in the downregulation of T cell and NK cell activity by the induction of apoptotic events, are decreased in pre-eclamptic patients (Le Bouteiller *et al*., 2003).

Notably, the Fas-FasL system could have a key role in the protection of the foetus from the maternal immune system (Pearson, 2002). Fas is a surface protein of the tumour necrosis factor (TNF) superfamily and can induce cell apoptosis upon binding to the ligand FasL (Griffith *et al*., 1995). FasL is expressed on the trophoblast of first-trimester placentas, and the Fas antigen is localized on decidual cells, specifically maternal leukocytes (Runic *et al*., 1996, Uckan *et al*., 1997). *In-vitro*, trophoblasts expressing FasL can induce lymphocyte apoptosis when exposed to activated lymphocytes (Hammer *et al*., 1999). In PE, decidual cells have a lower expression of Fas antigen (Ishihara *et al*., 2002), and a lower expression of FasL (Allaire *et al*., 2000) is noted on trophoblast cells resulting in the diminished deletion of lymphocytes in the decidua with increased apoptosis and trophoblast damage (Levy, 2005). As a consequence of exaggerated apoptosis of the invading trophoblasts, limited invasion of the spiral arteries occurs (Levy, 2005).

1.3.6 Immune maladaptation of pre-eclampsia and HIV infection

In contrast to HIV infection, an up-regulation of the immune response occurs in normal pregnancy, however this up-regulation is exacerbated in PE producing an excessive maternal inflammatory response. Therefore, in HIV associated PE, a neutralisation of the immune response is expected (Hall *et al*., 2014). Studies are however conflicting and report an increased, decreased and a neutralisation of PE development in HIV associated pregnancies (Boyajian *et al*., 2012, Hall, 2007a, Kalumba *et al*., 2013, Machado *et al*., 2014, Wimalasundera *et al*., 2002, Hall *et al*., 2014).

In 2002, Wimalasundera and colleagues found that HIV-1 infected women who received no antiretroviral therapy had a significantly lower rate of PE development compared to HIV infected

women who were on triple antiretroviral therapy (Wimalasundera *et al*., 2002). They therefore proposed that women with untreated HIV infection are protected against PE development due to their immunocompromised state, they suggest that these women are unlikely to induce an exaggerated inflammatory response during pregnancy due to the progressive loss of CD4+ T lymphocytes. The administration of HAART in HIV infected women re-establishes the mother's immune response to foetal antigens, and thus exacerbates the risk of PE development (Wimalasundera *et al*., 2002)**.**

Similarly, a cohort study by Suy and colleagues concluded that HIV infected women receiving HAART prior to pregnancy was associated with a higher risk of developing PE and was associated with greater foetal mortality (Suy *et al*., 2006)**.** In contrast, PE and gestational hypertension were less prevalent in women receiving mono- or triple anti-retroviral therapy (Hall *et al*., 2014).

Conflicting results were found in a study by Sansone and colleagues where HIV infected women had an increased risk of PE development, when receiving HAART (13%) and not receiving HAART (4.6%) compared to HIV uninfected women (4.1%) (Sansone *et al*., 2016). Whereas in a matched cohort study by Boyajian *et al*, no difference in the risk of developing PE was demonstrated between HIV infected women on HAART versus uninfected women (Boyajian *et al*., 2012). In South Africa, approximately 30% of antenatal patients are infected with HIV (Sebitloane *et al*., 2009). It is therefore rational to study the effect of HIV infection on PE development.

1.4 Oxidative stress

Reactive oxygen species (ROS) include; nitric oxide (NO), superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (·OH). These are signalling molecules implicated in the regulation of several functions in human physiology and are controlled by anti-oxidant host defences (Kalyanaraman, 2013). An increase in ROS generation during gestation is a physiological requisite (Yang *et al*., 2012). ROS are known as 'secondary messengers' in intracellular signalling cascades where the molecules control cell growth, proliferation and apoptosis (Thannickal and Fanburg, 2000).

A state of oxidative stress is observed when the relative pro-oxidant species is higher than the antioxidant defences (Sánchez-Aranguren *et al*., 2014). The increased generation of ROS could eventually trigger a redox signalling process that induces cell apoptosis (Sánchez-Aranguren *et al*., 2014).

1.5 Apoptosis and apoptotic pathways

Apoptosis (programmed cell death), is vital in the normal development of the placenta however, when exaggerated it is associated with placental disease (Sharp *et al*., 2010). Apoptosis can be initiated via the extrinsic or intrinsic pathway. The extrinsic pathway is regulated by members of the TNF death receptor family (Straszewski-Chavez *et al*., 2005). Cellular stresses like DNA damage and ROS can initiate the intrinsic pathway. Activation of the intrinsic pathway leads to an altered mitochondrial membrane permeability due to an imbalance in the relationship of pro- and anti-apoptotic Bcl-2 proteins (Cory and Adams, 2002). Anti-apoptotic Bcl-2 members include; Bcl-2, Bcl-xL and Mcl-1 whilst pro-apoptotic members include; Bax, Bak, tBid, Bim, Puma, Noxa and Bad (Vaseva and Moll, 2009). Bax and Bak convert into pore forming proteins by oligomerizing in the mitochondrial outer membrane. The lipid pores formed release fatal proteins from the mitochondrial inter-membranous space (Kroemer *et al*., 2007).

tBid and Bim are activators which trigger mitochondrial outer membrane permeabilization (MOMP) by the direct stimulation of Bax and Bak oligomerization (Vaseva and Moll, 2009). Puma, Noxa and Bad are de-repressors which inhibit the anti-apoptotic Bcl-2 family members allowing the release of pro-apoptotic members *e.g.,* Bax or tBid from Bcl-xL (Vaseva and Moll, 2009, Kroemer *et al*., 2007).

An increase in mitochondrial permeability results in pore formation and the leakage of cytochrome-c into the cytosol (Li *et al*., 1997). Cytochrome-c binds the apoptosis protease activating factor-1 (APAF-1) (Ott *et al*., 2002) and the initiator caspase 9 in the cytosol forming the apoptosome complex (Chinnaiyan, 1999). This stimulates caspase 9, which activates the effector caspases. Furthermore, the protein Smac/DIABLO is also released from the mitochondrion into the cytosol which block the effects

of a group of anti-apoptotic proteins known as inhibitor of apoptosis proteins (IAPs), thereby indirectly promoting apoptosis (van Loo *et al*., 2002).

Both the intrinsic and extrinsic pathways end in a terminal pathway involving the cleavage and activation of caspase-3, 6, and 7 initiating cell destruction by the activation of DNAses and cleaving DNA repair enzymes (Sharp *et al*., 2010, Tewari *et al*., 1995, Koh *et al*., 2005). Important cell signalling molecules involved in the regulation of apoptosis include tumour protein p53 (Prives and Hall, 1999) and c-Jun N-terminal protein kinase (JNK) (Lin and Dibling, 2002, Liu J and A., 2005).

1.6 p53

p53 is a transcription factor (Lane, 1992) mostly known for its activity as a tumour suppressor factor, however, it can also help to control a wide range of cellular processes and diseases. p53 has a critical role in the determination of cell fate by the activation of growth arrest, cellular senescence or apoptotic pathways (Brooks and Gu, 2010).

p53 is an unstable protein with a short half-life, it is stabilized by hypoxia and oxidative stress (Levy, 2005). Under normal conditions, the expression of p53 is kept at low levels to protect the cell from any uncontrolled activity. The ubiquitin ligase, murine double minute 2 (Mdm2) regulates the transcriptional activity of p53 by the direct inhibitory binding to p53, ubiquitination and enzymatic degradation of p53 by the proteasome as well as the nuclear export of p53 to the cytoplasm (Haupt *et al*., 1997, Kubbutat *et al*., 1997, Momand *et al*., 1992, Levine, 1997, Lu *et al*., 2000). p53 is further controlled by acting as a transcription factor for Mdm2, this promotes its own inhibition as part of an essential negative feedback loop (Harris and Levine, 2005).

1.6.1 Activation of p53 antigen

Cellular p53 increases rapidly post exposure to a variety of stressors including: heat, hypoxia, hyperoxia, and cytokines (Harris and Levine, 2005). These stresses promote DNA damage thereby activating repair enzyme activities (Harris and Levine, 2005). Following DNA damage, p53 becomes phosphorylated at several sites. ATM, JNK and ATR are some of the kinases able to phosphorylate

residues within the N-terminus of p53 *in-vitro* (Ashcroft *et al*., 1999). Phosphorylation of the N-terminus allows for the stabilization of p53 by inhibiting the p53-Mdm2 interaction (Kruse and Gu, 2008) which allows for the expression of cellular p53 to increase. A series of post translational events need to occur for the activation of p53, of which phosphorylation may be a part of (Brooks and Gu, 2010). The mechanism by which p53 either promotes cell growth arrest or apoptosis is poorly understood. Evidence suggests that the *in-vivo* function of p53 depends on the type and severity of damage that occurs (Brooks and Gu, 2010).

1.6.2 Apoptotic pathways of p53 antigen

p53 can induce apoptosis by the activation of gene expression in the nucleus, or by directly permeabilizing mitochondria in the cytoplasm (Wang *et al*., 2007). p53 is implicated in the induction of both the extrinsic and intrinsic apoptotic pathways (Susan Haupt *et al*., 2003).

1.6.2.1 The role of p53 within the nucleus

Activated p53 translocates from the cytosol to the nucleus (Liang and Clarke, 2001). p53 binds to a specific DNA target sequence, the responsive element (RE). Genes which contain the RE regulate the cell cycle and control cell fate (el-Deiry *et al*., 1992, Funk *et al*., 1992).

Key targets of p53 include genes for pro-apoptotic proteins; Bax, Noxa, Puma, Bid and APAF-1 (Sharp, 2011). p53 activates the intrinsic mitochondrial apoptotic pathway via the induction of the expression of at least three Bcl-2 pro-apoptotic members, disrupting the balance and allowing a shift towards pro-apoptotic effects (Susan Haupt *et al*., 2003). (Figure 2).

1.6.2.2 Mitochondrial effects of p53

Activated p53 can initiate apoptosis without transcription or protein synthesis by migrating to the mitochondria. A proposed mechanism for this involves the direct interaction of p53 with the mitochondria (Mihara *et al*., 2003) where it acts like a pro-apoptotic Bcl-2 member either as a direct activator of Bax and/or Bak or as a de-repressor (Green and Kroemer, 2009).

Eventually the activation of p53 determines whether a cell enters a state of cellular senescence for DNA repair (Bhana *et al*., 2008, Chen *et al*., 2006, Dahm-Daphi *et al*., 2005, Sengupta *et al*., 2003) or p53 initiates a cell death pathway leading to apoptosis. While controlled levels of p53 activity is needed for normal cell function, abnormal activation levels could be responsible for a variety of disease pathologies (Sharp, 2011) (Figure 2).

1.6.3 Association of p53 in pre-eclampsia

Placental p53 is present in normal pregnancy and in gestational trophoblast disease (Shi *et al*., 1996, Cheung *et al*., 1999). p53 has been found in cytotrophoblasts but hardly ever seen in the syncytiotrophoblast (Quenby S *et al*., 1998, Haidacher S *et al*., 1995). Whereas, Mdm2 is expressed within the cytotrophoblast and syncytiotrophoblast (Cheung *et al*., 1999, Fulop *et al*., 1998, Heazell *et al*., 2008b). An upregulation of p53 was found in placental and placental bed samples of pregnancies complicated by PE, with associated exaggerated apoptosis (Levy *et al*., 2002).

p53 expression in explanted human placental tissue is increased by hypoxia and is associated with exaggerated trophoblast apoptosis, which might reflect the *in-vivo* condition (Heazell and Crocker, 2008). Furthermore, in response to decreased oxygen tension and ROS, there is an associated increase in p53 expression and the induction of apoptosis (Heazell *et al*., 2009). This suggests that hypoxia and ROS could be the causative mechanism in exaggerated placental apoptosis by the induction of p53 activity in PE (Crocker, 2007) and that increased p53 expression in the placenta possibly plays a pathogenic role in PE development (Heazell *et al*., 2005).

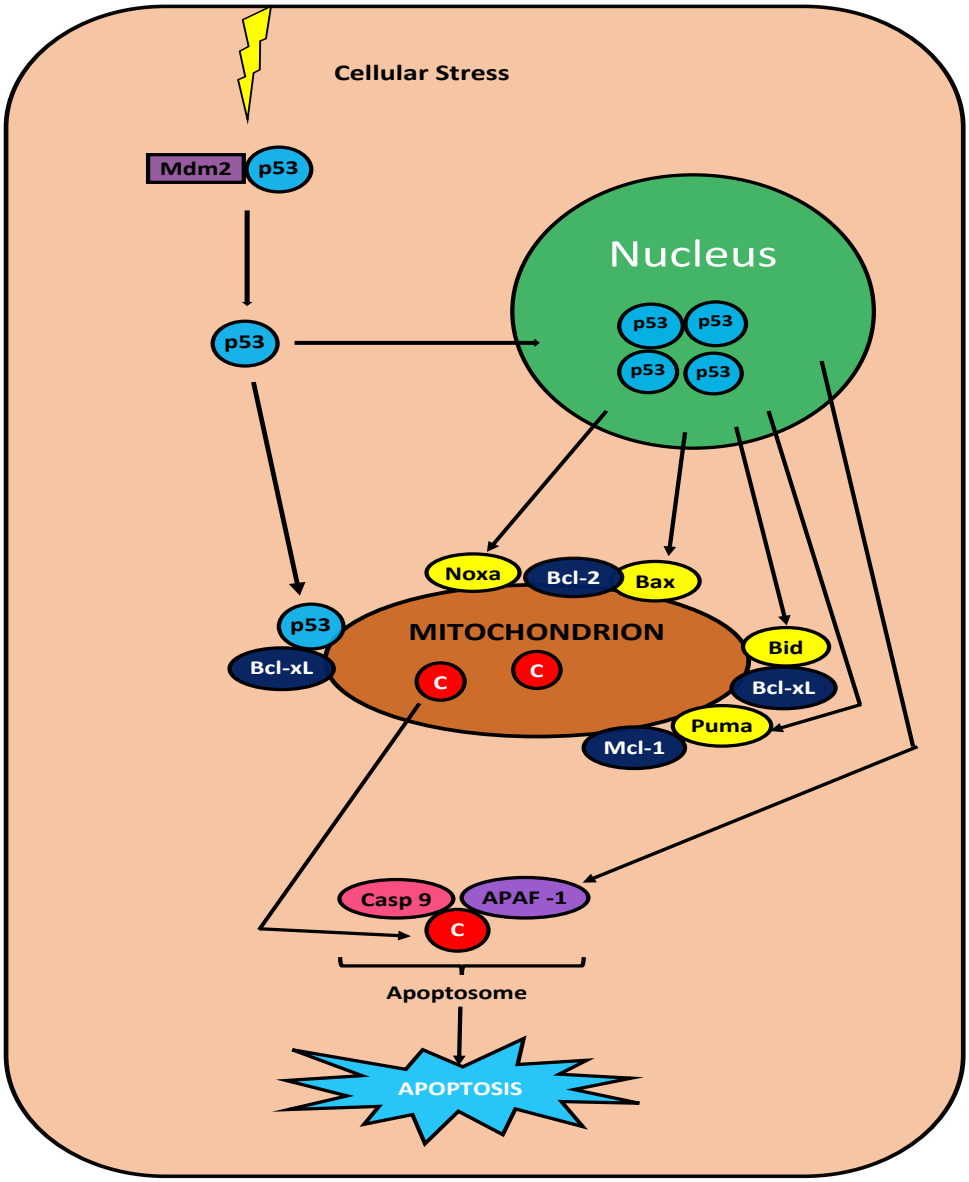

Figure 2: Apoptosis Pathway in p53.Under normal circumstances p53 is bound inactive to Mdm2 within the cytoplasm. Post exposure to stress can cause the dissociation of p53 from Mdm2, allowing the phosphorylation and translocation of p53 to the nucleus. P53 forms a tetramer within the nucleus which facilitates DNA binding and the initiation of transcription of pro-apoptotic proteins such as Bax, Noxa, Puma, Bid and APAF-1. Alternatively, activated p53 could translocate to the mitochondria where p53 has been suggested to act as a direct activator of Bax and/or Bak or as a de-repressor. This eventually leads to formation of the apoptosome and apoptosis takes place. Adapted from (Heazell and Crocker, 2008).

1.7 JNK

Mitogen activated protein kinases (MAPKs) are mediators in signal transduction pathways and are also involved in the differentiation of villous trophoblast (Qi and Elion, 2005, Vaillancourt *et al*., 2009). c-Jun N-terminal protein kinase (JNK) is a subfamily of the MAPK superfamily (Liu J and A., 2005). They are associated with the regulation of cell proliferation, differentiation, and apoptosis. JNKs has a vital role in the mediation of apoptotic signalling (Dhanasekaran and Reddy, 2008). Studies have shown JNKs can be activated by various stimuli which include; growth factors (Hibi *et al*., 1993), cytokines (Westwick *et al*., 1994), and stress factors (Cano *et al*., 1994).

JNK is able to phosphorylate and regulate the activity of transcription factors such as; c-Jun, ATF2, Elk-1, p53 and c-Myc (Chang and Karin, 2001, Davis, 2000, Karin, 1995, Lin, 2003) as well as other factors like the members of the Bcl-2 family (Bcl-2, Bcl-xL, Bim and BAD), when exposed to a wide range of extracellular stimuli (Liu J and A., 2005).

1.7.1 Activation of JNK

Specific stimuli trigger the activation of MAP3Ks, a member of MAP3K is primary apoptosis signalling regulated kinase 1 (ASK1) (Hayakawa *et al*., 2012) which can be activated by various stresses including, oxidative stress (Cindrova-Davies, 2009). This then phosphorylates and activates the MAP2K isoforms MKK4 and MKK7, which in turn phosphorylates and activates JNK. A key target of the JNK signalling pathway is the activator protein-1 (AP-1) transcription factor, which is activated partly due to the phosphorylation of c-Jun and associated molecules (Weston and Davis, 2002).

1.7.2 Signalling of JNK in the regulation of apoptosis

JNK signalling is a contributor to apoptotic signalling via phosphorylation of pro-apoptotic proteins. JNK participates in both the extrinsic and intrinsic signalling pathways that initiate apoptosis and can initiate apoptosis by nuclear and mitochondrial signalling (Dhanasekaran and Reddy, 2008).

1.7.2.1 Nuclear signalling of JNK in the regulation of apoptosis

Following activation, the phosphorylated JNK translocates to the nucleus and phosphorylates and trans-activates c-Jun (Davis, 2000, Chang and Karin, 2001). The phosphorylation of c-Jun results in the formation of AP-1. AP-1 is involved in the transcription of many proteins, including pro-apoptotic proteins (Dhanasekaran and L Johnson, 2007, Raman *et al*., 2007, Turjanski *et al*., 2007). The JNK-AP-1 pathway is associated with the increased expression of pro-apoptotic genes like, TNF-α, Fas-L, and Bak (Fan and Chambers, 2001). Therefore, JNK can promote apoptosis by the increased expression of pro-apoptotic genes (Dhanasekaran and Reddy, 2008). Studies have shown that JNK can modulate the expression of p53 directly or indirectly and can positively influence apoptotic cell death (Saha *et al*., 2012).

The phosphorylation of p53 at Ser6 by JNK stabilizes p53 by inhibiting ubiquitin-mediated degradation (Dhanasekaran and Reddy, 2008). Due to p53 being involved in the expression of pro-apoptotic proteins including Bax and PUMA, it is possible that the apoptotic pathway that is activated by JNK involves the upregulation of pro-apoptotic genes mediated by p53 (Dhanasekaran and Reddy, 2008). (Figure 3).

1.7.2.2 Mitochondrial signalling of JNK in the regulation of apoptosis.

JNK has a vital role in the modulation of the functions of the pro- and anti-apoptotic proteins found in the mitochondria. After activation by apoptotic stimuli, JNK translocates to the mitochondria (Kharbanda *et al*., 2000, Chauhan *et al*., 2003) where it modulates the activity of pro-apoptotic BH3-only subgroup Bim and Bmf (Lei and Davis, 2003) and also promotes apoptosis by the inhibition of anti-apoptotic proteins like Bcl2 (Dhanasekaran and Reddy, 2008). (Figure 3).

1.7.3 Association of JNK in Pre-eclampsia

A study conducted by (Xiong *et al*., 2013) showed that an increase in the phosphorylation of p38 and JNK occur in human placental explants when exposed to a range of PE associated stress factors such as hypoxia and inflammatory cytokines. *In vitro* experiments involving placental villous explants exposed to hypoxia-reoxygenation (H/R) activates p38 and JNK stress pathways, and is associated with the

induction of apoptosis and inflammation (Cindrova-Davies *et al*., 2007). Also, it has been shown that *in vitro*, H/R and H_2O_2 treatment of placental villi can induce the activation of ASK1 (Cindrova-Davies, 2009).

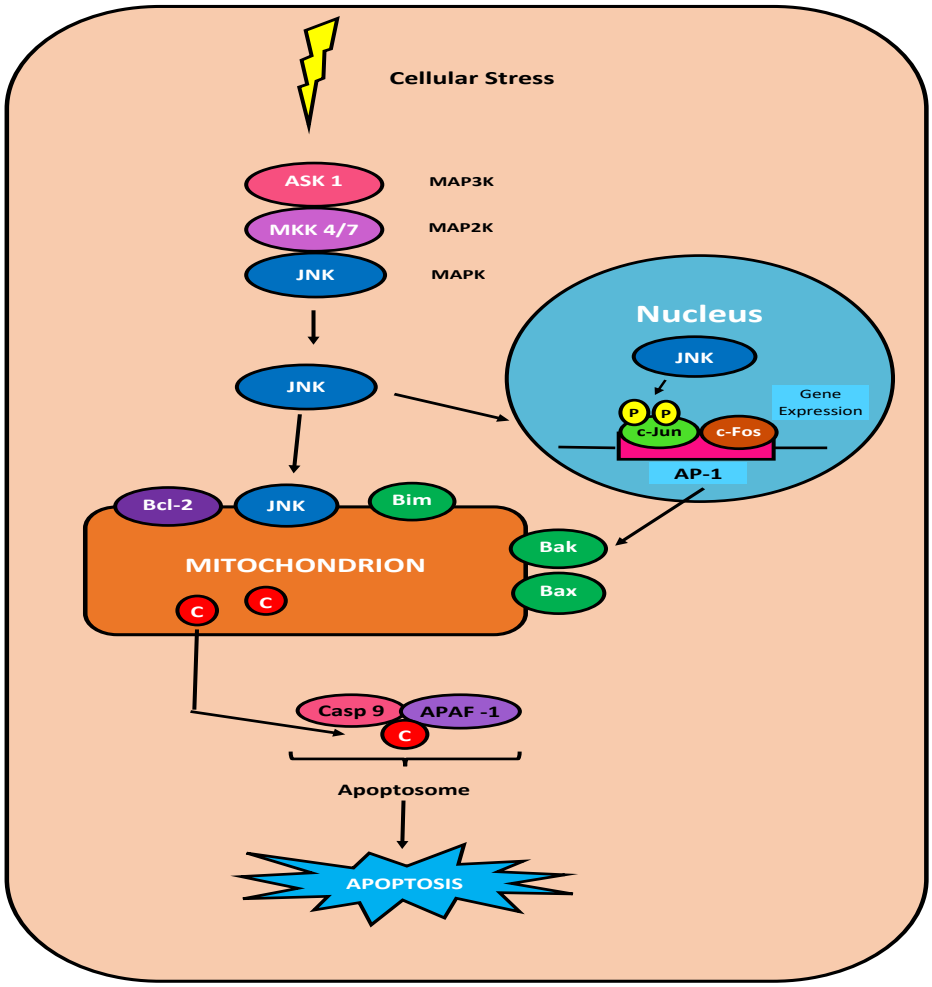

Figure 3: JNK apoptotic pathways. Specific stimuli trigger the activation of MAP3Ks (ASK-1), this then phosphorylates and activates the MAP2K isoforms MKK4 and MKK7, this in turn phosphorylates and activates JNK. Phosphorylated JNK translocates to the nucleus and phosphorylates and transactivates c- Jun which results in the formation of AP-1. The JNK-AP-1 pathway is associated with the increased expression of pro-apoptotic genes like Bak. Alternatively, JNK translocates to the mitochondria where it modulates the activity of pro-apoptotic BH3-only subgroup Bim. JNK can also promote apoptosis by the inhibition of anti-apoptotic proteins. Adapted from (Dhanasekaran and Reddy, 2008).

1.8 Association of apoptosis in pre-eclampsia

In severe PE, cell turnover is dysregulated with a decrease in syncytiotrophoblast area (Huppertz and Kingdom, 2004), increased apoptosis (Leung *et al*., 2001, Allaire *et al*., 2000, Heazell *et al*., 2008a), and an increased density of syncytial nuclear aggregates (SNAs) (Jones and Fox, 1980, Heazell *et al*., 2007). Morphometric image analysis has shown an increase in trophoblast cell apoptosis in PE (Naicker *et al*., 2013). Studies have shown that exaggerated apoptosis can be reproduced in trophoblast *in vitro* by exposure to hypoxia (Crocker *et al*., 2004) and reactive oxygen species (Moll *et al*., 2007).

The role of enhanced apoptosis in the pathology of PE remains unclear. It could eventually prevent replenishment of syncytiotrophoblast, increase syncytial degeneration and release vasoactive or inflammatory factors into the maternal circulation (Sharp *et al*., 2014).

1.9 Link between apoptosis and endothelial dysfunction

The relationship between placental ischemia and endothelial cell dysfunction in the pathogenesis of PE remains unknown. Systemic endothelial damage seems to be the main theme in the signs and symptoms seen in PE. A theory proposed by (Redman and Sargent, 2000) suggests that systemic endothelial damage is caused by the microdeposition of syncytiotrophoblast microvillous membrane particles (STBMs) which can be detected in the plasma of normal pregnancies but are increased in women with PE (Nelson, 1996, Huppertz B *et al*., 1998). Increased syncytiotrophoblast deportation in PE could be produced by increased apoptosis at the syncytium (Huppertz B *et al*., 1998, Nelson, 1996)

When STBMs are present within the maternal circulation, this has been associated with an altered immunological response, in specific, neutrophil activation (Von Dadelszen *et al*., 1999, Gupta *et al*., 2006). Maternal levels of IL-1β, IL-6, and IL-8 are increased in PE (Luppi P and JA, 2006). *In-vitro,* STBMs can disrupt endothelial cells, supporting the connection between placental apoptosis, syncytial microparticle release and the maternal vascular complications, typical of the pre-eclamptic syndrome (Cockell *et al*., 1997, Hoegh *et al*., 2006). (Figure 4).

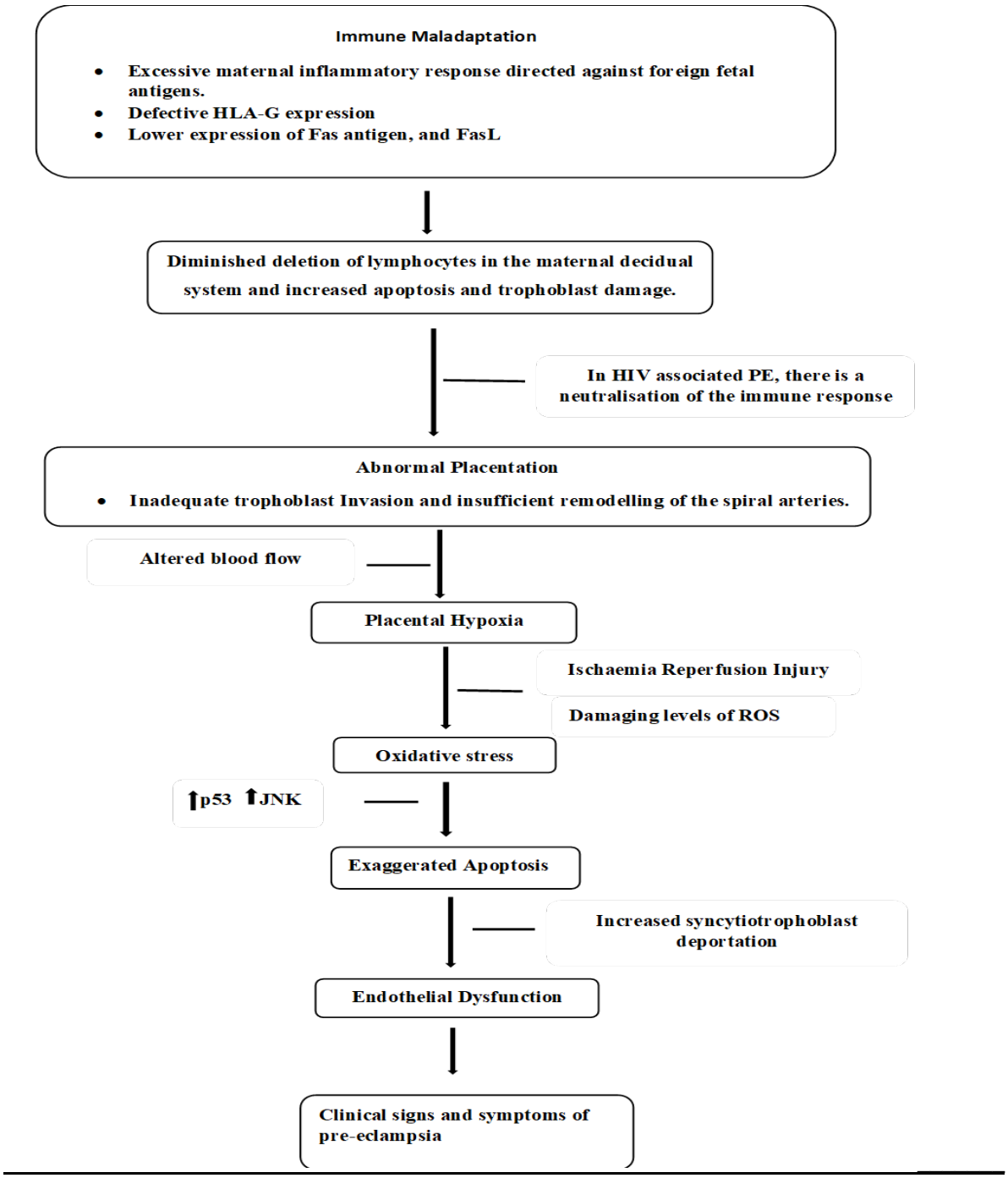

Figure 4. Sequence of events leading to pre-eclampsia.

The exact pathophysiology of PE remains unknown. A common theory used to explain the disease mechanism is immune maladaptation leads to abnormal placentation which in turn causes reduced perfusion which can lead to oxidative stress. This results in an increase of regulatory components of apoptosis p53 and JNK, which consequently results in exaggerated apoptosis. This has been proposed to result in endothelial dysfunction and the clinical signs and symptoms of pre-eclampsia. With regards to HIV associated PE, this can neutralize the immune system.

1.10 Aim and Objectives of this study

Aim of study

To compare and contrast JNK and p53 levels across the study population based on pregnancy type (pre-eclamptic *vs* normotensive) and HIV status (HIV Positive *vs* HIV negative).

Specific Objectives

•To determine the level of JNK in HIV associated normotensive and pre-eclamptic pregnant patients with the use of a BioPlex Multiplex immunoassay.

•To determine the level of p53 in HIV associated normotensive and pre-eclamptic pregnant patients with the use of a BioPlex Multiplex immunoassay.

•To compare and contrast JNK and p53 levels across the study population based on pregnancy type (pre-eclamptic vs normotensive) and HIV status (HIV Positive *vs* HIV negative).

CHAPTER TWO

HYPERTENSION
IN
PREGNANCY

CELL SIGNALLING FACTORS c-JUN N-TERMINAL KINASE (JNK) AND TUMOUR PROTEIN p53 IN HIV ASSOCIATED PRE-ECLAMPSIA

Journal:	*Hypertension in Pregnancy*
Manuscript ID	LHIP-2017-0088
Manuscript Type:	Original Article
Date Submitted by the Author:	03-Nov-2017
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Keywords:	pre-eclampsia, JNK, p53, HIV

CELL SIGNALLING FACTORS c-JUN N-TERMINAL KINASE (JNK) AND TUMOUR PROTEIN p53 IN HIV ASSOCIATED PRE-ECLAMPSIA

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Total word count: 5121

Total word count excluding abstract and references: 2679

Number of figures: 2

Number of tables: 2

Abstract

Objective: Exaggerated apoptosis is associated with HIV infection and the pathogenesis of pre-eclampsia, therefore the objective of this study was to determine expression of p53 and JNK in HIV associated pre-eclampsia.

Method: This study used a Bio-plex assay to quantify expression of phosphprylated-p53 and phosphorylated-JNK in the buffy coat of HIV positive pre-eclamptic (n=20), HIV negative pre-eclamptic (n=20), HIV positive normotensive pregnant (n=20), and HIV negative normotensive pregnant (n=20) women.

Results: The expression of p53 significantly differed according to pregnancy type (pre-eclampsia *vs* normotensive; p=0.0162) and HIV status (HIV positive *vs* HIV negative; p=0.0469). There was also a significant difference in the expression of p53 between HIV negative pre-eclamptic *vs* HIV negative normotensive group ($p < 0.05$) and the HIV positive normotensive *vs* HIV negative pre-eclamptic group ($p < 0.05$). The expression of JNK, however showed no significant differences by pregnancy type (p=0.8701), HIV status (p=0.2227) and across study groups.

Conclusion: An upregulation of p53 and JNK was found in pre-eclamptic women regardless of HIV status, suggesting a role in the pathogenesis of PE. Moreover, both p53 and JNK was downregulated in HIV infection regardless of pregnancy type. This suggests that HIV infection might confer a protective effect in pre-eclampsia development.

Keywords: Pre-eclampsia, HIV, p53, JNK.

Running title: p53 and JNK in HIV associated pre-eclampsia

Introduction

Pre-eclampsia (PE) is defined as new-onset hypertension, with proteinuria occurring in the second half of pregnancy (1). It is a major cause of perinatal and maternal morbidity and mortality globally (2). The exact pathophysiology of PE remains elusive. The most accepted concept is that immune maladaptation to the foetal allograft results in abnormal placentation, where cytotrophoblast invasion of the myometrium is shallow and remodelling of the uterine spiral arteries are incomplete (2-4). Abnormal placentation results in reduced perfusion which leads to hypoxia and subsequent ischemic reperfusion insult of the villus (2, 5), leading to the release of cytokines, other inflammatory factors and reactive oxygen species (ROS) such as superoxide dismutase (6).

The increased generation of ROS triggers a redox signalling process that induces cell apoptosis (6). Apoptosis is important in the normal development of the placenta however, when elevated it hinders trophoblast invasion (7, 8). Moreover, a theory proposed by Redman and Sargent (9) suggests that systemic endothelial damage, the characteristic lesion of PE is caused by the increased deportation of syncytiotrophoblast microvillous particles (STBMs) due to increased apoptosis (10, 11).

The precise mechanisms that stimulate apoptotic amplification in PE is unknown. Regulatory components of apoptosis include tumour protein p53 (12), and c-Jun N-terminal protein kinase (JNK) (13). p53 is a transcription factor (14) which has a critical role in determining cell fate by the activation of growth arrest, cellular senescence or apoptotic pathways (15). Under normal conditions, the expression of p53 is kept at low levels to protect the cell from any uncontrolled activity by the ubiquitin ligase, murine double minute 2 (Mdm2) (16, 17).

Post exposure to stresses including: heat and hypoxia and cytokines DNA damage and activate repair enzyme activities are promoted (18). Following DNA damage, p53 becomes phosphorylated at several sites. ATM, JNK and ATR are some of the kinases able to phosphorylate residues within the N-terminus of p53 *in-vitro* (19). This enables the stabilization of p53 by inhibition of the p53-Mdm2 interaction (20) thus allowing for the expression of cellular p53 to increase. Phosphorylation may be a part of a series of post-translational events that occur prior to p53 activation (15).

The intrinsic apoptotic pathway involves the Bcl-2 family of proteins which influence cytochrome-c release from the mitochondria (21, 22). They include anti-apoptotic (Bcl-2, Bcl-xL and Mcl-1) and pro-apoptotic (Bax, tBid, Bim, Puma, Bak, Noxa and Bad) members (23). p53 can induce apoptosis by either activating gene expression of pro-apoptotic proteins such as Bax, Noxa, Puma, Bid (16, 24) and Mtd (25) in the nucleus or by directly permeabilizing mitochondria in the cytoplasm by acting as a pro-apoptotic BH3-only protein (26, 27).

JNK is part of the mitogen activated protein kinases (MAPKs) superfamily, primarily involved in the regulation of cell proliferation, apoptosis and survival (28). They get activated through phosphorylation (29). JNKs can be activated by cytokines and exposure to environmental stress. Generally, the substrate of JNK is c-Jun however, they can also be involved in the activation of transcription factors like c-Myc, p53, and Elk-1 (28). JNK initiates apoptosis by the increasing the expression of pro-apoptotic genes in the nucleus like TNF-a, Fas-L, and Bak (30). Alternatively, activated JNK can translocate to the mitochondria where it modulates the activity of pro-apoptotic Bcl-2 proteins (31-34).

In PE, a dysregulation of JNK (35) and p53 (36-38) has been observed. Studies have reported that HIV-1 infection is capable of activating the MAPK signalling pathway (28). Activation of the MAPK signalling pathway can enhance the efficiency of HIV-1 infection (28). While p53 expression and activation has been associated with enhancing HIV disease progression, most likely through the induction of CD4 T cell death, and its cooperative effect in controlling viral gene transcription (39). There is however, a paucity of information on the level of both p53 and JNK in HIV associated pregnancy.

In contrast to HIV infection, an upregulation of the immune response occurs in normal pregnancy, however, this up-regulation is exacerbated in PE producing an excessive maternal inflammatory response. Therefore, in HIV associated PE, a neutralisation of the immune response is expected (40).
In light of the fact that the top three causes of maternal deaths in South Africa are non-pregnancy related infections (mainly HIV infected pregnant women complicated by tuberculosis and pneumonia; 34.7%,), obstetric haemorrhage (15.8%) and hypertension (14.8%) (41). The aim of this study is to determine the expression of JNK and p53 in HIV associated PE.

Methods and Materials

Study Population

This study received institutional ethical (BE204/17), Hospital manager's and the Department of Health approval. The study was conducted at a large regional hospital that serves a mainly low socio-economic black South African population group.

The study population consisted of a total of n=80 women categorized into HIV positive pre-eclamptic (n=20), HIV negative pre-eclamptic (n=20), HIV positive normotensive (n=20) and HIV negative normotensive (n=20). PE was defined as blood pressure reading of ≥ 140/90 mmHg on two or more occasions after the 20th week of pregnancy with the presence of significant proteinuria >300mg/dl in a 24-hour period (42). All HIV positive women received dual anti-retroviral therapy *i.e.*, highly active antiretroviral treatment (HAART) and nevirapine.

The exclusion criteria for the PE group included: unknown HIV status, polycystic ovarian syndrome, chorioamnionitis, eclampsia, chronic hypertension, intrauterine death, abruption placentae, gestational diabetes, chronic diabetes, systemic lupus erythematous, chronic renal disease, sickle cell disease, thyroid disease, antiphospholipid antibody syndrome, cardiac disease, pre-existing seizure disorders and asthma.

Bio-Plex Multiplex Method

Maternal blood samples were centrifuged at 1200g for 10 minutes at 4°C, the buffy coat layer was aliquoted and used for the quantification of phosphorylated-p53 (S15) and phosphorylated-JNK (T183/Y185). A Bio-plex Pro Cell Signalling MAPK customised Panel 2-Plex Assay kit (#LQ0-0000S6KL81S, Bio-Rad Laboratories, Inc., USA), was used according to the manufacturer's instructions. UV-treated HEK-293 was used as a positive control while detection antibody diluent was used as a negative control. Samples were prepared in a 1:4 dilution.

The immunoassay involved using capture antibody coupled beads, which were incubated with the antigen samples, i.e. phosphorylated-p53 and phosphorylated-JNK. Addition of biotinylated detection antibodies, and a reporter streptavidin-phycoerythrin conjugate (SA-PE) completed the interaction.

Samples were read using the Bio-Plex® MAGPIX™ Multiplex Reader (Bio-Rad Laboratories Inc., USA). The Bio-Plex Manager™ software version 4.1 was used, and presented data as median fluorescence intensity (MFI). The relative concentration of analyte bound to each bead is proportional to the MFI of the reporter signal.

Statistical Analysis

Analysis of the data was done using Graphpad Prism 5.00 for Windows (GraphPad Software, San Diego California USA). Data is presented by mean ± standard error mean. A t-test was used to determine statistical significance according to pregnancy type (pre-eclamptic and normotensive) and HIV status (positive and negative), whilst a One-way ANOVA in combination with Bonferroni multiple comparison post-hoc test was used to determine statistical significance across all groups. Statistical significance was established when $p < 0.05$.

Results

Clinical Characteristics

Table 1 shows a summary of the demographic data of the study population. Gestational age ($p= 0.0009$), parity ($p= 0.0410$) and systolic ($p < 0.0001$) and diastolic ($p < 0.0001$) blood pressures were statistically different between the PE *vs* normotensive pregnant groups (T-test). Maternal age ($p=0.8105$) and maternal weight ($p=0.1931$) were similar between the PE *vs* normotensive pregnant groups.

Table 1. Patient demographics in the normotensive pregnant (n=40) and pre-eclamptic (n=40) groups

	Normotensive (mean ± SEM)	PE (mean ± SEM)	p-value
Maternal Age (years)	28 ± 0.91	30 ± 2.50	0.8105 (ns)
Gestational Age (weeks)	38 ± 0.18	35 ± 0.70	0.0009 (***)
Parity	1.5 ± 0.19	2.0 ± 0.17	0.0410 (*)
Systolic BP (mmHg)	109 ± 3.70	154 ± 2.40	<0.0001(***)
Diastolic BP (mmHg)	69 ± 2.80	100 ± 1.30	<0.0001(***)
Weight (kg)	80 ± 2.00	85 ± 2.90	0.1931 (ns)

Results are represented as the mean ± standard error mean (SEM), ns: non-significant

* - $p < 0.05$

*** - $p < 0.001$

Median Fluorescent Intensity (MFI) of p53 and JNK.

The MFI of p53 and JNK are shown in Table 2 and figure 1 (A-C) and 2 (A-C).

p53 expression:

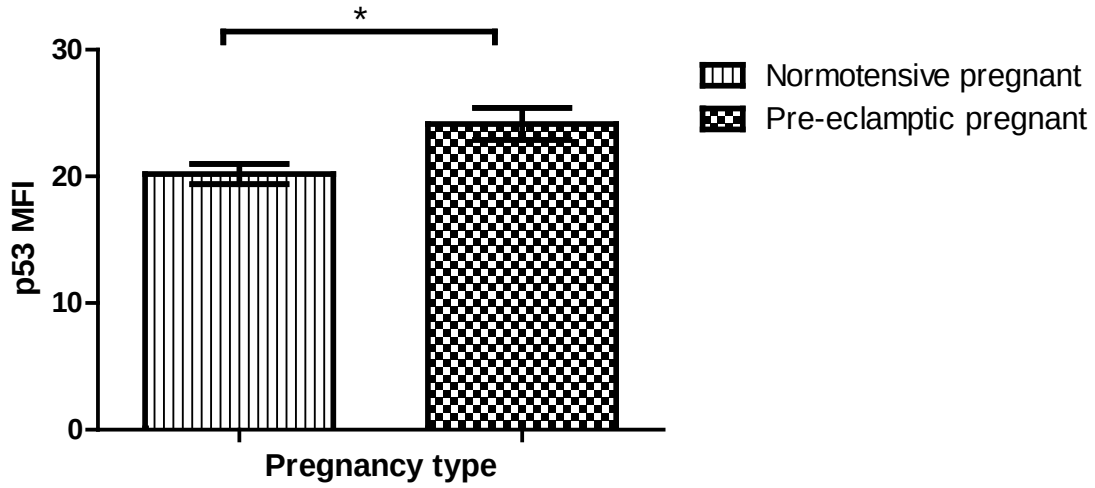
Based on pregnancy type, a significant difference in the expression of p53 was noted between the PE *vs* normotensive pregnant groups (p=0.0162), regardless of HIV status (HIV positive *vs* HIV negative). A higher expression was demonstrated in the PE (24±1.30 MFI) compared to the normotensive pregnant (20 ± 0.80 MFI) group (Figure 1A).

Based on HIV status, there was a significant difference in the expression of p53 between the HIV positive *vs* HIV negative groups (p=0.0469), irrespective of pregnancy type (pre-eclamptic *vs*

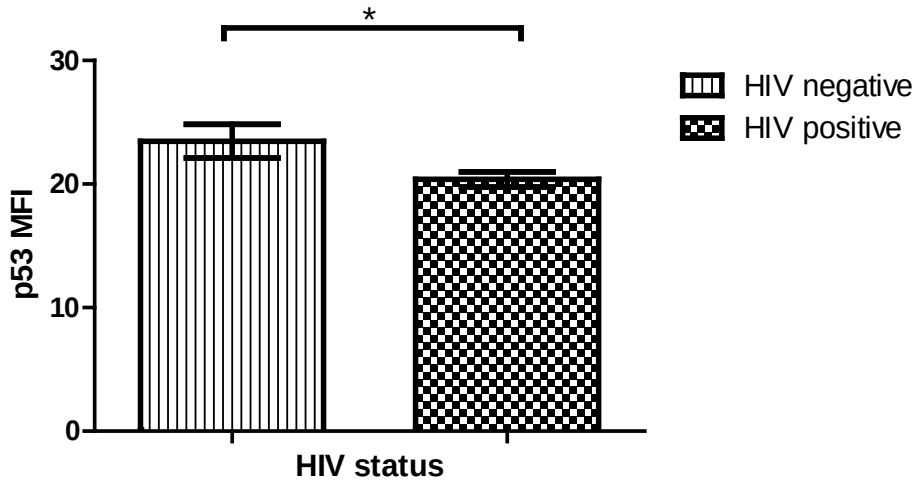
normotensive pregnant). A lower expression was observed in the HIV positive (20 ± 0.60 MFI) compared to the HIV negative (23 ± 1.40 MFI) group (Figure 1B).

There was a significant difference ($p < 0.05$) in the expression of p53 between the HIV negative pre-eclamptic (26 ± 1.60 MFI) *vs* HIV negative normotensive (20 ± 1.80 MFI) group and the HIV positive normotensive (20 ± 0.72 MFI) *vs* HIV negative pre-eclamptic (26 ± 1.60 MFI) group, ($p < 0.05$) (Figure 1C).

1A.



1B.



1C.

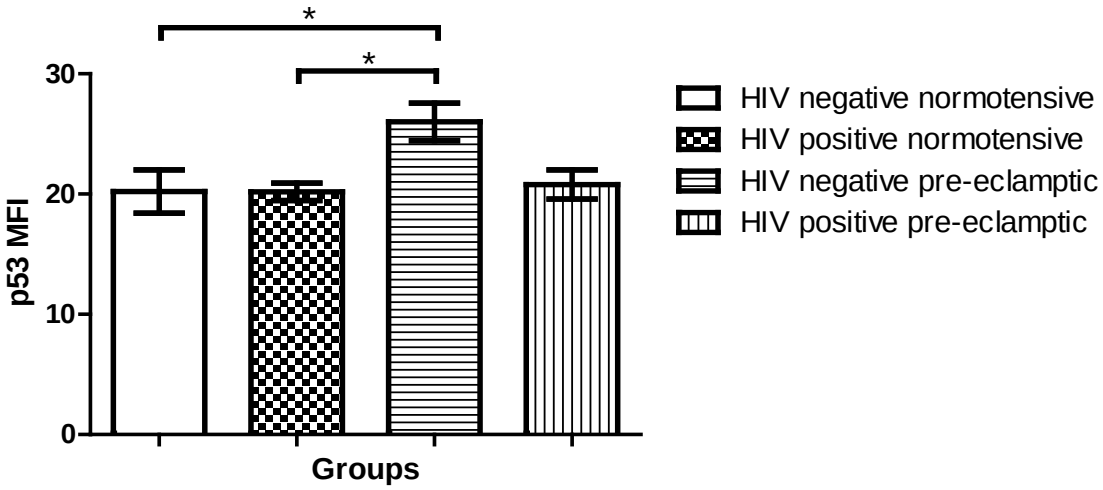


Figure 1. MFI of cell signalling factor p53: (A) p53 based on pregnancy type, (B) p53 based on HIV status and (C) p53 across all groups. The MFI of p53 was significantly different based on pregnancy type (*; pre-eclamptic *vs* normotensive) regardless of HIV status and based on HIV status (*; HIV positive *vs* HIV negative) regardless of pregnancy type. There was also a significant difference in the MFI of p53 between HIV negative pre-eclamptic *vs* HIV negative normotensive (*), and between HIV positive normotensive *vs* HIV negative pre-eclamptic (*).

Results are represented as mean ± standard error mean (SEM).

* - $p < 0.05$.

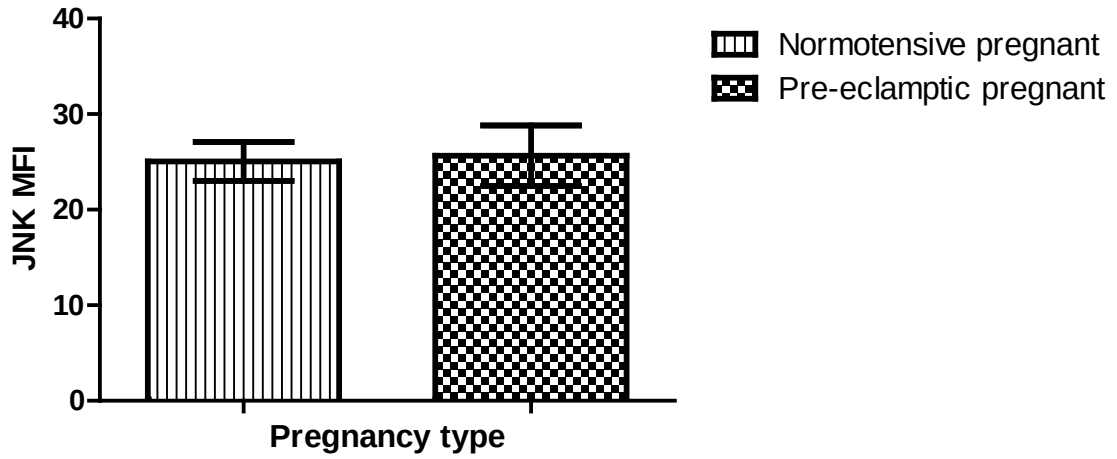
JNK expression:

Based on pregnancy type. The expression of JNK was similar between pre-eclamptic *vs* normotensive pregnant groups (p=0.8701), regardless of HIV status (HIV positive *vs* HIV negative). Despite having no statistical significance, the expression of JNK was increased in the pre-eclamptic (26 ± 3.20 MFI) compared to the normotensive pregnant group (25 ± 2.00 MFI) (Figure 2A).

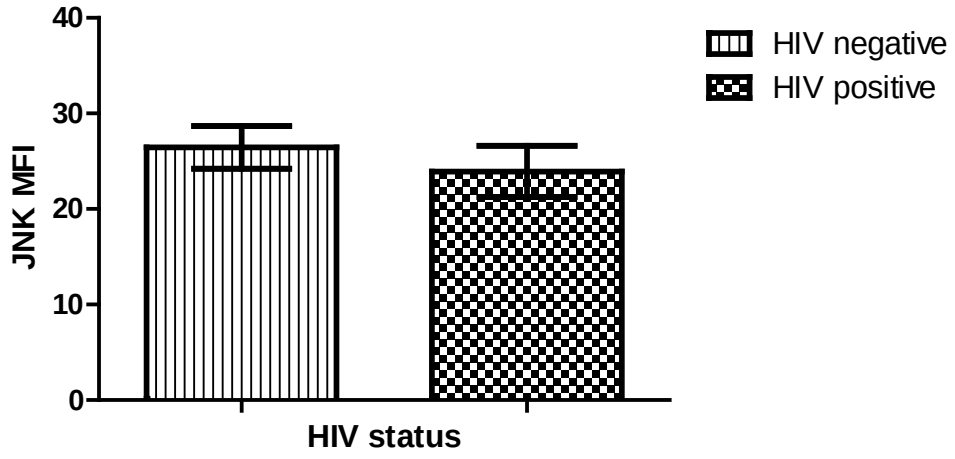
Based on HIV status, the expression of JNK was similar in the HIV positive *vs* HIV negative groups (p=0.2227), regardless of pregnancy type (pre-eclamptic *vs* normotensive). There was also a decrease in the expression of JNK in the HIV positive group (24 ± 2.70 MFI) compared to the HIV negative (26 ± 2.20 MFI) group (Figure 2B).

No significance in the expression of JNK was demonstrated across all study groups, HIV positive pre-eclamptic, HIV negative pre-eclamptic, HIV positive normotensive and HIV negative normotensive (Figure 2C).

2A.



2B.



2C.

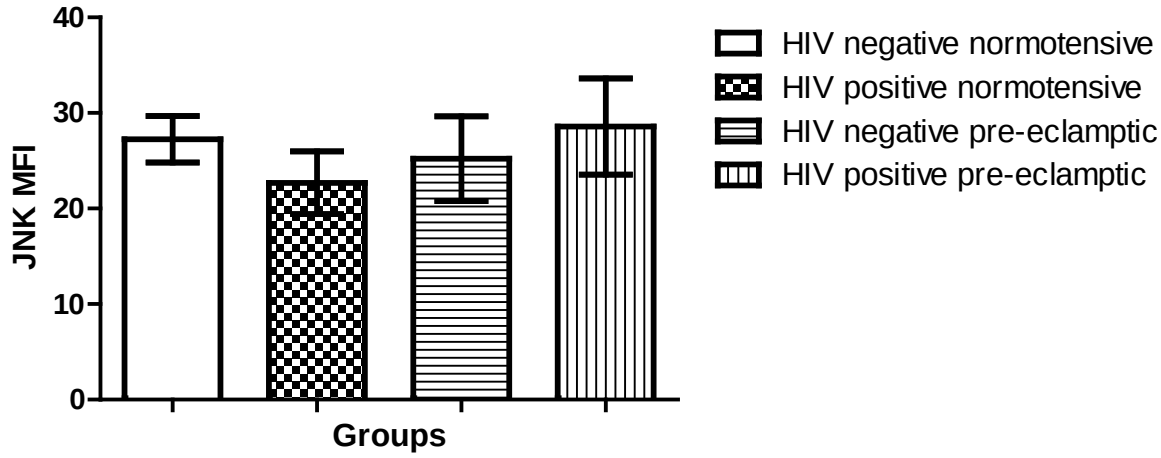


Figure 2. MFI of cell signalling factor JNK: (A) JNK based on pregnancy type, (B) JNK based on HIV status and (C) JNK across all groups. No significant difference was found in the MFI of p53 based on pregnancy type (pre-eclamptic *vs* normotensive) regardless of HIV status and based on HIV status (HIV positive *vs* HIV negative) regardless of pregnancy type. There was also no significant difference across all groups.

Results are represented as mean ± standard error mean (SEM).

Table 2 MFI of p53 and JNK across all study groups.

	Normotensive Pregnant (n=40)		**Pre-eclamptic Pregnant (n=40)**	
	HIV negative	HIV positive	HIV negative	HIV positive
p53	20 ± 1.80	20 ± 0.72	26 ± 1.60	21 ± 1.20
JNK	27 ± 2.40	23 ± 3.30	25 ± 4.40	29 ± 5.00

Results are represented as the mean ± standard error mean (SEM).

Discussion

This study has demonstrated a significant increase in the level of p53 in women with PE compared to normotensive pregnant women. This observation in pre-eclamptic women, is consistent with previous reports albeit using placental tissue samples (36, 38). Notably, the hypoxia and oxidative stress that occurs in PE, expounds the exaggerated syncytiotrophoblast autophagy and apoptosis that characterises PE (36). In support of this, studies have shown an association between exposure of trophoblasts to

hypoxia and enhanced apoptosis (43) with an increase in the expression of p53 (43), Bax (43), and Mtd-1 (44) with a simultaneous decrease in Bcl-2 expression (43, 45).

In our study, we also report no significant difference, albeit an upwards trend in JNK expression between pre-eclamptic compared to normotensive pregnancies. Our results are corroborated by Szabo *et al* (35) who evaluated JNK immunostaining of the villous trophoblast and demonstrated that there was no change in JNK immunoscores in preterm or term PE. However, an upregulated trend was noted in late-onset PE compared to term controls. This increase could be attributed to the increased hypoxia and oxidative stress secondary to defective placentation that occurs in PE (7). Studies in support of this theory show that placental explants challenged with different pre-eclamptic associated stresses such as hypoxia-reoxygenation (H/R) (46), hypoxia and inflammatory cytokines (47) lead to in increased oxidative stress and subsequent phosphorylation of JNK with the resultant activation of the JNK/MAPK pathway (46, 47). The antioxidant vitamins C and E effectively suppress markers of oxidative stress, apoptosis as well as phosphorylation of JNK induced by H/R (46).

Notably, plasma hydrogen peroxide (H_2O_2) levels are increased in women with PE (48). Tang and colleagues (49) found H_2O_2 treatment decreased the viability, triggered apoptosis and increased phosphorylation of JNK of human trophoblast like cell line JEG-3 cells whilst the addition of a JNK inhibitor SP600125 increased cell viability and decreased apoptosis (49). Thus, it is plausible to assume that both hypoxia and oxidative stress contribute to the increase of p53 and JNK in the causation of the exaggerated apoptosis in PE.

In our study, we demonstrate a significant decrease of p53 expression in HIV positive compared to HIV negative individuals, regardless of pregnancy type. This observation may be attributed to the neutralisation of the immune response expected in HIV associated PE (40). Furthermore, the HIV-1 accessory protein, Nef directly interacts with p53, with consequential destabilization of p53, decreasing its half-life, proapoptotic, transcriptional, and DNA binding activities (50). This demonstrates that Nef could supplement HIV replication by extending infected cell sustainability by blocking p53-mediated apoptosis (50). This indicates that HIV infection may have a protective effect by reducing the level of p53, hence preventing PE development.

However, in contrast to these observations, previous studies report that p53 is activated in HIV-1 infection (39). Our results may be due to the use of HAART which suppresses HIV replication (51). Moreover, a significant difference of p53 was found in HIV positive normotensive *vs* the HIV negative pre-eclamptic group.

Based on HIV status, we also report no significant difference in the JNK expression. Previous studies have shown that HIV-1 infection could activate the host cellular MAPK signalling pathway, this could enhance HIV-1 gene expression level, viral genome replication level and virus infection activity (52-54). A study by Gong *et al* (28) showed that a JNK pathway inhibitor SP600125 had an effect on HIV infection and HIV-1 LTR promoter activity (28).

One should also take cognisance of the fact that all HIV infected women in our study were on HAART or anti-retroviral drugs (nevirapine). The administration of HAART in HIV infected women has been linked to increased predisposition to PE development (55, 56) as it causes immune reconstitution (57). Lower activation levels of JNK, AKT and ERK kinases have been observed post HAART treatment (51), thereby indicating HAART may be responsible for the decreased p53 and JNK levels in HIV infected individuals.

Usually, the syncytiotrophoblast is protected against apoptosis, by the expression of various anti-apoptotic proteins including: Mdm2, Bcl-2, and Mcl-1 which antagonize the effects of pro-apoptotic p53, Bax, Bak, Mtd and smac (58-61). However, in PE, Sharp *et al* (36) have shown a substantial reduction in the anti-apoptotic protection of the syncytiotrophoblast with a decrease in Mdm2. This therefore facilitates an increase in p53 expression culminating in a pro-apoptotic environment. The loss of function of Mdm2 is also related to increased apoptosis and syncytial degeneration (36).

In PE, intrinsic cell damage has been proposed to lead to increased levels of apoptosis, hypothesized *in vivo* to stem from hypoxia-reperfusion injury (62), and replicated *in vitro* by hypoxia (38, 60, 63) and oxidative stress (64, 65). The role that exaggerated apoptosis plays in the pathogenesis of PE is uncertain, but could eventually prevent trophoblast migration, normal syncytiotrophoblast turnover, promote syncytial degeneration and release vasoactive or inflammatory factors into the maternal

circulation (7). PE is also associated with exaggerated levels of cell-free foetal DNA and syncytiotrophoblast microparticles (STBM) which show characteristics of apoptosis in the maternal circulation (66-69). It is hypothesised that STBMs as well as cell-free foetal DNA could be released as a result of apoptotic cell death, this is supported by studies demonstrating increased levels of STBMs and cell-free foetal DNA in trophoblast cultured in conditions of hypoxia and H/R (70-72). Moreover, STBMs found in the maternal circulation are associated with an altered immunological response. In specific, neutrophil activation (73, 74) and the release of superoxide radicals (75). Furthermore, *in vitro* STBMs can disrupt endothelial cells, providing a link between placental apoptosis, STBM liberation and the maternal vascular complications which are characteristic of the pre-eclamptic syndrome (76, 77).

The elevation of p53 in pre-eclamptic women suggest that p53 is being released into the maternal circulation. Furthermore, altered apoptosis in pre-eclampsia is not only found in the placenta but in the endothelial cells (78). This may occur from the toxicity effect of excessive syncytial fragments or from the local hypoxic environment, and this effect may occur via p53-mediated endothelial apoptosis as seen in other systems (79, 80). It was also shown that the upregulation of p53 induced apoptosis and suppressed endothelial cell proliferation in PE (37).

Conclusion

In summary, we demonstrate a significant increase in p53 expression with a slight increase in JNK in PE, suggesting a potential role in the pathogenesis of PE. A significant down regulation of p53 with concurrent decrease of JNK was noted in the HIV positive group, indicating that HIV infection might offer a protective effect against exaggerated levels of p53 and JNK, however altered levels might be associated with use of HAART, which merits further investigation. The increase in expression of p53 and JNK in PE elucidates their potential value as a risk indicator in the development of PE.

Declaration of Interest

There are no conflicts of interest.

Acknowledgements

The authors wish to thank the College of Health Sciences, UKZN for providing funding.

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CHAPTER THREE

SYNTHESIS

In South Africa, during 2011-2013 there were 4452 maternal deaths which is unacceptably high despite numerous health care improvements. The top 3 causes include non-pregnancy related infections (mainly HIV related complications) (34.7%), obstetric haemorrhage (15.8%) and 14.8% accounting for hypertension (South African Department of Health, 2013). Both, HIV infection and hypertensive disorders of pregnancies, especially pre-eclampsia (PE) are important conditions in developing countries (Hall *et al*., 2014).

Despite decades of research the exact mechanism underlying the cause and progression of PE remains unknown and no successful treatment for PE is available currently except delivery of the placenta and baby (Williamson *et al*., 2017). A common concept is that immune maladaptation to the foetal allograft can result in impaired trophoblast invasion and defective spiral arterial remodelling (Matthiesen *et al*., 2005, Dekker and Sibai, 1998). In normal placentation the extravillous cytotrophoblasts invade the uterine spiral arteries of the decidua and myometrium, replacing the endothelial layer of the maternal spiral arteries, transforming them from small, high-resistance vessels to high capacitance, low resistance vessels that can sustain the growth of the foetus (Wang *et al*., 2009). In PE remodelling of the spiral arteries is inadequate and limited to the decidua (Wang *et al*., 2009).

The reduced utero placental perfusion secondary to abnormal placentation leads to placental ischemia and hypoxia (Levy, 2005). Retention of vasoreactivity of the spiral arteries could persist, resulting in intermittent perfusion, transient hypoxia, and ischemia-reperfusion insult of the villus (Hung *et al*., 2001), which in turn releases cytokines as well as other inflammatory factors and damaging levels of reactive oxygen species (ROS) (Sánchez-Aranguren *et al*., 2014). Oxidative stress is observed when the relative pro-oxidant species (ROS) are higher than the antioxidant defences (Sánchez-Aranguren *et al*., 2014), this can trigger a redox signalling process that induces cell apoptosis (Sánchez-Aranguren *et al*., 2014).

Apoptosis (programmed cell death), is important in the normal development of the placenta, however apoptosis is exaggerated in PE and initiated by various stimuli, including hypoxia and oxidative stress

(Sharp *et al*., 2010). Important cell signalling molecules involved in the regulation of apoptosis include tumour protein p53 (Prives and Hall, 1999) and c-Jun N-terminal Kinase (JNK) (Lin and Dibling, 2002, Liu J and A., 2005).

p53 is a transcription factor (Lane, 1992) and contributes to the regulation of proliferation, DNA repair, apoptosis, and survival (Sharp *et al*., 2014, Vousden and Ryan, 2009). Under normal conditions, the expression of p53 is kept at low levels, restrained by the ubiquitin ligase, murine double minute 2 (Mdm2) which targets it for ubiquitination via the proteasome and removes it from the nucleus (Haupt *et al*., 1997, Kubbutat *et al*., 1997, Momand *et al*., 1992, Levine, 1997, Lu *et al*., 2000).

Furthermore, p53 also promotes the transcription of Mdm2 (Harris and Levine, 2005). Stresses including heat, hypoxia, and cytokines promote DNA damage (Sharp, 2011, Harris and Levine, 2005), subsequently causing the phosphorylation of p53 at several sites, allowing for the stabilization of p53 by inhibition of the p53-MDM2 interaction (Kruse and Gu, 2008). This results in the rapid increase of cellular p53 (Sharp, 2011).

The results of this study demonstrate an upregulation of p53 in women with PE compared to normotensive pregnant women regardless of HIV status (HIV positive vs HIV negative). Similar findings of elevated p53 in PE has been observed in previous studies using placental tissue samples (Levy *et al*., 2002, Sharp *et al* 2014). Hypoxia and oxidative stress could explain the stimulation and increase of p53 expression associated with apoptosis in PE in our study (Sharp *et al*., 2014). In support of this, studies have reported an association between exposure of trophoblasts to hypoxia and enhanced apoptosis (Levy *et al*., 2000), with an increase in the expression of p53 (Levy *et al*., 2000), Bax (Levy *et al*., 2000), and Mtd-1 (Soleymanlou *et al*., 2005) and a simultaneous decrease in anti-apoptotic Bcl-2 expression (Levy *et al*., 2000, Hu *et al*., 2006).

Apoptosis is initiated via the intrinsic or extrinsic pathway. Hypoxia (Levy, 2005), and ROS are able to trigger apoptosis via the intrinsic pathway, the activation of which leads to an altered mitochondrial membrane permeability due to the imbalance in the relationship of pro-apoptotic (Bax, Bak tBid, Bim, Puma, Noxa and Bad) and anti-apoptotic (Bcl-2, Bcl-xL and Mcl-1) Bcl-2 proteins (Cory and Adams,

2002, Vaseva and Moll, 2009). The resulting mitochondrial membrane permeability causes pore formation and cytochrome-c to leak into the cytosol where it binds with apoptosis protease activating factor-1 (APAF-1) and the initiator caspase 9 in the cytosol to form the apoptosome complex (Li *et al*., 1997, Ott *et al*., 2002, Chinnaiyan, 1999). Both pathways culminate in a terminal pathway involving the activation of caspase-3, 6 and 7 that initiate cell destruction (Sharp *et al*., 2010, Tewari *et al*., 1995, Koh *et al*., 2005).

p53 can induce apoptosis by promoting the downstream transcription of elements involved in apoptosis such as Bax, Noxa, Puma, Bid (Sharp, 2011), Mtd (Yakovlev *et al*., 2004) and APAF-1 in the nucleus or by directly permeabilizing mitochondria in the cytoplasm by acting as a pro-apoptotic BH3-only protein (Green and Kroemer, 2009).

Mitogen activated protein kinases (MAPKs) are well known mediators in signal transduction pathways (Qi and Elion, 2005). These mediators are activated by phosphorylation (Cindrova-Davies, 2009). JNK is part of the MAPK family which are associated with the regulation of cell proliferation, differentiation, and apoptosis (Dhanasekaran and Reddy, 2008). JNK can be activated by various stimuli which include; growth factors (Hibi *et al*., 1993), cytokines (Westwick *et al*., 1994) and ROS (Chambers and LoGrasso, 2011).

When activated JNK can phosphorylate and regulate the activity of transcription factors such as; c-Jun, ATF2, Elk-1, and p53 (Chang and Karin, 2001, Davis, 2000, Karin, 1995, Lin, 2003) and other factors like the members of the Bcl-2 family (Bcl-2, Bcl-xL, Bim and BAD) (Liu J and A., 2005). JNK initiates apoptosis by increasing the expression of pro-apoptotic genes in the nucleus like TNF-a, Fas-L, and Bak (Fan and Chambers, 2001). Alternatively, activated JNK can translocate to the mitochondria where it modulates the activity of pro-apoptotic Bcl-2 proteins. (Dhanasekaran and Reddy, 2008).

In this study, the expression of JNK was similar according to pregnancy type regardless of HIV status. Similar to our findings, Szabo *et al* evaluated JNK immunostaining of the villous trophoblast and showed no change in the JNK immunoscore in preterm or term PE, however it tended to be higher in late-onset PE compared to term controls (Szabo *et al*., 2015). A limitation of our study was the

heterogeneity of the pre-eclampsia cohort. Further studies are underway to sub-stratify PE into early-onset pre-eclampsia (EOPE) and late-onset pre-eclampsia (LOPE) to maintain a homogenous population.

EOPE is considered more severe than LOPE and originates from poor placentation, while LOPE seems to be exaggerated by predisposing risks for maternal endothelial dysfunction in the second half of pregnancy. Both types show enhanced systemic inflammatory responses (Herzog *et al*., 2017). Separate pathogenic paths for EOPE and LOPE have been proposed. The distinction could thus lead to different approaches in clinical management (Chen *et al*., 2012). A study has shown that the shedding of cell-free foetal DNA syncytiotrophoblast microparticles (STBMs) into the maternal circulation occurs in greater amounts in EOPE vs LOPE (Chen *et al*., 2012).

The upregulation in our study observed may be attributed to the increased hypoxia and oxidative stress in PE (Sharp *et al*., 2010). Studies supporting this show that placental explants challenged with different pre-eclamptic associated stresses such as hypoxia-reoxygenation (H/R) (Cindrova-Davies *et al*., 2007), hypoxia and inflammatory cytokines (Xiong *et al*., 2013) lead to increased oxidative stress and subsequent phosphorylation of JNK with the resultant activation of the JNK/MAPK pathway (Cindrova-Davies *et al*., 2007, Xiong Y *et al*., 2013).

In our study, a significant difference in the expression of p53 based on HIV status (HIV positive vs HIV negative) was observed, with a downregulation in the level of p53 in the HIV positive compared to the HIV negative group. These results could be due to the neutralisation of the immune response expected in HIV associated PE (Hall *et al*., 2014). Furthermore, the HIV accessory protein, Nef interacts directly with p53, Nef could therefore supplement HIV replication by extending infected cell sustainability by blocking p53-mediated apoptosis (Greenway *et al*., 2002). This indicates that HIV infection might have a protective effect in the pathogenesis of PE by reducing the level of p53. However, previous studies report that p53 may be activated in HIV-1 infection (Eduardo Pauls *et al*., 2006). Therefore, our results might be due to the use of HAART which suppresses HIV replication and exacerbates inflammatory response (Kusdra *et al*., 2002).

In normal pregnancy there is an upregulation of the immune response, in PE this upregulation is exacerbated leading to an excessive maternal inflammatory response. Therefore, in HIV infection which is a condition of immune deficiency, a neutralisation of the immune response is expected in HIV associated PE. (Hall *et al*., 2014) which could possibly explain our results. We also demonstrate a significant difference of p53 between HIV negative pre-eclamptic vs HIV negative normotensive.

There was no significant difference demonstrated in the expression of JNK based on HIV status. However, a downregulation trend was seen in the HIV positive vs the HIV negative group. Other studies have reported that HIV-1 infection could activate the host cellular MAPK signalling pathway, enhancing the HIV-1 gene expression level, viral genome replication level and virus infection activity (Cohen *et al*., 1997, Jacque J M *et al*., 1998, Xiaoyu Yang and Gabuzda., 1998). Furthermore, a study by Gong *et al* showed that a JNK pathway inhibitor SP600125 had a slight effect on HIV infection and HIV-1 LTR promoter activity (Gong *et al*., 2011) supporting our results. A limitation of our study was that all HIV positive women in our study were on HAART or anti-retroviral drugs, as it is the standard of care in South Africa. HAART can re-constitute the mother's immune response, and thus increase the risk of developing PE (Wimalasundera *et al*., 2002).

Both hypoxia and oxidative stress may contribute to the increase of p53 and JNK which are implicated in the causation of the exaggerated apoptosis in PE. In PE, increased apoptosis is believed to result from intrinsic cell damage, hypothesized *in vivo* to result from hypoxia-reperfusion injury (Hung and Burton, 2006), and replicated *in vitro* by hypoxia (Heazell *et al*., 2008b, Soleymanlou *et al*., 2007, Levy *et al*., 2002) and oxidative stress (Hung *et al*., 2012, Hung *et al*., 2008) which could be the underlying cause in the pathophysiology of PE (Sharp *et al*., 2010).

Exaggerated apoptosis could eventually prevent normal replenishment of the syncytiotrophoblast, promote syncytial degeneration and release vasoactive or inflammatory factors into the maternal circulation (Sharp *et al*., 2010). PE is associated with an elevation in STBMs which have features of apoptosis in the maternal circulation (Johansen *et al*., 1999, Goswami *et al*., 2006, Knight *et al*., 1998, Zhong *et al*., 2002). It is hypothesised that STBMs and cell-free foetal DNA may be released due to apoptotic cell death, supported by studies demonstrating increased levels of STBMs and cell-free foetal

DNA in trophoblast cultured in hypoxia and H/R (Abumaree *et al*., 2006, Orozco *et al*., 2006, Tjoa *et al*., 2006).

STBMs found in the maternal circulation are associated with an altered immune response specifically, neutrophil activation (Von Dadelszen P *et al*., 1999, Gupta *et al*., 2006) and the release of superoxide radicals (Aly AS *et al*., 2004). Additionally, *in vitro* STBMs can disrupt endothelial cells, providing a connection between placental apoptosis, syncytial microparticle liberation and the maternal vascular complications which are characteristic of the pre-eclamptic syndrome (Cockell *et al*., 1997, Hoegh *et al*., 2006).

Placental apoptosis as well as cytotrophoblast proliferation is enhanced. This imbalance may contribute to the pathogenesis of PE. Increased syncytiotrophoblast apoptosis counters cytotrophoblast fusion, thereby promoting the release of syncytial material which can disrupt the maternal vascular endothelium (Crocker, 2007)**.** A study by Jeschke *et al*, shows elevated proliferation of villous trophoblastic cells in HELLP placentas is accompanied with an elevation of p53 expression (Jeschke *et al*., 2006).

Endothelial cells have a role in fluid filtration, homeostasis, hormone trafficking and regulating vascular tone. In a study by Gao *et al*, an upregulation of p53 induced apoptosis and suppressed endothelial cell proliferation in PE, therefore, these mechanisms could result in impaired regulation of vasodilation and vasoconstriction, as well as angiogenesis which could aggravate PE (Gao *et al*, 2016).

These results suggest that elevated levels of p53 and JNK could be released from the placenta into the maternal circulation in PE, and inhibited by HIV infection as a downregulation trend of p53 and JNK was seen, Further studies need to be conducted examining the expression of p53 and JNK in HIV associated PE using placental tissue samples.

The constant generation of potentially damaging oxygen radicals are controlled by anti-oxidants like vitamin C, vitamin E and glutathione. However, these defences are not flawless, and when there is an imbalance between the relative pro-oxidant species (ROS) and anti-oxidants, a state of oxidative stress is reached (Cindrova-Davies, 2009, Sánchez-Aranguren *et al*., 2014). Also, antioxidant vitamins C and E effectively suppressed markers of oxidative stress, apoptosis as well as phosphorylation of JNK

induced by H/R (Cindrova-Davies *et al*., 2007). Due to multiple different mechanisms contributing to the development of apoptosis, further studies are needed to clarify the signalling mechanisms of apoptosis in PE before therapeutic treatments can be employed (Levy, 2005).

In conclusion, this study demonstrates a significant increase in p53 expression whilst JNK showed an up-regulation trend in PE, regardless of HIV status. These results support the role for these cell signalling molecules in the pathogenesis of PE. However, based on HIV status a significant down regulation of p53 and a slight decrease of JNK was noted in HIV positive compared to HIV negative group indicating that HIV infection may offer a protective effect against the exaggerated apoptosis associated with PE, however these altered levels may be attributes to HAART. Notably the increase in expression of p53 and JNK in PE, embraces their potential value as a risk indicator in the development of PE, hence further research in this regard is required.

CHAPTER FOUR

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APPENDIX

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

10 April 2017

Ms Y Pillay (213515235)
Discipline of Optics and Imaging
School of Laboratory Medicine and Medical Sciences
yazira123@gmail.com

Dear Ms Pillay

Protocol: The role of c Jun N-terminal kinase (JNK) and tumour protein p53 in HIV associated pre-eclampsia.
Degree: MMedSc
BREC reference number: BE204/17

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 14 March 2017.

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

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

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

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

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

The sub-committee's decision will be **RATIFIED** by a full Committee at its next meeting taking place on **09 May 2017.**

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

Yours sincerely

Professor Joyce Tsoka-Gwegweni
Chair: Biomedical Research Ethics Committee

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