EXPRESSION OF PLASMA NUCLEAR FACTOR KAPPA-B (NFκB) AND INHIBITORY SUBUNIT KAPPA B ALPHA (IκB-α) IN HIV-ASSOCIATED PRE-ECLAMSIA

By

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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, Doris Duke Medical Research Institute, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa, under the supervision of Professor Thajasvarie Naicker.

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Professor Thajasvarie Naicker
(Supervisor)
DECLARATION

I, Bambanani Selunathi Zozo 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, 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.

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(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: 30 November 2017
DEDICATION

To my late mother Minazana Zozo,

*I can just imagine how proud you would be of me right now; you are forever and always in my heart.*

To my father Mlindeni, my brother Yanga and my sister Nqabomzi,

*Thank you for your support, love and faith in me throughout my studies.*
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<td>AIDS</td>
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<td>Human Immunodeficiency Virus</td>
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ABSTRACT

Objective: The relationship between pre-eclampsia and HIV-1 infection is one of the most unexplored relationships in research. Pre-eclampsia is characterized by inflammation and HIV is characterized by a decline in immune activity. The NFκB pathway is involved in pre-eclampsia and in HIV-1 infection as a transcriptional factor in both conditions. Previous literature has showed that NFκB is upregulated in both pre-eclampsia and HIV-1 infection. Therefore, the aim of this study was to investigate the level of plasma NFκB and the inhibitory subunit IκB-α in HIV associated pre-eclampsia.

Method: This retrospective study examined plasma NFκB and IκB-α expression in normotensive (n =32) and preeclamptic (n = 34) HIV positive and HIV negative pregnant women. Quantification of plasma NFκB and IκB-α expression were done using a Bio-Plex Multiplex immunoassay.

Results: Our results demonstrated a significant decrease in the level of plasma NFκB expression in pre-eclamptic compared to normotensive pregnancies (p< 0.05), irrespective of HIV status. However, the level of plasma NFκB expression was not significantly different between HIV positive and HIV negative women, irrespective of pregnancy type. Moreover, a significant difference in NFκB expression across all the study groups was observed, specifically a significant decrease in NFκB expression in HIV positive pre-eclamptic women compared to normotensive women (p < 0.05). Furthermore, based on pregnancy type, a significant decrease in the level of plasma IκB-α expression was noted in pre-eclamptic compared to normotensive pregnancies, irrespective of HIV status (p< 0.05). However, our results showed no significant difference across all groups. Although no significance was
observed, a downwards trend in plasma IκB-α expression was observed in HIV positive pre-eclamptic compared to normotensive women.

Conclusion: Our study demonstrates decreased plasma NFκB and IκB-α expression in preeclamptic women irrespective of HIV status. This could be attributed to oxidative stress as an underlying factor subsequently leading to decreased plasma NFκB and IκB-α in preeclamptic women. HIV status had no effect on Plasma NFκB and IκB-α expression. The similarity in plasma NFκB and IκB-α expression based on HIV status may be due to antiretroviral therapy.
BACKGROUND AND LITERATURE REVIEW

1.0 HIV and Pre-eclampsia epidemiology

In 2017, approximately 7.06 million individuals of the South African population were HIV positive (Statistics South Africa, 2017). This notably high prevalence makes it the epicentre of the global HIV pandemic. For adults aged 15–49 years, an estimated 18.0% of the population is HIV positive (Statistics South Africa, 2017). In this group, HIV incidence is disaggregated into a ratio of female to male prevalence (5:1). This high prevalence in women of reproductive age is a serious obstetric problem.

Global proportions of maternal deaths of HIV-infected women are estimated to range between 7%-21% (Zaba et al., 2013). The effect of HIV infection on the risk of maternal deaths has not been studied extensively therefore research in this area is vital (Zaba et al., 2013). In South Africa, the main cause of maternal deaths are non-pregnancy-related infections associated with HIV/AIDS (40%), followed by obstetric haemorrhage (14.1%) and hypertension (14%) (National Department of Health, 2015b). Importantly, pre-eclampsia accounts for 83% of the maternal deaths emanating from hypertension in pregnancy. Furthermore, approximately 30% of antenatal patients are infected with HIV (Moran and Moodley, 2012, Kalumba et al., 2013). Therefore, both conditions of HIV and pre-eclampsia (PE) are associated with significant maternal and neonatal morbidity and mortality (Moran and Moodley, 2012).

Pre-eclampsia (PE) is a pregnancy specific condition characterized clinically with hypertension ($> \frac{140}{90}$ mmHg) and proteinuria ($> 300$mg/d) after 20 weeks of gestation. Other clinical symptoms include sudden weight gain, headaches, oedema, epigastric pain and
blurred vision (Li et al., 2014). PE is also associated with fetal complications such as growth restriction and stillbirth (Li et al., 2014). One of the identified risk factors for PE development is primigravidae (Moran and Moodley, 2012). However, in South Africa there is a paucity of data on the incidence of PE in Black African primigravidae (Moran and Moodley, 2012).

PE complicates 2%–10% of pregnancies, and is directly associated with 10%–15% of maternal deaths worldwide (Duley, 2009). According to a report by the National Confidential Enquiries on Maternal Deaths in South Africa, PE together with other hypertensive disorders of pregnancy and HIV/AIDS are implicated as the leading causes of maternal deaths (National Department of Health, 2015b).

Interestingly, the prevalence of PE may be affected by immunosuppressive conditions such as HIV/AIDS (Hall, 2007, Wimalasundera et al., 2002, Frank et al., 2004). Yet, the relationship between HIV infection and PE remains unclear, as there is still no definite information as to whether HIV-infected women are at a lower, equal or higher risk of developing PE than the uninfected population (Kalumba et al., 2013). Antiretrovirals have been implicated in PE development. Frank et al. (2004) showed no difference in the prevalence of PE between untreated HIV positive women and HIV negative women in South Africa (Frank et al., 2004). In contrast, Suy et al. (2006) found an increased risk of PE among HIV positive women in Spain whilst Kalumba et al. (2013) found that the prevalence of PE was lower amongst HIV positive women (Kalumba et al., 2013, Suy et al., 2006). The reduced rate of PE amongst HIV positive women could possibly be due to the immune insufficiency stimulated by HIV together with the normal immune changes of pregnancy (Govender et al., 2013). Therefore this may minimize the susceptibility to immune hyper-reactivity that is associated with PE (Govender et al., 2013).
2.0 Pre-eclampsia

The placenta plays an active role in fetal development within a protective maternal environment. Proper development and maintenance of the placental vasculature is very important during pregnancy, as failure results in complications such as miscarriage and PE (Cerdeira and Karumanchi, 2012). During normal placental development, maternal spiral arteries are transformed from small-caliber high resistance vessels to high capacitance low resistance vessels, capable of providing adequate placental perfusion to sustain the growing fetus (Cerdeira and Karumanchi, 2012). In PE, trophoblast invasion is inadequate, and the physiological conversion of the spiral arteries is limited to the decidua (Cerdeira and Karumanchi, 2012). Also, endovascular cytotrophoblast cells fail to obtain an endothelial-like phenotype (Kadyrov et al., 2006, Naicker et al., 2003, Zhou et al., 1997). The myometrial spiral arteries remain of small calibre, high resistance vessels hence do not supply adequate oxygen and nutrients to meet the growing needs of the baby (Khaw et al., 2008, Lin et al., 1995).

There are two proposed pathophysiological stages of PE viz., placental stage and a maternal stage (Sargent et al., 2006):

During the placental stage, poor placentation results in a limited maternal blood supply to the fetus (Raghupathy, 2013). This impaired trophoblast invasion and lack of physiological transformation of the myometrial arteries results in ischaemia-reperfusion injury, with consequential placental oxidative and endoplasmic reticulum stress (Steegers et al., 2010). The maternal stage consists of the classic maternal manifestation of widespread endothelial dysfunction (endotheliosis), hypertension, proteinuria, and edema (Raghupathy, 2013). At this stage, the systemic maternal circulation releases soluble form of the vascular endothelial
growth factor (sFlt-1) and other mediators accompanied by an enhanced maternal intravascular systemic inflammatory response. Widespread endotheliosis with complement abnormality and clotting activation manifests (Khan et al., 2015). Eventuating in an overall decrease in intravascular volume and increased vascular reactivity (Steegers et al., 2010).

Figure 1: Proposed pathophysiological stages of pre-eclampsia.

The placental maladaptation and the reduced blood flow leads to subsequent intracellular hypoxia, with consequential release of various substances including trophoblastic debris and apoptotic cells into circulation (Kalumba et al., 2013). Consequentially, multiple organ endothelial dysfunction occur due to an imbalance between anti-angiogenic and angiogenic factors (Eastabrook et al., 2011). This initiates a vasospasm, hypertension and multiple organ affectation and dysregulation of immunological factors (Kalumba et al., 2013).
During PE there is excessive activation of leukocytes which is associated with exaggerated innate and adaptive immune response that restrict normal pregnancy progression (Lok et al., 2009). As mentioned, systemic and diffuse endothelial cell dysfunction is one primary feature of PE (Raghupathy, 2013). This is caused by an increase in the production of pro-inflammatory cytokines with resultant activation of maternal endothelial cells (Raghupathy, 2013). Additionally, oxidative stress caused by the up-regulated production of reactive oxygen species (ROS), and lipid peroxidation, is also a prominent feature of PE (Karabulut et al., 2005). In mononuclear lymphoid cells of pre-eclamptic women, an increased level of activation of nuclear transcription factor-κB (NF-κB) occur (Giorgi et al., 2012). This results in an increased release of the primary pro-inflammatory cytokines, tumour necrosis factor-alpha (TNF-α) and interleukin-1b (IL-1b), which play a role in PE development (Peraçoli et al., 2011).

3.0 NFκB

Normal pregnancy is a pro-inflammatory state, whereas PE represents an exaggerated immune response. In PE increased lipid peroxidation and decreased anti-oxidant protection is manifested (Kaur et al., 2008). Activation of the immune system in PE is dysregulated by the oxidative stress resulting in the activation of NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) (Vaughan and Walsh, 2012). In contrast, during normal pregnancy, NFκB activity is only significantly increased during the onset of labour (Lindstrom and Bennett, 2005). NFκB is a protein transcription factor involved in the transcription of a variety of genes, including cytokines, growth factors, adhesion molecules, immunoreceptors, and acute-phase proteins (Giuliani et al., 2001, Kim et al., 2006). NFκB is a heterodimer composed of p50 and p65 subunits bound to its inhibitory subunit IκB-alpha (IκB-α ) (Giorgi et al., 2012). NFκB plays a key role in regulating primary pro-inflammatory
cytokines found in PE development, viz., tumour necrosis factor-alpha (TNF-α) and interleukin-1b (IL-1b) (Peraçoli et al., 2011).

Figure 2: NFκB basic pathway for regulation of inflammatory proteins in the cytoplasm and nucleus.

Modified from: (Yu, 2012)

4.0 NFκB, IκB-α and HIV

NFκB translocation into the nucleus is induced by many different agents such as viral infection, microbial components and cytokines that signal via toll-like-receptors that prime the degradation of IκB (Lin and Karin, 2007). Research has shown that NFκB is a master regulator of pro-inflammatory genes and is upregulated in HIV-1 infection (Fiume et al., 2012). NFκB activity in HIV-1 infected cells can be regulated by a number of mechanisms.
Upon binding of HIV-1 to CD4 T cells, NFκB is activated (Hiscott et al., 2001). This leads to a series of events that activate the IkB kinase complex directly via HIV-1 regulatory proteins or by the release of cytokines (Hiscott et al., 2001). Once phosphorylation and degradation of IkB-α and IkB-β occur, NFκB is then released and translocated to the nucleus where it transactivates responsive genes (Hiscott et al., 2001). IkB-β then enters the nucleus and prevents IkB-α-mediated termination due to NFκB response (Hiscott et al., 2001). This maintains constitutive NFκB activity at the protein-DNA level and creates an intracellular environment that promotes viral replication (Hiscott et al., 2001). Additionally, in HIV-1 infected immune cells, NFκB is required for both elongation of initial, Tat-independent viral transcripts and also for producing the high levels of Tat-dependent viral gene expression needed for productive infection (Karn, 1999). Moreover, in chronically infected immune cells, NFκB is activated, therefore assuring the abundant expression of the viral genes (Roulston et al., 1995).

Figure 3: Proposed mechanisms of NFκB and IkB-α activation in HIV-1–infected cells. Modified from (Hiscott et al., 2001)
Furthermore, overexpression of the p65 subunit of NFκB due to retroviral infection causes an increase in the endogenous inhibitory subunit IκB-alpha (IκB-α) (Scott et al., 1993). IκB-α is a regulatory protein inhibitor of NFκB and the NFκB p65 subunit is bound to IκB-α. The release of IκB-α from NFκB is mediated by reactive oxygen intermediates serving as both direct or indirect messengers, that trigger activation of the NFκB pathway (Schreck et al., 1991). Research evidence has suggested that NFκB is specifically activated if T cells are exposed to oxidant stress (Schreck et al., 1991).

5.0 NFκB, IκB-α and Pre-eclampsia

The effect of oxidative stress on NFκB activation examined in vitro show that placental oxidative stress in PE could result in the activation of NFκB by demonstrating that oxidizing (Ox) solution enriched with linoleic acid (OxLA) strongly activates NFκB (Vaughan and Walsh, 2012). However, anti-oxidants are known to inhibit NFκB activation (Gupta et al., 2010).
Previous studies have shown that the activity of NFκB is significantly higher in women with PE compared to normotensive women (Giorgi et al., 2016, Giorgi et al., 2012). Luppi et al. (2006) suggested that PE can be demonstrated by activation of the NFκB pathway when compared with both non-pregnant females and to women with uncomplicated pregnancy at term (Luppi et al., 2006). Moreover, NFκB activation in pre-eclamptic patients is higher than that observed in normotensive patients during labour (Luppi et al., 2004, Gervasi et al., 2001, Luppi et al., 2006). Overall, this shows a relationship between cellular activation and provides evidence of NFκB signal transduction activation in leukocytes in pre-eclamptic women (Luppi et al., 2006). This suggests that monocytes and T lymphocytes are the prospective cell types accounting for the high level of NFκB activation in pre-eclamptic women (Luppi et al., 2006). As a result, there could exist a cycle of leukocyte and endothelial hyperactivation, fuelled by a continuous inflammatory response in PE (Luppi et al., 2006).
6.0 Aims and Objectives

Both NFκB regulation and IκB-α in HIV associated PE is urgent due to their roles in HIV infection and in PE, hence the duality of their response in HIV associated PE is urgently required.

Hypothesis

We hypothesize that the expression of plasma NFκB and IκB-α will increase in HIV preeclamptic women.

Aim

The aim of the study is to investigate the expression of plasma NFκB and IκB-α in HIV associated PE.

Objectives

- To compare the expression of plasma NFκB and IκB-α between normotensive and pre-eclamptic woman irrespective of HIV status.

- To compare the expression of plasma NFκB and IκB-α between HIV positive and HIV negative woman irrespective of pregnancy type (normotensive pregnant vs pre-eclamptic women).

- To compare the expression of plasma NFκB and IκB-α across all the study groups.
CHAPTER TWO
Expression of Plasma Nuclear Factor-kappa B (NFkB) and Inhibitory subunit kappa B alpha (IkB-α) In HIV-Associated Pre-eclampsia

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Expression of Plasma Nuclear Factor-kappa B (NFκB) and Inhibitory subunit kappa B alpha (IκB-α) In HIV-Associated Pre-eclampsia

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Abstract

Objective: The relationship between pre-eclampsia and HIV-1 infection is poorly explored in research. Pre-eclampsia is a hyper-inflammation state whilst HIV is characterized by a decline in immune activity. NFκB is a transcriptional factor in both conditions; pre-eclampsia development and in HIV-1 infection. Therefore, the aim of this study was to investigate the level of plasma NFκB and the inhibitory subunit IκB-α expression in the duality of HIV associated pre-eclampsia.

Method: This retrospective study examined plasma NFκB and IκB-α expression in HIV positive and negative normotensive pregnant (n=32) and pre-eclamptic (n=34) women. Quantification of plasma NFκB and IκB-α expression were done using a Bio-Plex Multiplex immunoassay.

Results: Plasma NFκB and IκB-α NFκB expression were decreased in pre-eclamptic compared to normotensive pregnancies (p < 0.05). HIV status had no effect on plasma NFκB and IκB-α NFκB expression; however, a decrease in NFκB expression between HIV positive normotensive pregnant versus HIV positive pre-eclamptic women was observed (p< 0.05).

Conclusion: Our study demonstrates decreased plasma NFκB and IκB-α expression in pre-eclamptic women irrespective of HIV status. This could be attributed to oxidative stress as an underlying factor subsequently leading to decreased plasma NFκB and IκB-α in pre-eclamptic women. The similarity in plasma NFκB and IκB-α expression based on HIV status may be due to antiretroviral therapy.

Keywords: NFκB; IκB-α; pre-eclampsia; HIV

Running title: NFκB & IκB-α in HIV-Associated Preeclampsia
1. Introduction

The prevalence of the HIV epidemic continues to increase despite efforts to decrease the rate of infection globally, with about 36.7 million people living with HIV by the end of 2016\(^1\). South Africa is considered the epicentre of the HIV pandemic with an astounding 7.1 million HIV infected individuals\(^2\). Women are at a greater risk of HIV infection than males\(^3\). In fact, approximately one-fifth of women in their reproductive age are HIV positive, a serious obstetric predicament\(^4\). A notably high prevalence of HIV infection occur in pregnant women with approximately 30% of antenatal patients been HIV positive\(^5, 6\). The latest National Confidential Enquiries on Maternal Deaths in South Africa has reported that HIV infection accounts for 34.7% of maternal deaths\(^7\). Furthermore, hypertensive disorders of pregnancy is the commonest direct cause of maternal deaths, of which the majority is due to pre-eclampsia (PE) development\(^7\). Both HIV infection and pre-eclampsia (PE) are associated with significant maternal and neonatal morbidity and mortality\(^8\).

Clinical symptoms and signs of PE are high blood pressure (\(>140/90\) mmHg) and proteinuria (\(>300\) mg/d) occurring after 20 weeks of gestation. Fetal complications include growth restriction and stillbirth\(^9\). PE represents an exaggerated immune response and reactive oxygen species activity emanating from a hypoxic microenvironment. The oxidative stress causes activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) which is implicated in the dysregulation of the immune response in PE\(^10\).

NFκB is a protein transcription factor involved in the transcription of a variety of genes related to inflammation, including cytokines, growth factors, adhesion molecules, immunoreceptors and acute-phase proteins\(^11, 12\). NFκB is a heterodimer composed of p50 and p65 subunits present in an inactive form bound to its inhibitory subunit IκB-\(\alpha\) within the
cytosol\(^{(13)}\). In fact IκB-α keeps cytosolic NFκB inactive by concealing nuclear localization sequences\(^{(14)}\). Increased NFκB activation results in an increased release of the primary pro-inflammatory cytokines, \textit{viz.}, tumour necrosis factor-alpha (TNF-α) and interleukin-1b (IL-1b), which also contribute to PE development\(^{(15)}\).

Interestingly, it has being suggested that the prevalence of PE may be affected by immunosuppressive conditions such as HIV/AIDS\(^{(16-18)}\). NFκB is known to be upregulated during HIV-1 infection\(^{(19)}\). Notably, the binding of HIV-1 to a CD4 T cell activates NFκB, leading to a series of events that activates the IκB kinases complex\(^{(20)}\). The translocation of NFκB into the nucleus is induced directly via HIV-1 regulatory proteins, by the release of cytokines and/or by microbial components that signal via toll-like-receptors which also primes the degradation of IκB\(^{(21)}\). A number of studies have investigated the relationship between NFκB and IκB-α regulation in PE as well as in other HIV related diseases. However, there is a paucity of data on NFκB and IκB-α regulation in HIV-associated pre-eclampsia. Therefore, we hypothesized that the expression of plasma NFκB and IκB-α will increase in HIV pre-eclamptic women.

2. Methods

\textit{Study Population:}

Institutional ethical approval was obtained (BE208/17). The study population (n=66) consisted of pre-eclamptic (n=34) and normotensive (n=32) Black pregnant women (primigravid and multigravid) recruited at RK Khan Hospital, a regional and district hospital in Chatsworth, a suburb in the eThekwini health district, South Africa. Both groups were further divided according to their HIV status. The control group was composed of healthy normotensive pregnant women with blood pressure at or below 120/80 mmHg. Pre-eclamptic women was defined as new onset blood pressure of at or above 140/90 mmHg and
proteinuria. The exclusion criteria for the pre-eclamptic group included women who did not have antenatal care, chronic hypertension, gestational diabetes, chorioamnionitis, polycystic ovarian syndrome, sickle cell disease, eclampsia, thyroid disorders, chronic renal disease, antiphospholipid antibody syndrome, cardiac failure, pre-existing seizure disorders, intrauterine death, abruptio placentae as well as those with unknown HIV status.

Maternal blood samples were collected at venipuncture and centrifuged at 3000 rpm for 10 min at 4°C. Plasma samples were stored at -80°C until analysis.

Bio-Plex multiplex method:

To determine the expression of NF-κB and IκBα in plasma, a 6-plex NFκB Signaling Magnetic Bead Kit (EMD Millipore Corporation) was used according to manufacturer’s instructions. The MILLIPLEX® MAP NF-κB Magnetic Bead Signaling 6-plex kit detected changes in phosphorylated NF-κB (Ser536) and IκBα (Ser32) in plasma using the Luminex® system. The immunoassay involved an overnight incubation (16-20 hours) of the control cell lysates and sample lysates with the capture antibody-coupled beads. Incubation was carried out at 2-8°C on a plate shaker (600-800 rpm) protected from light. Subsequently, a MILLIPLEX® MAP Detection Antibody was added and further incubated with agitation on a plate shaker for 1 hour at room temperature (20-25°C). Thereafter, StreptavidinPhycoerythrin (SAPE) coupled with amplification buffer was added to complete the interaction. The 96-well plate was read using the Bio-Plex® MAGPIX™ Multiplex Reader (Bio-Rad Laboratories Inc., USA) and a Bio-Plex Manager™ software version 4.1 was used to obtain the data.

Statistical analysis:

Results were analysed using Graph Pad Prism version 5 software. Shapiro-Wilk test was used to test the data for normality. Data was analysed using Kruskal-Wallis test and Dunn's
Multiple Comparison Test post hoc test was used for comparison between groups. Mann Whitney test was used for Pregnancy type and HIV status. A $p$ value of less than 0.05 was considered statistically significant.

3. Results

Table 1. Patient demographics in the normotensive pregnant (n = 32) and pre-eclamptic pregnant (n = 34) groups.

<table>
<thead>
<tr>
<th></th>
<th>Maternal age (years)</th>
<th>Maternal weight (kg)</th>
<th>Gestational age (weeks)</th>
<th>Systolic blood pressure (mmHg)</th>
<th>Diastolic blood pressure (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normotensive Pregnant</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>24.00</td>
<td>67.50</td>
<td>38.50</td>
<td>108.0</td>
<td>66.00</td>
</tr>
<tr>
<td>Q1–Q3</td>
<td>19.00- 32.00</td>
<td>62.50- 73.15</td>
<td>37.00- 40.00</td>
<td>99.75- 120.8</td>
<td>61.00- 77.75</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>26.06 ± 7.179</td>
<td>68.31±8.414</td>
<td>38.38±1.809</td>
<td>110.5±13.40</td>
<td>68.22±10.36</td>
</tr>
<tr>
<td><strong>Pre-eclamptic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>29.00</td>
<td>75.85</td>
<td>30.00</td>
<td>160.0</td>
<td>102.0</td>
</tr>
<tr>
<td>Q1–Q3</td>
<td>26.00- 34.00</td>
<td>72.00- 98.50</td>
<td>27.00- 35.25</td>
<td>144.8- 173.8</td>
<td>96.00- 111.0</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>30.03±5.987</td>
<td>82.39±16.23</td>
<td>30.47±5.832</td>
<td>161.3±18.51</td>
<td>104.4±10.03</td>
</tr>
</tbody>
</table>

Clinical characteristics

Table 1 presents a summary of the clinical demographics of the study population. A statistical difference in maternal age ($p$<0.05) between the normotensive pregnant (NT) and pre-eclamptic (PE) groups was observed. Gestational ages, maternal weight, systolic and diastolic blood pressures (BP) were statistically different between the NT pregnant and PE groups ($p$ < 0.0001 each).
**NFkB expression:**

A Mann Whitney test showed a significant decrease in the level of plasma NFkB expression in PE compared to NT women, irrespective of HIV status (Mann-Whitney U = 379.5; \( p = 0.0353 \)). However, the level of plasma NFkB expression was not significantly different between HIV positive and HIV negative women, irrespective of pregnancy type (NT vs PE; \( p = 0.7729 \)). A Kruskal-Wallis test demonstrated a significant difference in NFkB expression across all the study groups (Kruskal–Wallis H = 8.143, \( p = 0.0432 \)) whilst a Dunn's Multiple Comparison post hoc Test showed a significant decrease in NFkB expression in HIV positive pre-eclamptic women compared to normotensive women (\( p < 0.05 \)).

**IκB-α expression:**

Based on pregnancy type, a significant decrease in the level of plasma IκB-α expression was noted in PE compared to NT, irrespective of HIV status (Mann-Whitney U = 386.0; \( p = 0.0433 \)). In contrast, plasma IκB-α expression was not significantly different between HIV positive versus HIV negative woman, irrespective of pregnancy type (\( p = 0.8827 \)). Lastly, Kruskal-Wallis test showed no significant difference for IκB-α expression across all the study groups (\( p = 0.1399 \)). Moreover, the Dunn's Multiple Comparison post hoc Test showed similar plasma IκB-α expression across groups (\( p > 0.05 \)). Although no significant difference was observed, a downwards trend in plasma IκB-α expression was observed in HIV positive pre-eclamptic compared to normotensive women.
Figure 1: Median Fluorescence Intensity (MFI) of NFkB (nuclear factor kappa-light-chain-enhancer of activated B cells): (A) Pregnancy type (normotensive versus pre-eclamptic) irrespective of HIV status, (B) HIV Status (HIV negative versus HIV positive) irrespective of pregnancy type, (C) across all pregnant groups (HIV negative normotensive, HIV positive normotensive, HIV negative pre-eclamptic, and HIV positive pre-eclamptic). Results are median± IQR of 66 patients.
Figure 2: Median Fluorescence Intensity (MFI) of IkB-a (inhibitory subunit IkB-alpha): (A) Pregnancy type (normotensive versus pre-eclamptic) irrespective of HIV status, (B) HIV Status (HIV negative versus HIV positive) irrespective of pregnancy type, (C) across all pregnant groups (HIV negative normotensive, HIV positive normotensive, HIV negative pre-eclamptic, and HIV positive pre-eclamptic). Results are median± IQR of 66 patients.

4. Discussion

The NFκB pathway is a master regulator of pro-inflammatory genes. Notably whilst normal pregnancy is a pro-inflammatory state, PE is considered a hyper-inflammatory disorder. Our findings show a decrease in plasma NFκB expression in PE compared to NT pregnant women, irrespective of their HIV status. These results corroborate previous findings from McCracken et al. (2003) who demonstrated suppression of NFκB activity in PE. PE is linked to increased sensitivity to angiotensin II. Angiotensin II triggers NFκB activation,
however, angiotensin-converting-enzyme inhibitors, have been proven to be successful in preventing NFκB activation (23, 24). Therefore high blood pressure treatment could possibly have an effect on both the decreased plasma NFκB and IκB-α expression in pre-eclamptic women. In contrast, PE is characterized by activation of the NFκB pathway in peripheral blood mononuclear cells (PBMC) when compared to normotensive women (13, 25, 26). Furthermore, a significant increase in NFκB activation in pre-eclamptic placentas compared to normal placentas have been reported (10). One of the prominent features of PE is oxidative stress caused by the up-regulated production of reactive oxygen species (ROS) and lipid peroxidation (27). It is therefore plausible to hypothesize that ROS activation and its correlation with pro-inflammatory cytokines contribute to PE development (15). Also, ROS induces a decrease in IκB-α degradation subsequently leading to a decrease in the nuclear translocation of NFκB (28).

Similar to NFκB, our findings demonstrate decreased plasma IκB-α expression in PE compared to NT women irrespective of their HIV status. Furthermore, a decreasing trend in plasma IκB-α expression was observed in HIV positive pre-eclamptic compared to normotensive women albeit with no significant difference. Since NFκB is downregulated by IκB-α, the decrease in plasma NFκB expression in pre-eclamptic women noted in our study is unexpected. Again McCracken et al. (2003) demonstrated a suppression of IκB-α activity in PE (22).Moreover, based on HIV status and regardless of pregnancy type we report that plasma NFκB and IκB-α expression was similar. However previous studies have shown that HIV Tat protein stimulates NFκB induction therefore upregulating NFκB in HIV-1 infection (20). Research has indicated that HIV transcription is closely related to elevation of TNF-a, IL-1b, IL-6, TGF-b, and IFN-g expression, thus activating NF-kB via IκB Kinase (20).

This similarity in our findings may be due to the effect of antiretroviral treatment, ARV duration of therapy (pre-pregnancy or intra-pregnancy) or severity of HIV infection. All HIV-
infected women in our study received antiretroviral drugs for prevention of mother to child transmission as part of the standard of-care treatment regimen in South Africa \(^{(29)}\). This regimen consists of Azidothymidine (AZT), a nucleoside-analogue reverse transcriptase inhibitor (NRTI) \(^{(29)}\). AZT is phosphorylated to AZT mono-, di- and triphosphate within the placenta \(^{(30)}\). AZT monophosphate has been shown to inhibit phosphorylation and degradation of IκB-α through the IkB kinase complex, suggesting that antiretroviral treatment could possibly have an effect on both the decreased plasma NFκB and IκB-α expression\(^{(31)}\).

Previous studies have shown that NFκB is upregulated in HIV-1 infection and as well as in PE, however our results show a down regulation in NFκB when comparing across all groups. Extensive research on the specific molecular mechanisms underlying this effect need to be further investigated.

One of the limiting factors in our study was that the viral load was not obtained for each of the women; therefore the biomarkers in our study were not associated with the severity of HIV infection. In conclusion, our study demonstrates decreased plasma NFκB and IκB-α expression in preeclamptic women irrespective of HIV status. This could be attributed to oxidative stress as an underlying factor subsequently leading to decreased plasma NFκB and IκB-α in preeclamptic women. The similarity in plasma NFκB and IκB-α expression based on HIV status may be due to antiretroviral therapy.

**Acknowledgments**
The authors wish to thank the College of Health Sciences (CHS) from the University of KwaZulu-Natal (UKZN) for their financial support as well as the institutional biostatistician, Dr. C Connolly.

**Disclosure**
We declare no conflict of interest.
References


2. UNAIDS. Ending AIDS: progress towards the 90–90–90 targets 2017.


CHAPTER 3
SYNTHESIS

South Africa has the highest incidence of HIV infection globally, with a notably high prevalence of HIV infection amongst pregnant women (Moran and Moodley, 2012). Approximately 35.8% of maternal deaths are attributed to non-pregnancy related infections, most of which are HIV-related (National Department of Health, 2015b). KwaZulu-Natal province has a prevalence rate of approximately 37.7% of HIV in pregnancy (Department of Health, 2012). Approximately 14.8% of maternal deaths in SA are due to hypertension in pregnancy, with 83% attributed to PE (National Department of Health, 2015b). Furthermore, in KwaZulu-Natal the prevalence of PE is 12% (National Department of Health, 2015b). One of the identified risk factors for PE development is first pregnancy (Moodley et al., 2016). However, the aetiology of PE remains unclear, therefore, treatment of this condition is still empirical (Waugh and Smith, 2012).

According to Govender et al., (2015), the pathogenesis of PE is divided into two stages. The first stage is characterised by impaired spiral artery remodeling, this induces cellular hypoxia, creating an imbalance between anti-angiogenic and pro-angiogenic factors (Moodley et al., 2016). Moreover, an increase in soluble anti-angiogenic factors leads to widespread endothelial dysfunction as well as to the clinical signs of hypertension, proteinuria, and intrauterine fetal growth restriction (Govender et al., 2015). During normal pregnancy, there is an increase in production of free radicals whilst anti-oxidant defences contribute to the maintenance of a balance in the redox state (Kaur et al., 2008). In contrast, women with PE show increased production of free radicals as well as a decrease in several important anti-oxidants (Kurlak et al., 2014, Vanderlelie et al., 2008). The generating oxidative stress consequently damages important molecules such as DNA, proteins, unsaturated fatty acids, and overstimulation of lipid peroxidation (Vanderlelie et al., 2008, Kurlak et al., 2014).
The NFκB pathway is a master regulator of pro-inflammatory genes. Notably whilst normal pregnancy is a pro-inflammatory state, PE is considered a hyper-inflammatory disorder. Many of the pro-inflammatory cytokines implicated in PE such as TNF-α, IL-1 and toll like receptors (TLRs), influence receptor-mediated signalling pathways that are mainly routed through NFκB (Keelan and Mitchell, 2007, Chatterjee et al., 2012, Tinsley et al., 2009). The importance of NFκB activation in PE has been underlined in several previous studies (Bidwell and George, 2014). NFκB activation in PE is important in systemic endothelial dysfunction (Jiang et al., 2010). Furthermore, Centlow et al., (2011) showed that NFκB is upregulated in PE compared to the control group. The expression of NFκB in PE correlates with increased trophoblast apoptosis within the placenta (Aban et al., 2004). Moreover, systemic NFκB activation is also present in the vasculature of preeclamptic mothers and is specifically associated with neutrophil infiltration (Shah and Walsh, 2007). This subsequently results in the release of toxic substances such as TNF-α, ROS and thromboxane, therefore promoting vasoconstriction and vascular dysfunction (Shah and Walsh, 2007).

Our first objective was to compare the level of NFκB and IκB-α between normotensive and pre-eclamptic woman irrespective of HIV status. We demonstrate a decrease in plasma NFκB expression in pre-eclamptic compared to normotensive pregnant women, irrespective of HIV status. In contrast, other studies reported that PE is characterized by activation of the NFκB pathway in PBMC’s when compared to normotensive women (Giorgi et al., 2016, Giorgi et al., 2012, Luppi et al., 2006). Furthermore, a significant increase in NFκB activation in pre-eclamptic placentas compared to normal pregnancy placentas have been reported (Vaughan and Walsh, 2012). Similar to NFκB, our findings demonstrated decreased plasma IκB-α expression in pre-eclamptic compared to normotensive women irrespective of their HIV status. Again these results corroborate previous reports demonstrating suppression of IκB activity in PE (McCracken et al., 2003). This could be an effect of ROS, known to induce a
decrease in IκB-α degradation subsequently leading to a decrease in nuclear translocation of NFκB (Strassheim et al., 2004).

Our second objective was to compare the level of NFκB and IκB-α between HIV positive and HIV negative women irrespective of pregnancy type. Our findings show no significant difference between HIV positive and HIV negative women for both plasma NFκB and IκB-α expression. However, previous reports have demonstrated that in HIV-1 infection, phosphorylation and degradation of IκB-α occur and NFκB is up-regulated (Fiume et al., 2012, Hiscott et al., 2001). In South Africa, as part of the standard-of-care treatment regimen, HIV positive women receive anti-retroviral drugs for prevention of Mother-to-child transmission (National department of health, 2015a). This regimen consists of Azidothymidine (AZT), a nucleoside-analogue reverse transcriptase inhibitor (NRTI) (National department of health, 2015a). AZT is phosphorylated to AZT mono-, di- and triphosphate within the placenta (Pornprasert et al., 2006). AZT monophosphate has been shown to inhibit phosphorylation and degradation of IκB-α through the IκB kinase complex (Ghosh et al., 2003). Therefore this suggests that anti-retroviral treatment could possibly have an effect on both the decreased plasma NFκB and IκB-α expression.

Finally, we compared the expression of plasma NFκB and IκB-α across all the study groups. Our results clearly established a decrease in plasma NFκB expression in HIV positive pre-eclamptic compared to HIV positive normotensive women. Moreover, a decreasing trend in IκB-α expression was noted in HIV positive pre-eclamptic compared to HIV positive normotensive women albeit with no significance.

One of the limitations of our study was that we did not sub-stratify pre-eclampsia into early and late-onset, this would have ensured a more homogenous PE population. Another limitation is that all HIV positive women in our study received either HAART or anti-
retroviral drugs for the prevention of mother-to-child transmission as part of the standard of care treatment regimen in South Africa.

In conclusion this study demonstrates that plasma NFkB and IκB-α expression is dysregulated in HIV associated pre-eclampsia. Our results demonstrate decreased plasma NFkB and IκB-α expression in preeclamptic women irrespective of HIV status. This could be attributed to oxidative stress as an underlying factor subsequently leading to decreased plasma NFkB and IκB-α in preeclamptic women. Furthermore, we demonstrate decreased plasma NFkB and IκB-α expression in the duality of pre-eclamptic HIV positive women compared to HIV negative women. However, HIV status had no effect on plasma NFkB and IκB-α expression. The similarity in plasma NFkB and IκB-α expression based on HIV status may be due to antiretroviral therapy.
CHAPTER 4
REFERENCES


13 March 2017

Ms BS Zonse (21601449)
Department of Optics and Imaging
School of Laboratory Medicine and Medical Sciences
belumane2000@gmail.com

Dear Ms Zonse,

Protocol: The role of Nuclear Factor-kappa B cells p65 (NF-κB p65) and Nuclear Factor kappa B cell alpha inhibitor (IKB - α) in HIV-Associated Fricamentosis.

Degree: MMedSci

BREC reference numbers: BE268/17

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 10 March 2017.

The conditions have been met and the study is given full ethics approval and may begin as from 13 April 2017.

This approval is valid for one year from 13 April 2017. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for re-certification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

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


BREC is registered with the South African National Health Research Ethics Council (REC 290408-009).

BREC has an Office for Human Research Protection (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 Tsaka-Gwegweni
Chair: Biomedical Research Ethics Committee

cc: Supervisor: yakwaza2000@gmail.com
cc: Chair: belumane2000@gmail.com

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