ROLE OF BIOMARKERS, KIDNEY INJURY MOLECULE-1, INTERLEUKIN-18, CALBINDIN AND MONOCYTE CHEMOTACTIC PROTEIN-1, IN EVALUATING AND DIAGNOSING KIDNEY INJURY IN PRE-ECLAMPSIA

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, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa under the supervision of Professor T. Naicker.

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DECLARATION

I, Dr. Soumaya Eltounali declare that:

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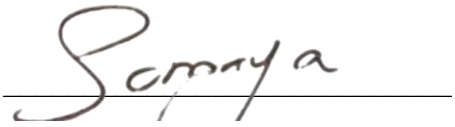
Signed: 

Date: 14-03-2017

DEDICATION

To My Parent

Strong and gentle souls who taught me to trust in Allah. Believe in hard work and that so much could be done with so little;

My Husband

For earning an honest living for us and for supporting and encouraging me to believe in myself;

My Children

Hoping that this work will inspire them in the future and encourage them to work hard and be ambitious.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS AND SYMBOLS

ANOVA	Analysis of Variance
BREC	Biomedical Research Ethics Committee
BUN	Blood urea nitrogen
EGF	Epithelial growth factor
GBM	Glomerular basement membrane
GFR	Glomerular filtration rate
HAART	Highly active antiretroviral therapy
HDP	Hypertensive disorders of pregnancy
HIV	Human immunodeficiency virus
IL-18	Interleukin-18
KIM-1	Kidney injury molecule-1
MCP-1	Monocyte chemoattractant molecule-1
mRNA	Messenger ribonucleic acid
NGAL	Neutrophil gelatinase–associated lipocalin
PAS	Periodic acid-Schiff
PE	Pre-eclampsia
PLGF	Placenta growth factor
sEng	Soluble endoglin
sFLT-1	Soluble Fms-like tyrosine kinase-1
VEGF	Vascular endothelial growth factor
VEGFR1	Vascular endothelial growth factor receptor-1

PUBLICATION

Eltounali, Soumaya Moodley, Jagidesa, and Naicker, Thajasvarie (2016): Role of kidney biomarkers [Kidney injury molecule-1, Calbindin, Interleukin-18 and Monocyte chemo-attractant protein-1] in HIV associated pre-eclampsia. Hypertension in Pregnancy (LHIP-2016-0102).

ABSTRACT

Objective: This study was designed to measure the level of kidney injury molecule-1, calbindin, interleukin-18, and monocyte chemoattaractant protein-1 in pre-eclampsia, since the kidney is an organ of target in PE. This study was aimed at measuring the levels of these markers of kidney injury in the urine of HIV infected and uninfected women with normal pregnancy and pre-eclampsia.

Study Design: The following study groups was included in the study; women with normal pregnancy who were HIV negative (n=19), women with normal pregnancy who were HIV positive (n=19), HIV negative pre-eclamptics (n=19) and HIV negative pre-eclamptics (n=19).

Results: The concentration of KIM-1 in PE pregnancy was significantly higher than the level in normal pregnancy ($p < 0.05$). Urinary KIM-1 level in the HIV negative women with PE was significantly higher than the level in the HIV negative women with normal pregnancy ($p < 0.05$). The urinary calbindin level was significantly higher in the PE group when compared to the level in the control group ($p < 0.05$). The urinary IL-18 levels in the PE group did not show a significant deference when compared to the control group ($p > 0.05$). There was no significant difference in the urinary MCP-1 in PE compared to the level in the controls ($p > 0.05$).

Conclusion: Our results show an increase in the urinary concentration of kidney injury molecule-1 and calbindin in PE, while the urinary Interleukin-18 and monocyte chemoattractant molecule -1 in PE did not show any difference compared to the level in the

controls. We propose that studies with larger sample sizes and case control studies using these markers be conducted to establish their use as markers of diagnosing i kidney injury in PE.

Key words: Kidney injury molecule-1 (KIM-1), Calbindin, Interleukin-18(IL-18), Monocyte chemoattaractant protein-1(MCP-1).

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.0 Pre-eclampsia

Pre-eclampsia (PE) is an obstetric complication of gestation which manifests after the 20th week of pregnancy, and one of the diagnostic feature aside the elevated blood pressure is the presence of protein in the urine (Watanabe *et al*., 2013). The cause of PE continues to be a question without answers as the cause is unknown. It affects women in both developed and developing countries (Hutcheon *et al*., 2011).

The enormousness of the problems caused by PE is felt more by underdeveloped nations, where it is responsible for a greater percent of maternal and fetal deaths (Barra *et al*., 2012). Preventing and finding the cure of this disorder has been a topmost priority health agencies and the United Nations, the possibility of attaining this objective is still not feasible due to lack of adequate healthcare facilities in third world countries. PE is associated with grave complications such as preterm delivery, fetal and maternal death (Barra *et al*., 2012).

During the course of normal gestation, the process of placental development and formation is highly regulated, this process involves the cytotrophoblast cells invading the maternal uterine wall thereby causing the placental spiral artery to be remodelled and converted into larger blood supplying channels (Eastabrook *et al*., 2011). Aberration in this process and altered spiral artery remodelling is associated with PE and this manifest as diminished blood flow to the placenta, followed by ischaemia of the placenta (Naljayan and Karumanchi, 2013).

There are other associated factors known to contribute to the pathogenesis of PE, these include an imbalance in the concentration of pro-inflammatory substances in maternal circulation, endothelial damage, placental hypoxia and resulting endoplasmic reticulum stress (Tannetta and Sargent, 2013). .The corner stone of treatment is the delivery of the baby and the placenta, since this ailment terminates with expulsion of the placenta (Magee *et al*., 2014).

Women affected with PE may come down with severe complications such as seizures leading to coma and may result in death (Souza *et al*., 2013). This complication of pregnancy (PE) affects almost all the organs and systems in the body, it causes unfavourable changes in the intrauterine environment and also causes activation of adverse cellular signalling which may be harmful to foetal development (Goulopoulou and Davidge, 2015).

Pre-eclampsia may be superimposed on existing hypertension. This occurs in women who were hypertensive before getting pregnant but didn't manifest proteinuria (Magee *et al*., 2014). Superimposed PE may be initiated by an underlying condition of the kidney (Magee *et al*., 2014). Severe cases of PE may result in seizures, this manifest before the onset of labour, during labour or after labour (Watanabe *et al*., 2013). Associated with the development of eclamptic seizures in PE includes; vasospasm of the cerebrum, as well as cerebral oedema (Sibai *et al*., 2005).

1.1 Epidemiology of pre-eclampsia

Hypertensive disorders of pregnancy (HDP) causes about 62,000 to 77,000 maternal deaths globally each year, this translates to approximately 18% of all the cases of maternal deaths (Abalos *et al*., 2013). Underdeveloped economies are the worst hit as the probability of pregnant woman dying from HDP is 1 out of 39 compared to the ratio of 1:3800 in the advanced countries (Abalos *et al*., 2013). It has also been stated that PE accounts forE in pregnancy globally (Jeyabalan, 2013).

In South Africa, a total of 4452 maternal deaths was reported from 2011 – 2013 ,the percentage of maternal deaths due to hypertension associated with pregnancy being 14.8% of this total figure. The value in KwaZulu – Natal Province stood at 8.3 % of the national figure (Saving Mothers Report, 2011-2013).

1.2 Risk factors associated with the development of pre-eclampsia

Pre-eclampsia development is known to be facilitated by some risk factors. Risk factors involved in PE development include history of vascular diseases and high blood pressure, maternal over weight, current or previous history of renal complications, presence of metabolic disorders such as diabetes (Villar *et al*., 2006). It has also been stead that maternal age at the time of conception, nulliparity, an extended period between pregnancies, and multiple gestations are additional factors which predispose women to developing PE (Villar *et al*., 2006; Lin *et al*., 2015).

Pre-eclampsia may also result from an underlying genetic condition which may predispose women to developing this complication of pregnancy (Powe *et al*., 2011; Lin *et al*., 2015). Also implicated in the development of PE is change in paternity and change in partners by woman from a previous pregnancies, the use of condoms and other barrier contraception resulting in a decreased maternal exposure to paternal antigens, this thus poses a danger to women and predisposes the woman to developing pre-eclampsia (Powe *et al*., 2011).

Pre-eclampsia can be classified either based on the onset or on the severity of this hypertensive complication of pregnancy. Based on the severity of PE, it is classified as mild PE or severe PE (Watanabe *et al*., 2013).

- Mild form of PE is defined by a blood pressure of ≥ 140/90 mmHg and < 160 mmHg, a urine protein concentration of ≥ 300 mg but not above a value of 2.0 g in 24 hour urine (or 2 pluses (+) on a dipstick) (Watanabe *et al*., 2013).
- Severe form PE is defined by a blood pressure of ≥ 160/110 mmHg, with a urine protein concentration > 2.0g in a 24 hour urine (or 3 pluses (+) on a dipstick) (Watanabe *et al*., 2013).

The second classification of PE is based on onset of the disorder. With this, PE is classified as early onset PE which occurs before the 34^{th} week of gestation (≤ 33 weeks 6 days). The late form of PE occurs 34 or after 34 weeks post conception (Lisonkova and Joseph, 2013).

1.3 The kidney in pregnancy

During the course of pregnancy, glomerular filtration rate (GFR) is increased by almost 40% to 60% in the first trimester. In pregnancy with complications like PE, there is a decrease in both GFR and renal plasma flow by 30% to 40% as compared to that in a normal pregnancy (Moran *et al*., 2003; Karumanchi *et al*., 2005). In PE, blood urea nitrogen (BUN) and creatinine levels may be within the normal physiological range for non-pregnant women notwithstanding the decrease in GFR (Karumanchi *et al*., 2005).

In PE impaired kidney function is associated with podocytopathy (Garovic *et al*., 2007). Podocyte injury results in loss of podocyte integrity and function, this activates a dedifferentiation process characterised by insufficient adherence to the glomerular basement membrane (GBM). This inadequate adherence of the popdocytes to the GBM results in its loss in the urine (podocyturia) (Hara *et al*., 2005). Disorientation of the GBM lends support to the progress of synechiae and glomerulosclerosis, which presents clinically as protein in the urine (proteinuria) (Facca *et al*., 2012).

Endotheliosis is a glomerular capillary lesion associated with PE. This lesion can be seen using light microscopy, the cells of the glomeruli will appear are enlarged than normal, another distinguishing feature seen with light microscopy is a bloodless glomerular capillary lumen due the characteristic hypertrophy of endothelial and mesangial cells (Karumanchi *et al*., 2005). Though endotheliosis is also seen in other conditions, it is more prominent in PE. Podocytes mostly appear swollen ‘’with periodic Acid-Schiff (PAS)-positive hyaline droplets’’. The presence of endotheliosis and loss of the integrity of the endothelial

fenestrae can be established with electron microscopy (Churg and Grishman, 1976). Studies have shown that mild glomerular endotheliosis is seen in about 50% of patients with pregnancy-induced hypertension without proteinuria (Nochy *et al*., 1980), this therefore suggest that PIH in some cases may be a reflection of a milder form of the same condition (Karumanchi *et al*., 2005). It has also been stated that endothelial swelling seen in PE usually resolve within 8 weeks post-delivery, this also coincides with the termination high blood pressure and proteinuria (Karumanchi *et al*., 2005).

The cause of PE remains unknown but studies have shown that altered spiral artery remodelling and subsequent reduction in uteroplacental blood flow which results in impaired trophoblast invasion of the placental spiral artery is a factor responsible for the development of PE (Barra *et al*., 2012). The development of this obstetric complication of pregnancy occurs in two stages as seen in Figure 1.

1.4 Pathogenesis of pre-eclampsia

The first stage of PE is the pre-clinical stage and the second stage of the disorder is the clinical stage (Tannetta and Sargent, 2013). The preclinical stage of PE development is associated with poor placenta development. Poor placentation results from shallow invasion of the uterine wall by cytotrophoblast cells, alteration in the process of placenta spiral artery remodelling and a diminished perfusion of the trophoblast cells (Tannetta and Sargent, 2013).

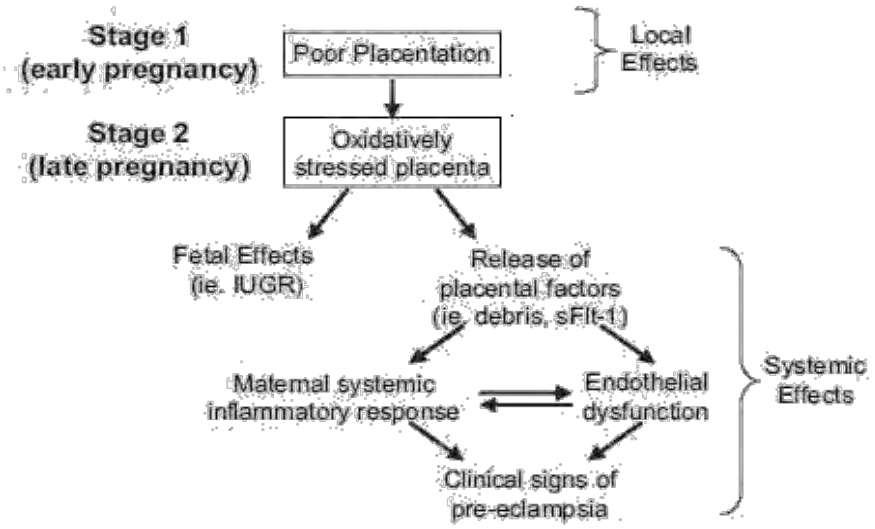

Figure 1. Diagram showing the two stages of PE.

Placenta dysfunction starts in the period of gestation (stage 1). Second stage of PE is associated with the release of trophoblast derived factors into maternal circulation, these particles stimulate inflammatory response and cause dysfunction of maternal vascular endothelium (Borzychowski *et al*., 2006).

The key event in normal placentation is the invasion of the maternal spiral arteries to the decidua and the inner third of the myometrium by the foetal trophoblast cells. Early in normal placental development, cytotrophoblast cells of foetal origin breaks through the syncytium and migrates into the endometrium (Figure 2) (Karumanchi *et al*., 2005). During this process the spiral artery is invaded and converted into larger conduits for the supply of blood to the placenta.

Research shows that placental ischaemia in PE occurs as a result of defective placental spiral artery remodelling. In PE, invasion of the spiral artery is shallow, and the spiral arteries remains

as small resistance channels, resulting in reduced utero-placental perfusion and subsequently placental hypoxia, which may be the initiating event in PE (Figure 2) (Redman and Sargent, 2003).

The hypoxic placenta is thought to synthesize and release elevated amounts of sFlt-1 and sEng (Young *et al*., 2010). Furthermore, an ischaemic placenta contributes to endothelial cell dysfunction in the maternal vasculature by inducing an alteration in the balance of circulating levels of angiogenic/antiangiogenic factors (Karumanchi and Bdolah, 2004).

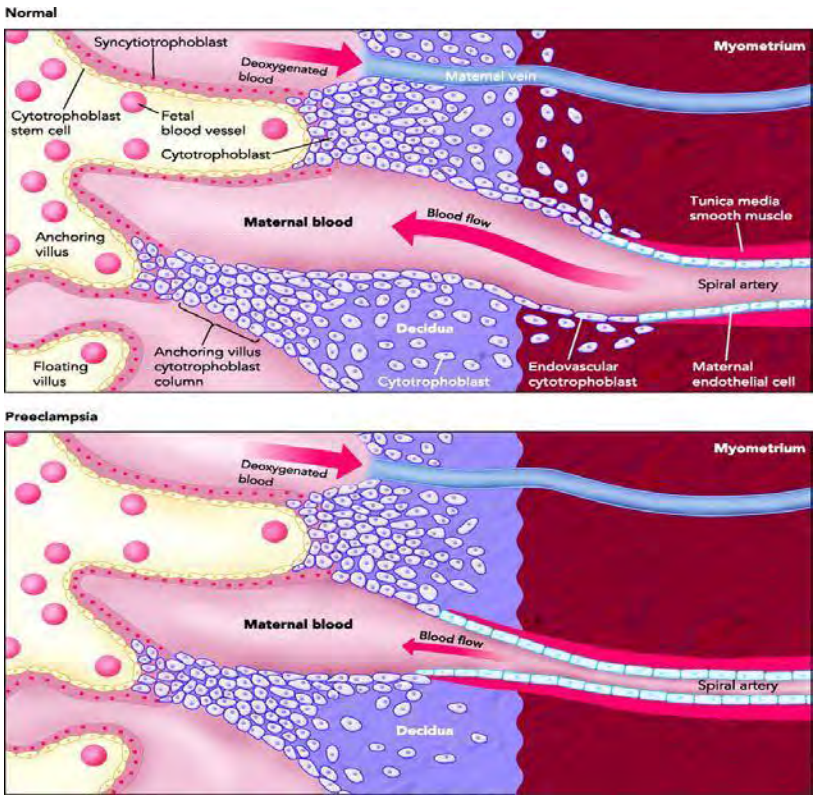

Figure 2: Spiral artery remodelling during normal and pre-eclamptic pregnancy (Wang *et al*., 2009).

The second stage of PE (Figure 1) is characterised by damaged syncytial architecture, with the release of trophoblast derived factors/ debris into the circulation. This stage of the disorder is also associated with increased peripheral vasoconstriction, and loss of arterial compliance (Tannetta and Sargent, 2013). In the clinical stage of PE, the placenta is stressed and it releases molecules such as the soluble vascular endothelial growth factor receptor-1 (VEGFR1 or the sFlt-1) and also soluble endoglin (sENG), which are both known to cause a disorganization of fenestrated endothelium. The molecule vascular endothelial growth factor (VEGF) is needed for maintaining the integrity of fenestrated endothelium. In PE, there is an imbalance in the circulating levels of angiogenic and ant-angiogenic molecules. There is up regulation of and over production of anti-angiogenic (sEng, sFlt-1) molecules in PE compared to the level in normal pregnancy important for its integrity (Redman, 2011). While the level of angiogenic (VEGF) molecules is diminished. Alteration in the orientation of the fenestrated endothelium manifests as the leakage of protein into the urine (Craici *et al*., 2013).

1.5 The role of angiogenic/antiangiogenic factors in pre-eclampsia

Ischemia of the placental cells results in them secreting soluble factors in to circulation, these factors which are anti-angiogenic may cause maternal endothelial dysfunction (Maynard *et al*., 2003). Disruption in the balances between angiogenic and antiangiogenic factors is critical to the pathogenesis of PE. Two antiangiogenic factors, soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng) have been shown to play a great role in the pathogenesis of PE.

The anti-angiogenic molecule sFlt-1 is a splice variant of the membrane bound VEGF receptor Flt1, it is mainly secreted and released into the circulation by syncytiotrophoblast cells (Young

et al., 2010). Elevated levels of sFlt-1 is associated with diminished placental growth factor (PLGF) and vascular endothelial growth factor (VEGF) signalling (Levine *et al*., 2004). Soluble fms-like tyrosine kinase-1 prevents VEGF and PLGF from carrying out their normal function by binding to them in circulation and inhibiting their interaction with receptors meant for their function (Maynard *et al*., 2003).

Another peptide molecule indicted in the pathogenesis of PE is soluble endoglin (sEng), it is an antiangiogenic protein. This molecule is highly expressed on endothelial cell membranes, syncytiotrophoblast cells and invading cytotrophoblast cells (Powe *et al*., 2011). Soluble endoglinng may combine with sFlt-1 and cause amplification of vascular injury (Wang *et al*., 2009).

1.6 Kidney Biomarkers in pre-eclampsia

In PE, renal injury with release of protein into the urine is a characteristic feature, but the extent of insult to different segments of the nephron is not well known. Kidney injury in PE can also be defined by total urinary protein excretion in a 24 hour period to the concentration of creatinine randomly collected urine samples (Burwick *et al*., 2014). Renal injury is characterised by changes occurring at both cellular levels and molecular levels which result in kidney insufficiency and also structural renal damage. The conventional biomarker used in the diagnosis of renal injury clinically is creatinine. The use of creatinine alone is not able to predict kidney nephrotoxicity, is limited (Peres *et al*., 2013), hence there is need for novel biomarkers to be used in the diagnosis of kidney injury. An ideal biomarker for the diagnosis

of kidney injury should be a marker that is easy to quantify and also able to establish insult to the kidney at an early stage of the condition (Cullen *et al*., 2012).

Extensive research employing the use of several kidney injury biomarkers have been carried out and these biomarkers have proven to be of great value in cases of ischemic injury, in both experimental and clinical scenario (Endre *et al*., 2011). Studies have been designed on the different urinary markers associated with PE, this involves the use of urinary biomarker panels to measure different markers of kidney injury which are specific for the different segments of the renal nephron (Burwick *et al*., 2014). These markers include albumin, β2 macroglobulin (B2M), C, epithelial growth factor (EGF), kidney injury molecule-1 (KIM-1), neutrophil gelatinase–associated lipocalin (NGAL), osteopontin (OPN), MCP-1, IL-18, calbindin and uromodulin (UMOD) (Burwick *et al*., 2014). These markers have shown to be of great value in the diagnosis of kidney injury. These study is designed to quantify the level of urinary KIM-1, IL-18, calbindin, and MCP-1 in pre-eclampsia.

1.7 Kidney Injury Molecule-1 (KIM-1)

The kidney injury molecule -1 (KIM-1) is a transmembrane glycoprotein molecule (type-one), it has an immunoglobulin domain and mucin. This molecule is not detectable in physiological kidney conditions as well as in the urine in normal conditions (Peres *et al*., 2013). This biomarker is expressed in the proximal tubular cells of the kidney post ischemic or toxic damage. The KIM -1 concentration is markedly elevated in human as well as in rodents 24-48 hours in the cells of the kidney proximal tubule after an ischemic injury (Peres *et al*., 2013). Studies has shown that a soluble form of human KIM-1 can be seen in the urine of patients

with proximal tubular injury, hence this molecule can be used as a marker for the diagnosis of injury to the kidney. It also has potential as an early marker of kidney injury (Ichimura *et al*., 2008).

Studies have revealed that the urinary level of KIM-1 correlated with the clinical outcome of the disease. In normal kidneys, the gene expression of the profile of KIM-1 is diminished or completely down regulated, but in cases of kidney injury the messenger ribonucleic acid (mRNA) of KIM-1 is produced at a fast rate (Slocum *et al*., 2012). Information obtained from the structure and expression data of KIM-1 shows that this molecule novel epithelial cell adhesion molecule (CAM) which is up-regulated in proximal tubular cells which are redeveloping, these regenerating cells are known to fix the injured section of the kidney nephron in a post ischemic kidney (Ichimura *et al*., 2008).

In conditions of ischemic or toxic acute kidney injury in humans, KIM-1 is expressed on cells of all three sections of the proximal tubules, but what makes this molecule unique and good marker of kidney injury is the fact that KIM-1 is not expressed on normal kidney cells (Slocum *et al*., 2012; Peres *et al*., 2013). Another peculiar feature is the increased ‘‘expression and in insertion into the apical membrane of the proximal tubule, and its persistence in the epithelial cell until the cell’s full recovery’’ (Peres *et al*., 2013).

In PE urinary KIM-1 level is markedly increased, this is supported by the fact that KIM-1 is up regulated by epithelial cells of the proximal tubule in reaction to ischemia of the renal cells (Burwick *et al*., 2014). Studies have also shown that expression of this molecule transforms

proximal tubule epithelial cells into cells that act like macrophages to eliminate cellular debris from injured tissues (Ichimura *et al*., 2008). It has also been shown in mouse models that up regulation of KIM-1 post ischemic injury mediates activation of the complement system in the proximal tubule (Peng *et al*., 2012). Burwick *et al*., (2014) have established that there exist a correlation between increased urinary KIM-1 level and activated complement components PE.

1.8 Interleukin-18 (IL-18)

Interleukin-18 (IL-18) is a pro-inflammatory molecule with interspersed expression in cells of the distal convoluted tubule as well as in cells of the collecting tubule in normal physiological conditions (Peres *et al*., 2013). These renal cells expressing IL-18 possess three major components needed for release of the active form of this cytokine, these are ‘‘pro-IL-18: the P2X7 and caspase-1 intracellular cysteine protease” (Figure 3; Dinarello, 1989), they convert the pro-form of this cytokine into the active form. The active form of IL-18 then leaves the tubular cell to the lumen thus impacting on the urinary levels in acute kidney injury (Ichimura *et al*., 2008).

The urinary concentration of IL-18 is elevated in human studies and it has a high sensitivity and specificity to detect injury and damage to tubular cells, thus it can serve as a marker of kidney toxicity. This is a unique marker as its level is markedly increase before an increase in the level of serum creatinine becomes visible (Peres *et al*., 2013). The concentration of urinary IL-18 early in kidney injury conditions correlate with the severity of damage. The concentration of urinary IL-18 may be considered as a marker of diagnosing injury to the kidney, but the pro-inflammatory nature of this cytokine and its

concentration in several inflammatory diseases may limit the use as this would affects its sensitivity (Urbschat *et al*., 2011).

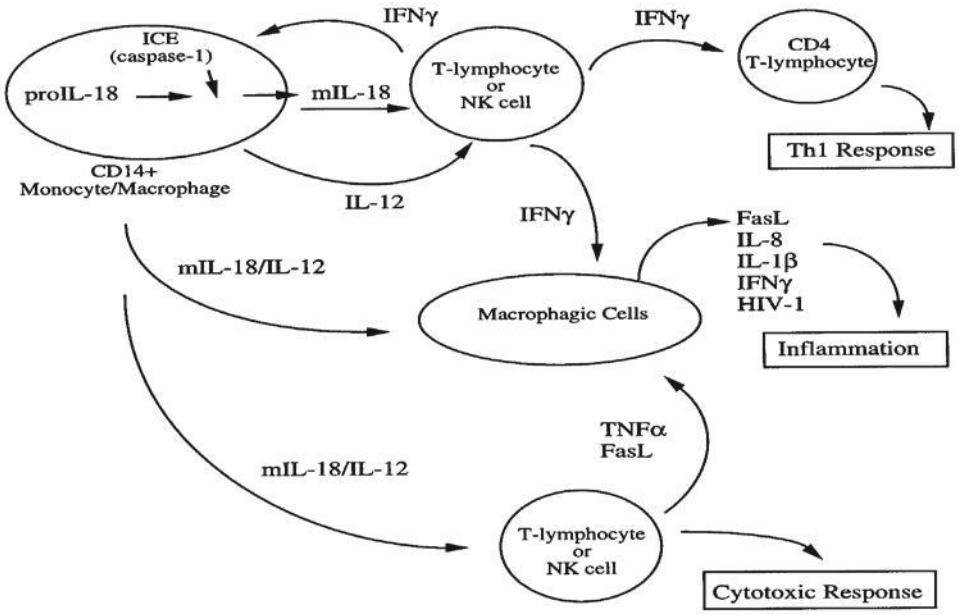

Figure 3. Pathways of IL-18 in the TH1 response (Dinarello, 1989)

IL-18 is a mediator of renal ischemic reperfusion injury that induces monocyte and neutrophil infiltration of the renal parenchyma, it is well know that PE is associated with ischaemia. Also, IL-18 increases in the urine during apoptosis (Adiyanti and Loho, 2012), PE is considered to be associated with increased apoptosis (Tannetta and Sargent, 2013).

1.9 Monocyte chemotactic peptide-1 (MCP-1)

The kidney marker, MCP-1 plays crucial role in part pathogenesis of renal injury. This has been shown by various studies in animal models as well as in humans. It is implicated in driving tubulo-interstitial injury (Viedt and Orth, 2002), and the expression of MCP-1 by cells of the glomerulus correlates with the extent of injury to kidney cells (Panzer *et al*., 2001). It has been shown that in humans with crescentic glomerulonephritis, this molecule (MCP-1) expressed

both the epithelial cells of the kidney tubules and the leukocytes infiltrating the interstitium of the tubules (Segerer *et al*., 2000). Experimental research has shown that the degree of proteinuria diminishes with the administration of MCP-1 antibodies in crescentic glomerulonephritis and ameliorates renal kidney (Wada *et al*., 1996).

Elevated level of urinary MCP-1 is seen in patients with a disorder of the kidney, the urinary MCP-1 Concentration shows a strong correlation with the extent of albuminuria/ proteinuria and kidney injury (Stephan *et al*., 2002). Monocyte chemotactic peptide-1 mRNA was shown to be up regulated in ischemia-reperfusion injury, and MCP-1 has been described as a marker of ‘‘mononuclear inflammatory processes’’(Peres *et al*., 2013) which occurs post-ischemia in kidney injury. This molecule is a powerful chemokine which is secreted by renal cells and is a mediator of kidney toxicity (Peres *et al*., 2013).

Studies in murine models have shown that the MCP-1 protein as well as the mRNA were elevated in intra-renal injuries in a high concentration. Urinary MCP-1 level may be of value in the diagnosis of kidney injury (Munshi *et al*., 2011). Monocyte chemoattractant peptide-1 has been shown in-vitro to activate renal tubular epithelial cells, the mechanism behind this is not known (Viedt and Orth, 2002). This ability of MCP-1 to activate renal tubular epithelial cells is in line with the fact it plays a role in tubulo-interstitial inflammation, a mark of progressive kidney injury (Remuzzi and Bertani, 1998).

1.10 Calbindin

Calbindin also known as calbindin-D28K, is an intracellular calcium binding protein, localised in the distal tubular cells and also in the proximal part of the collecting duct. Injury to the distal segment of the kidney nephron may affect the expression of this molecule and thus altering the urinary concentration of calbindin (Hemmingsen, 1999; Iida *et al*., 2014).

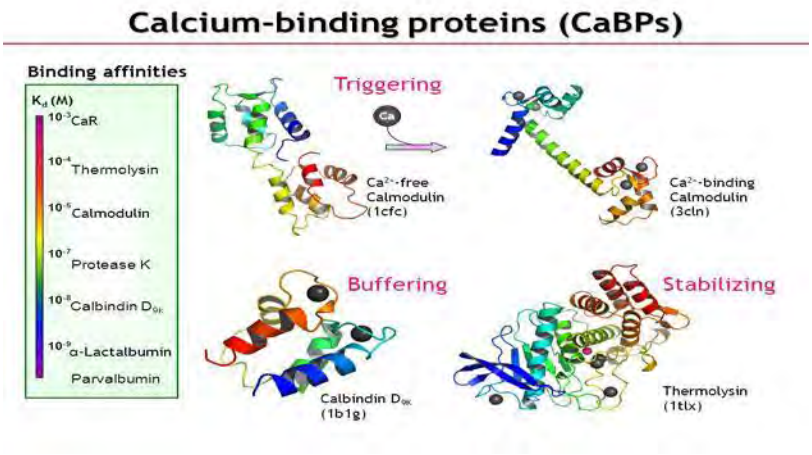

Figure 4: Ca $^{2+}$ -binding Calmodulin (3cln) Ca 2+ -free Calmodulin (1cfc) Triggering Calbindin D 9K (1b1g) Thermolysin (1tlx). (lithium.gsu.edu/faculty/Yang/Protein/lecture2_tertiary%20structure.ppt)

Though the exact role of this molecule is yet to be established, calbindin is as a carrier molecule that participates in the transportation of transcellular Ca^{2+}. It also acts as buffer keeping the level of Ca^{2+} normal and not allowing the level to be toxic.Additionally, it modulates the release of insulin from cells of the pancreas, and acts as an inhibitor of cell death (Figure 4; Timurkaan and Tarakci, 2004).

It has been reported in rat models with unilateral urethral obstruction that the calbindin protein expression level was noticeably down regulated in the kidney distal nephron, these authors also found a mark decrease in calbindin mRNA expression in kidney that are hydronephrotic (Iida*et al*., 2014). Morphological alteration in the kidney cellular arrangement explains the diminished calbindin expression in the distal nephron, emanating from increased stress, dilation of renal tubular lumen and the degeneration of renal epithelial cells (Iida *et al*., 2014). Altered calbindin production can further be explained the generalised cellular disturbance of the distal nephron. The concentration of calbindin excreted is dependent on the time of disease onset, patient age and the serum calcium level. Low concentration of urinary calbindin is linked to a diminished expression of this molecule on the renal cells resulting from damaged renal distal nephron (Iida *et al*., 2014).

1.11 Kidney injury in HIV infection

Kidney injury in HIV infected people is occurring more frequently than in uninfected individuals (Campos *et al*., 2016). Renal injury in individuals infected with HIV is associated with the severe opportunistic infections just as it is with the drugs used in for anti-retroviral therapy (Kalim *et al*., 2008; Ibrahim *et al*., 2010). Common kidney biopsy features in HIV infected persons with acute kidney injury include glomerular disorder and presence of drug-induced microtubular obstruction (Rao and Friedman, 1995). Studies have shown that in some settings nephrotoxicity is responsible for approximately 30% of cases of acute kidney disease in HIV infection (Randall *et al*., 2014).

Treatment regimen used in the treatment of HIV associated infections have been linked to the development of kidney conditions, an example of such ‘‘drugs are aminoglycosides, pentamidine, amphotericin and trimethoprim/sulfamethoxazole, and antivirals such as acyclovir and foscarnet’’(Campos *et al*., 2016).

Though the chance of a single or particular antiretroviral drug causing renal toxicity is low, so many factors are implicated in making HIV infected individuals susceptible in developing kidney injury (Roe *et al*., 2008; Wikman *et al*., 2013). Infection with HIV and the use of anti-retroviral drugs are linked to the development of chronic kidney disease. The prevalence of chronic kidney injury in HIV-infected persons in ‘‘North America and Europe ranges from 4.7 to 9.7%’’ (Campos *et al*., 2016), this prevalence increases to about 33% if chronic kidney disease is defined by diminished glomerular filtration rate (GFR) or the manifestation of pathological proteinuria (Szczech *et al*., 2002). The chance of HIV infected patients developing chronic kidney conditions isincreased in conditions underlying HIV infection such as diabetes as well as high blood pressure (Mocroft *et al*., 2010). In Africans infected with HIV the prevalence of chronic kidney injury is about 3.5 and 48.5% (Stanifer *et al*., 2014).

Kidney cell injury may occur from direct infection or from the response of immune cells to the antigenic stimulation of viral particles. The combination of anti-retroviral drugs, effects of therapeutic drugs used to treat associated infection with HIV, together pose a great risk in the development of kidney disorder in HIV patients (Campos *et al*., 2016).

1.12 Highly active antiretroviral therapy (HAART)

The use of highly active antiretroviral therapy (HAART) has helped reduce mortality and morbidity resulting infection with the human immunodeficiency virus (Izzedine *et al*., 2009). A number of adverse kidney side effects are associated with the use of this drugs. Individuals infected with HIV may develop chronic metabolic conditions associated with the use of HAART like diabetes and dyslipidaemia. These metabolic disorders may lend support to vascular and renal dysfunction (Izzedine *et al*., 2009).

Acute kidney injury seen in HIV infection mostly are a product of opportunistic infections than the toxic effect antiretroviral drugs. Studies have revealed that nephrotoxicity from antiretroviral therapy accounts for approximately 14% of late-onset cases of acute kidney injury (Roe *et al*., 2008). A major cause of kidney injury in HIV infection could be due to is acute interstitial nephritis which be due HIV infection itself, or presence of an opportunistic infection or direct drug cytotoxicity (Rho and Perazella, 2007; Izzedine *et al*., 2009).

Normal kidney function is crucial to metabolism and excretion of antiretroviral drugs. This exposes the kidney to insult from some of these therapeutic agents (Kalyesubula and Perazella, 2011). The clinical presentation of HAART induced kidney injury includes alteration in electrolyte and acid-base balance, lactic acidosis, both acute and chronic kidney conditions. HAART induced kidney injury occurs through numerous mechanism, which may be direct tubular injury or may be in the form of allergic reactions, accumulation of dug precipitates within the kidney tubule lumen (Kalyesubula and Perazella, 2011).

Apart from nephrotoxicity associated with the use of antiretroviral drugs, the HIV particle can directly cause injury to the renal cells, this can be seen in cases of HIV-associated nephropathy (Campbell *et al*., 2009; Choi *et al*., 2009). Other conditions which result from HIV infection that affect kidney function include HIV immune-complex kidney disease and thrombotic microangiopathy resulting from infection with the virus (Kalyesubula and Perazella, 2011).

1.13 Hypothesis

The urinary biomarkers of kidney toxicity; KIM-1, IL8, calbindin and MCP-1 are elevated in pre-eclampsia.

1.14 Aim

To identify and quantify the urine level of KIM-1, IL-18, calbindin and MCP-1 in HIV infected and uninfected women with normal pregnancy and in pre-eclampsia.

1.15 Objective

- To measure urinary levels of MCP-1, IL-18, KIM-1 and calbindin across all the study groups.
- To compare levels of MCP-1, IL-18, KIM-1 and calbindin based on the type of pregnancy (pre-eclamptic and normotensive pregnant women).
- To compare urine levels of MCP-1, IL-18, KIM-1 and calbindin based on the HIV status (HIV +ve vs HIV -ve).

CHAPTER 2

MANUSCRIPT SUBMITTED TO HYPERTENSION IN PREGNANCY

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Role of kidney biomarkers [Kidney injury molecule-1, Calbindin, Interleukin-18 and Monocyte chemoattractant protein-1] in HIV associated pre-eclampsia.

Abstract

Objective: Both HIV infection and pre-eclampsia (PE) are associated with considerable maternal mortality in South Africa. This study was designed to compare the urinary levels of kidney injury molecule-1 (KIM-1), calbindin, interleukin-18 (IL-18), and monocyte chemoattractant protein-1 (MCP-1) in HIV associated normotensive and pre-eclamptic pregnancies.

Methods: Following ethical approval and written consent, urine samples were collected from HIV negative (HIV –ve) normotensive pregnant (n=19), HIV positive (HIV +ve) normotensive pregnant (n=19), HIV –ve pre-eclamptic (n=19) and HIV +ve pre-eclamptic (n=19) women. The concentrations of KIM-1, calbindin, IL-18 and MCP-1 were assessed using the Bioplex technology.

Results: In contrast to IL-18 ($p > 0.05$) and MCP-1 ($p > 0.05$), the concentrations of KIM-1 ($p = 0.02$) and calbindin ($p = 0.02$) were significantly higher in PE compared to normotensive pregnancies, irrespective of HIV status. Based on HIV status, all 4 analytes were similar between HIV +ve and HIV -ve groups. Urinary KIM-1 levels in the HIV –ve pre-eclamptics were significantly higher than those in the HIV –ve normal pregnancies ($p = 0.007$).

Conclusion: Our results demonstrate an increase in the urinary level of kidney injury molecule-1 and calbindin in PE, implicating their possible value as biomarkers of kidney injury. We observed no differences in the levels of KIM-1, IL-18, MCP-1 and calbindin based on HIV

status. We suggest that studies with larger sample sizes using these markers be conducted to establish their use as markers of diagnosing kidney injury in PE.

Key words: Kidney injury molecule-1, calbindin, interleukin-18, Monocyte chemoattractant protein-1.

Running title: Kidney injury in HIV associated pre-eclampsia

Introduction

Pre-eclampsia (PE) is a hypertensive disorder of pregnancy characterized by an elevation in blood pressure and proteinuria after the 20^{th} week of gestation (1). Pre-eclampsia (PE) is a major public health problem and is a leading cause of maternal and foetal morbidity and mortality globally (1). The exact cause of PE remains unknown despite extensive research to understanding its pathophysiology and mechanism of development (2).

Pre-eclampsia is associated with inadequate trophoblast invasion and with spiral artery remodelling limited to the decidua (3, 4). The subsequent decreased blood flow results in the placental hypoxia and oxidative stress with consequential elevated apoptosis and necrosis (5). This leads to release of placental-derived debris into the circulation, which stimulate an exaggerated maternal systemic inflammatory response and results in injury to maternal endothelial tissue (6). Endothelial damage is widespread and affects different organ systems in the body such as the kidney, liver, brain, heart and lungs (7).

In normal pregnancy, there is a change in renal and systemic hemodynamic, the mechanisms responsible for this physiologic change plays important roles in the response of the renal system to the new changes in fluids and electrolytes (7). Glomerular filtration rate (GFR) increases post conception, reaching around 50% above the baseline value, this results in significant hyperfiltration during the second trimester; after which GFR then falls by approximately 20%, returning to the levels at which it was during the antepartum period 3 months post-delivery (7, 8). Pregnancies complicated with PE are associated with renal hyperfiltration, elevated urinary protein and a hypercoagulable state that influence kidney function (7). Also renal tubular function and glomerular charge is altered (9). Urinary protein

excretion is associated with pathologic alteration in renal glomerular endotheliosis, a hallmark of PE, although the underlying mechanism for this lesion is elusive (10-12)

The kidney injury molecule –1 (KIM-1) is produced by cells of the renal tubular epithelium and interstitial macrophages (19, 20). The level of KIM-1 is low in concentration during normal physiological state but its expression is significantly elevated in kidney diseases (21, 22). An increase in the level of urinary KIM-1 concentrations is indicative of ischemic injury of the kidney (12).

IL-18 is a biomarker specific for kidney injury depicting ischemic injury of the proximal tubules. In the absence of kidney injury, only traces of urinary IL-18 is seen, yet its concentration is raised by several folds during injury (19).

Calbindin also known as calbindin-D28K, is an intracellular calcium binding protein, localised in the distal tubular cells and also in the proximal part of the collecting duct (23, 24). Injury to the distal segment of the nephron affects the expression of calbindin thus altering the concentration of urinary calbindin (23).

The molecule monocyte chemoattractant protein-1 (MCP-1) correlates with renal function outcome and albuminuria (25). This molecule is expressed in the tubular epithelial cells and in white cells which infiltrate the interstitium of the tubules, hence they are the source of MCP-1 in the urine (26).

Since the kidney is a target organ in PE, it is important to study the urinary biomarkers of kidney injury (KIM-1, IL-18, calbindin, and MCP-1). This study aims to evaluate the level of these markers of kidney injury in the urine of HIV positive and negative women with normotensive and pre-eclamptic pregnancies.

Methods

Post ethical approval (BREC- No.BE302/16) and informed consent was obtained. Urine samples were obtained from normotensive (N) and pre-eclamptic (PE) women in the antenatal ward of a large regional hospital in Durban, South Africa. These 2 study groups were further stratified by HIV status (HIV +ve and HIV -ve). Demographic and clinical characteristics were collated onto a structured datasheet.

Women who had no history of pregnancy complications were used as controls. Pre-eclampsia was defined by a blood pressure ≥ 140/90 mm Hg and proteinuria ≥ 300 mg in a 24-hour urine sample. Women with a history of chronic hypertension, cardiovascular or renal disease, diabetic mellitus, bleeding disorders, and previous history of any medical conditions were excluded.

Spot urine samples was obtained from patients. Importantly, less than 20 minutes after collection, the urine samples were centrifuged at 1500 rpm for 10 min to remove residual cells and then stored at -80°C until Bioplex assay.

Urinary KIM-1, calbindin, IL-18 and MCP-1 were quantified using a BIO-RAD Human Kidney Toxicity Assay Panel 2 (BIO-RAD Laboratories). The assays are built on magnetic beads to enable robust quantification of multiple proteins in human urine samples. Samples were analysed based on the manufacturer's instructions and guidelines.

Data obtained in this study was analysed using a BioPlex 200 instrument equipped with Bio-Plex Manager™ analysis software version 4.1. A standard curve was produced using the known concentration (ng/ml) of each analytes by plotting the median fluorescent intensity (MFI) signal against concentration. These standards were used to interpolate the concentration of the unknown samples.

Intra plate variability was determined using determined with CV <20% and ($\frac{(Observed\ concentration}{Expected\ concentration}\ X\ 100$) between 70-130% (r=0.8, p=0.05).

Statistical analysis

GraphPad Prism, V 5.03 was used for data analysis (Graph-Pad Software Inc., California). One-way analysis of variance and t-test were used to compare the experimental results between the different study groups. A p-value less than 0.05 was considered significant.

Demographic and clinical data:

The patient demographic and clinical data is presented in Table 1. There was a significant difference in maternal age (p = 0.003) across the study groups. Gestational age, parity and maternal weight were non-significant amongst the study groups. A significant difference was observed in both systolic (p = 0.0001) and diastolic (p = 0.0001) blood pressure between the normotensive and pre-eclamptic groups.

Analyte quantification:

Pregnancy type: Data obtained from the analysis of the urinary levels of KIM-1, IL-18, calbindin, and MCP-1 are presented in Tables 2a, 2b, and 2c and the specificity of each of the markers are illustrated in figures 1A, 2A, 3A and 4A respectively. Urinary IL-18 and MCP-1 levels were not statistically significant according to pregnancy type (pre-eclamptic vs normotensive) respectively (p = 0.90, p = 0.50). There was a significant difference in the concentration of KIM-1 and calbindin between PE and N respectively (p = 0.02, p = 0.02).

HIV status; There was no significant difference in the urinary levels of KIM-1, IL-18, calbindin, and MCP-1 concentration in the HIV-ve compared to the HIV +ve women (p = 0.47, p = 0.28, p = 0.99, p = 0.78).

Comparison across the study groups; Urinary KIM-1 levels was significantly different across the study groups (p = 0.0001) whilst IL-18, calbindin, and MCP-1 were not significant respectively (p = 0.99, p = 0.28, p= 0.73). These observations are pictorially illustrated in figures 1B, 2B, 3B and 4B respectively.

Discussion

This study demonstrates that there was a significant difference in the concentrations of KIM-1 and calbindin between PE and N pregnancies. However, based on pregnancy type, IL-18 and MCP-1 were not statistically significant.

We report a twofold higher level of KIM-1 in PE compared to that in N pregnancy irrespective of the HIV status. This increase in urinary KIM-1 concentration is in accordant with findings from similar studies that have demonstrated an increase in urinary KIM-1 concentrations in PE (7). Wang *et al.*, (2015) (12) have shown that the urinary concentration of KIM-1 was affected by the severity of PE, as the level was significantly higher in severe PE compared to the urinary KIM-1 concentration in patients with mild form of the disorder. It is well known that PE is associated with the characteristic glomerular endotheliosis, widespread ischemic injury as well as altered functional integrity of the glomerular and proximal tubular cells (7, 22).

In this study, irrespective of HIV status, urinary calbindin level was significantly higher in PE compared to N pregnancies. This increase is corroborated in a previous study (23). Notably, urinary calbindin meets the criteria for use as an ideal biomarker for the prediction of kidney

injury because it is released by the distal tubular cells of injured kidney, where it is involved in Ca^{2+} reabsorption (23). Moreover, calbindin can be used to monitor the development and progression of injury to the distal convoluted tubule of the nephron (31).

Based on pregnancy type, the results of this study demonstrate similar urinary IL-18 levels between the PE and N groups. These results are in keeping with the findings of Lopez-Hernandez *et al*., (2016) (27). Also, another study has highlighted that urinary IL-18 is not beneficial as a urinary biomarker for the detection of acute kidney injury (28). IL-18 is a mediator of renal ischemic reperfusion injury that induces monocyte and neutrophil infiltration of the renal parenchyma, it is well know that PE is associated with ischaemia. Also, IL-18 increases in the urine during apoptosis (29), PE is considered to be associated with increased apoptosis (5). Furthermore, IL-18 activity is enhanced in a number of inflammatory diseases (Gracie *et al.,* 2003) (37) and PE represents an exaggerated inflammatory response.

The role for IL-18 in various pathological conditions are currently of great interest and its role in acute or chronic inflammatory conditions such as PE remains ambiguous. IL-18 is a pro-inflammatory cytokine and IL-18 is widely considered as a biomarker of tubular cell injury/necrosis in a kidney exposed to ischemic injury (29, 30). In this study, urinary IL-18 levels in the PE group were similar to the N group. These results are in keeping with the findings of Waanders *et al* (2009) (21). However, the circulating levels of cytokines particularly IL-18 in pre-eclampsia are however, conflicting. In contrast to PE, the shift to a Th2 immune response does not occur resulting in an increase in Th1 cytokines. The elevation of a pro-inflammatory cytokine (IL-18) may trigger the exaggerated inflammatory process in PE, however, we were unable to show this effect. Importantly, in HIV +ve patients there is a decrease in the Th1 and an increase in Th2 cytokines (38). Therefore, it is plausible to assume

that the HIV infection may explain the similarity in the IL-18 levels observed between our study groups. In our study, urinary IL-18 level in the HIV negative and positive women with PE did not statistically vary from that in the HIV-ve and HIV +ve women with normal pregnancy. The unexpected similarity of IL-18 levels between PE and N pregnancies may be attributed to the fact that all HIV +ve women in this study received anti-retroviral drug (ARV) therapy, a side effect of which is nephrotoxicity (12).

This study does not report a significant difference in the urinary MCP-1 level in PE compared normotensive pregnancy. These findings are similar to that of Lopez-Hernandez (2016) (27). Although MCP-1 is known to mediate acute ischemic kidney injury (32), it is confirmed by an in-vitro study that MCP-1 specifically activates cells of the tubular epithelium (26).

Based on HIV status, we report no significant difference in urinary KIM-1, calbindin, IL-18 and MCP-1 concentration in the study population. This lack of significance may be attributed to the fact that it is a national standard of care within antenatal facilities in South Africa for HIV +ve women to receive antiretroviral therapy and prevention of mother-to-child transmission of HIV (PMTCT). The use of highly active antiretroviral therapy (HAART) has helped reduce mortality and morbidity (13). However, individuals with HIV +ve may develop chronic metabolic conditions associated with the use of HAART like diabetes and dyslipidaemia. These metabolic disorders may lend support to vascular and renal dysfunction (13). Acute kidney injury seen in HIV infection mostly are a product of opportunistic infections than the toxic effect of ART drugs. Studies have demonstrated that nephrotoxicity from antiretroviral therapy accounts for approximately 14% of late-onset cases of acute kidney injury (14). A major cause of kidney injury in HIV infection is acute interstitial nephritis which

may occur as a result of HIV infection itself, or the presence of an opportunistic infection or direct drug cytotoxicity (13, 15).

Normal kidney function is crucial to the metabolism and excretion of antiretroviral drugs, this exposes the kidney to insult from some of these therapeutic agents (16). Asides nephrotoxicity associated with the use of ART drugs, the HIV can directly cause injury to the renal cells, this can be seen in cases of HIV-associated nephropathy (17, 18). Other conditions which result from HIV infection that affects kidney function include HIV immune-complex kidney disease and thrombotic microangiopathy resulting from infection with the virus (16).

Though studies have shown that HIV +ve patients have an increased chance developing acute kidney injury and also chronic disorder of the kidney (33). The chance of HIV +ve individuals suffering nephrotoxicity and nephron angiosclerosis is also increased compared to that in HIV –ve persons (34, 35). It is estimated that approximately 30% of acute kidney injury episodes in HIV +ve people is due to nephrotoxicity which may be due to treatment with anti-retroviral therapy (36). Drugs used in the treatment of HIV associated infections have been linked to the development of kidney conditions. Though the chance of a single or particular antiretroviral drug causing renal toxicity is low, so many factors are implicated in making HIV +ve individuals susceptible in developing kidney injury (33). The plasma levels of MCP-1 correlates with virus load in HIV infection (39, 40). Moreover, MCP-1 levels are diminished in HIV-ve patients after Indinavir (a viral protease inhibitor) treatment (41) and all HIV+ve women in this study were on ARV therapy.

In conclusion, our results demonstrate an increase in the urinary concentration of KIM-1 and calbindin, whilst the urinary IL-18 and MCP-1 in PE did not show any significant difference when compared to normotensive pregnancies.

We proposed that the biomarkers could be established as an indicator of renal injury and should be used on a more larger sample size of normotensive and pre-eclamptic patients in diagnosing kidney damage.

Conflict of interest

Authors have no conflict of interest to declare.

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Tables and Figures

Table 1. Demographic and clinical data for the different study groups.

	Normotensive pregnant women (HIV-ve)	Normotensive pregnant women (HIV +ve)	Pre-eclamptic pregnant women (HIV-ve)	Pre-eclamptic pregnant women (HIV +ve)	p-value
Sample size	n=19	n=19	n=19	n=19	
Age (years)	28 (23 – 32)	30 (23 – 36)	23 (19 – 32)	33 (30 – 34)*	p = 0.03
Gestational age (weeks)	36 (34 – 37)	36 (30 – 39)	38 (36 – 39)	37 (36 – 38)	ns
Parity	1 (1 – 2)	2 (1 – 2)	1 (1 – 3)	2 (1-3)	ns
Maternal weight (kg)	0.15 (0.02 – 0.99)	0.14 0.01 – 1.6	0.22 (0.0 – 4.5)	0.11 (0.01 – 2.7)	ns
Systolic BP (mmHg)	109 (106 – 117)	104 (98 – 120)	162 (152 – 173)*	154 (145 – 159)	p < 0.001
Diastolic BP (mmHg)	69 (63 – 72)	65 (60 – 74)	97 (92 – 108)	96 (90 – 100)	p < 0.001

Values expressed as median (range). Not significant (ns). Blood pressure (BP).

Table 2a. Urinary level of kidney toxicity biomarkers normal pregnancy and in pre-eclampsia

	Normotensive pregnant women	Pre-eclamptic pregnant women	p-value
Sample size	n=38	n=38	
KIM-1(ng/ml)	0.04 (0.01 – 0.2)	0.06 (0.01 – 1.1)*	$p < 0.05$
IL-18 (ng/ml)	0.02 (0.01 – 0.14)	0.01 (0.01 – 0.25)	ns
Calbindin (ng/ml)	44.1 (4.0 – 556.1)	68.5(7.5 – 3022)*	$p < 0.05$
MCP-1(ng/ml)	0.13(0.01 – 2.7)	0.17 (0.0 – 4.4)	ns

Values expressed as median (range). Not significant (ns). *significant at $p<0.05$

Table 2b. Urinary level of kidney toxicity biomarkers in HIV –ve and HIV +ve groups

	HIV –ve	HIV+ve	p-value
Sample size	n=38	n=38	
KIM-1(ng/ml)	0.07, (0.01 – 0.41)	0.03, (0.01 – 1.1)	ns
IL-18 (ng/ml)	0.02 (0.01 – 0.14)	0.01 (0.01 – 0.25)	ns
Calbindin (ng/ml)	44.8(4.0 – 609.1)	61.0(7.5 – 3022)	ns
MCP-1(ng/ml)	0.18(0.0 – 4.5)	0.13(0.01 – 2.7)	ns

ns= not significant

Table 2c. Urinary level of kidney toxicity biomarkers across study groups.

	Normotensive pregnant women (HIV-ve)	Normotensive pregnant women (HIV+ve)	Pre-eclamptic pregnant women (HIV-ve)	Pre-eclamptic pregnant women (HIV+ve)	p-value
Sample size	n=19	n=19	n=19	n=19	
KIM-1(ng/ml)	0.03 (0.01 – 0.16)	0.05 (0.01 – 0.2)	0.09 (0.01 – 0.41)*	0.04(0.01 – 1.1)	$p < 0.05$
IL-18 (ng/ml)	0.02 (0.01 – 0.14)	0.01 (0.01 – 0.09)	0.01(0.01 – 0.06)	0.02 (0.01 – 0.25)	ns
Calbindin (ng/ml)	46.0 (4.0 – 556.1)	42.5 (7.5 – 246.9)	36.7 (7.5 – 609.1)	36.7(7.5 – 609.1)	ns
MCP-1(ng/ml)	0.15 (0.02 – 0.99)	0.14, 0.01 – 1.6	0.22 (0.0 – 4.5)	0.11 (0.01 – 2.7)	ns

Values expressed as median (range). Not significant (ns).

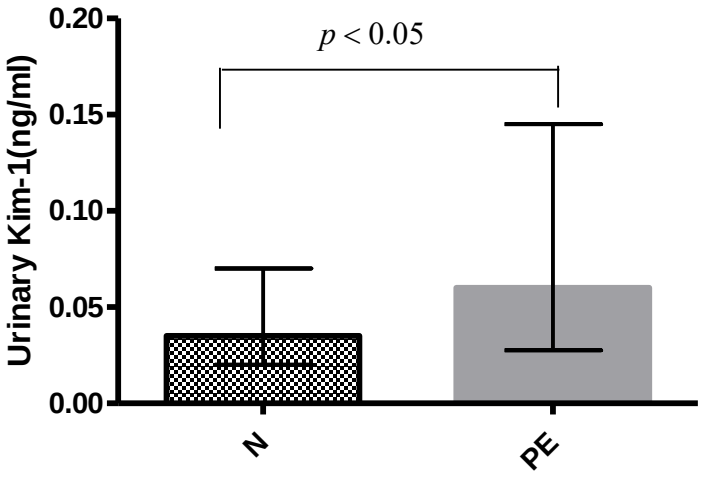

Figure 1A. Urinary KIM-1 concentration in normal Pregnancy and in PE.

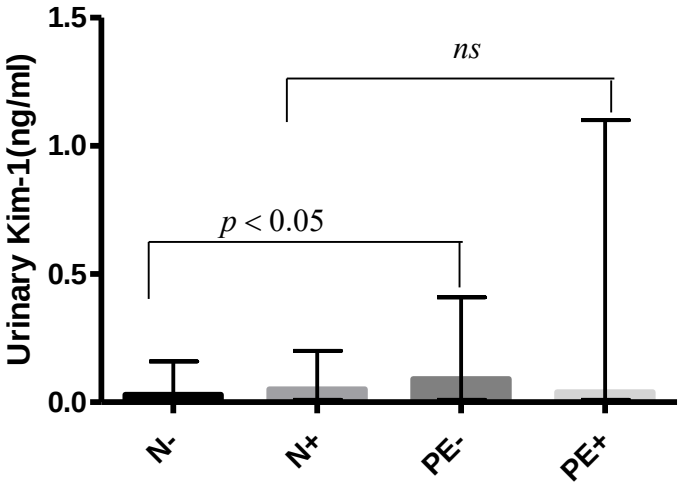

Figure 1B. Urinary KIM-1 level in HIV infected and uninfected women with normal pregnancy and with PE.

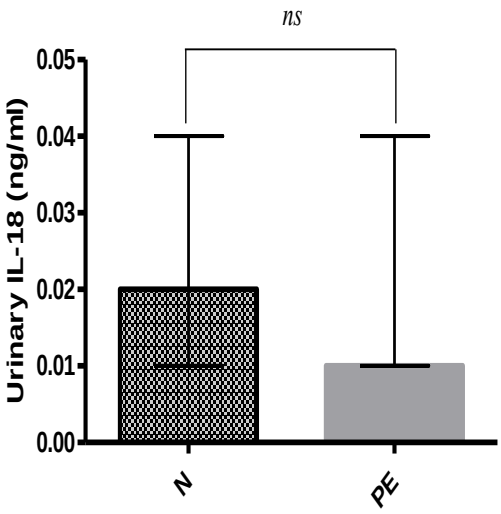

Figure 2A. Urinary IL-18 in normal pregnancy and in PE.

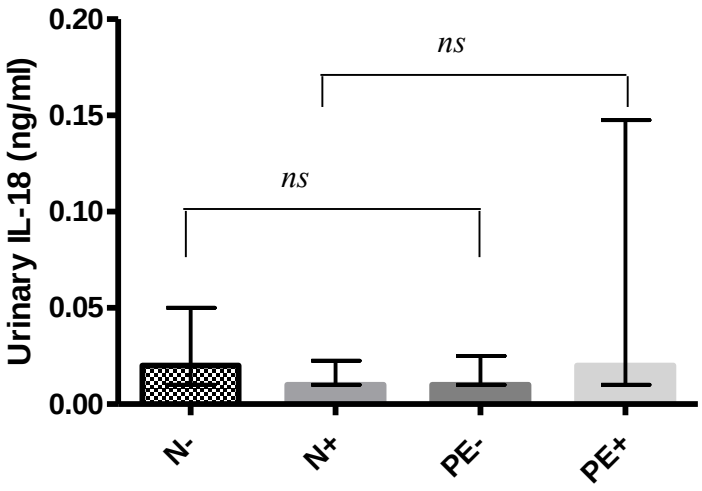

Figure 2B. Urinary IL-18 level in HIV infected and uninfected women with normal pregnancy and with PE.

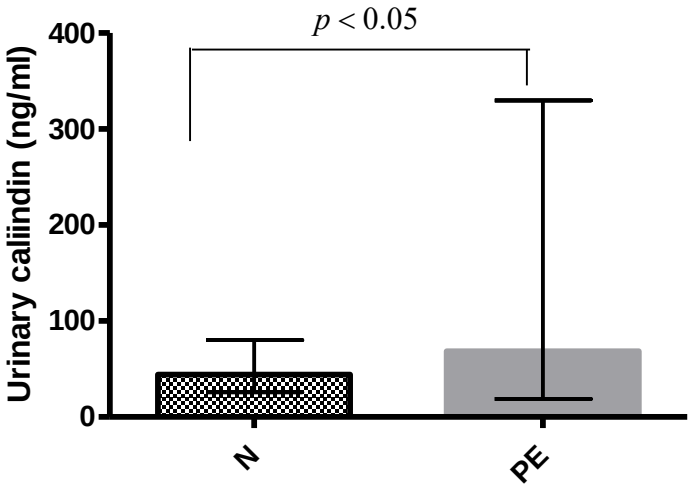

Figure 3A. Urinary calbindin in normal pregnancy and in pre-eclampsia.

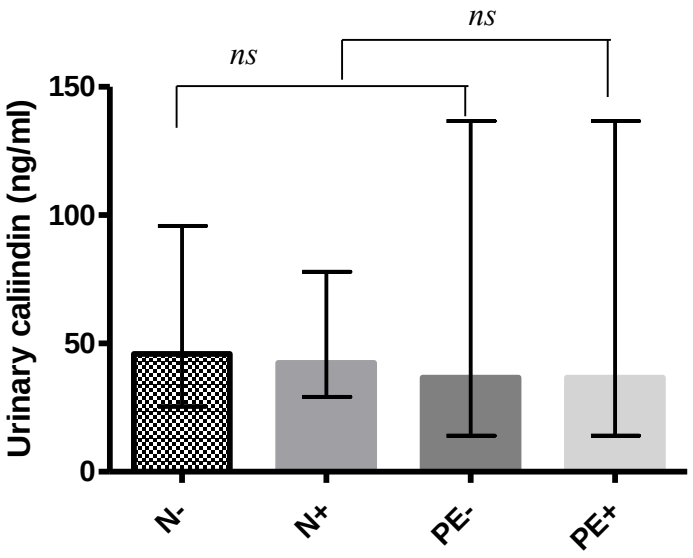

Figure 3B. Urinary calbindin level in HIV infected and uninfected women with normal pregnancy and with PE.

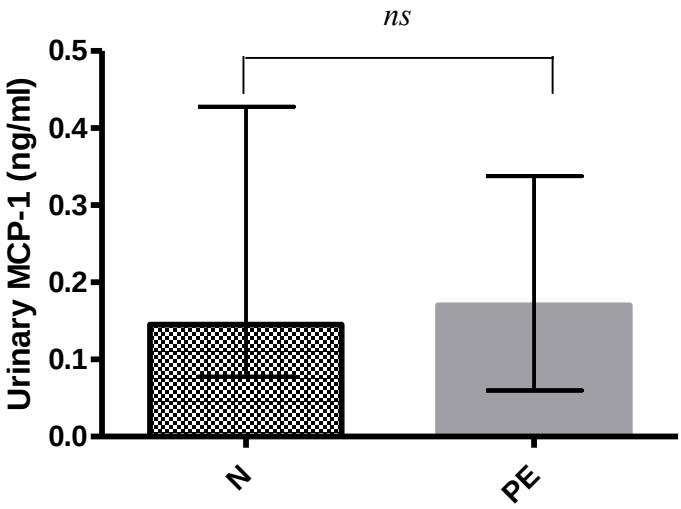

Figure 4A. Urinary MCP-1 in normal pregnancy and in PE.

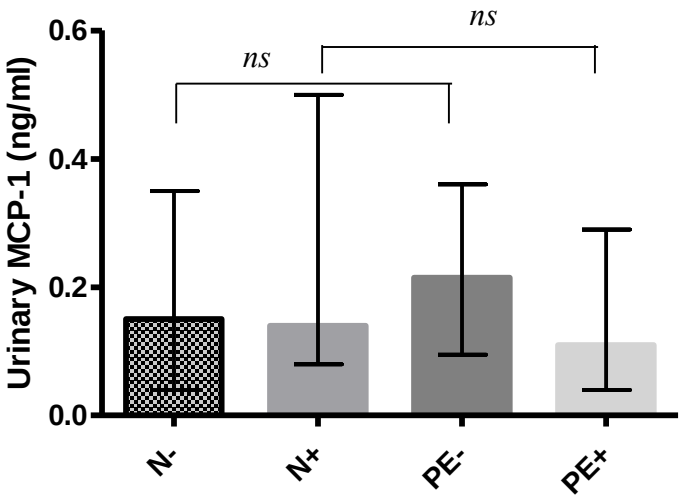

Figure 4B. Urinary MCP-1 level in HIV infected and uninfected women with normal pregnancy and with PE

CHAPTER 3

SYNTHESIS, CONCLUSION AND RECOMMENDATION

3.1 Synthesis

Extensive research has led to a sound understanding of the kidney's response in physiologic and pathologic conditions. This has led to the detection of certain genes and molecules that play a critical role in kidney function. Of note, is the fact that several of these proteins are present hence measurable in the urine, thereby serving as non-invasive biomarkers for the study of the kidney disorders (Hristova and Dimitrova, 2015). Studies have implicated the urinary concentration of these markers to the state of the kidney (Xiao *et al*., 2013; Wang *et al*., 2012). However, further research is warranted to establish the different tissues where these proteins are located in the kidney so that the urinary concentration can serve as a mirror image of the kidney function.

Kidney tubular function is altered in PE (Machado *et al*., 2012). Associated with PE is renal hyper filtration, elevated urinary protein and a hypercoagulable state which all contribute to influencing kidney function in PE (Xiao *et al*., 2013). The knowledge of the kidney being a target organ in hypertensive complications of pregnancy has progressed. Kidney injury in PE is characterised by lesions of the glomerulus, endotheliosis and loss of glomerular function. This has led to PE being considered a common glomerular disease (Reckelhoff, 2012; Stillman and Karumanchi, 2007).

A study by Wang *et al.,* has shown that the urinary protein concentration of specific proteins of glomerular origin are elevated in the urine of women with PE compared to their level in normotensive pregnant women (Wang *et al*., 2012), and are also lower compared to women who were hypertensive prior to getting pregnant. The same study report that these proteins are significantly higher in PE irrespective of the measurement, if it was absolute value measured or if they were factored for urinary creatinine (Wang *et al*., 2012). The current study attempts to quantify the urinary level of KIM-1, IL-18, calbindin and MCP-1 as biomarkers for the detection of kidney injury in HIV associated normotensive and pre-eclamptic pregnant women.

In this present study, we observed the urinary KIM-1 concentration in PE was significantly higher than normotensive pregnancies. This increase in urinary KIM-1 concentration is in keeping with findings from similar studies that have also shown an increase in urinary KIM-1 concentration in PE (Xiao *et al*., 2013). Wang *et al.*, (2015) suggests that the urinary concentration of KIM-1 is affected by the severity of PE, as the level was significantly higher in severe PE compared to the mild form of the disorder. This increase in urinary KIM-1in PE is associated with the increased incidence of endotheliosis, ischemia reperfusion injury of the kidney, and damage to cells of the kidney proximal tubules (Xiao *et al*., 2013, Zhang *et al*., 2007). In a normal kidney, the expression of KIM-1 is low, but it is intensely up- regulated after injury to the kidney.

This molecule is a phosphatidylserine receptor which converts epithelial cells into phagocytic cells via the recognition of cell surface-specific epitopes which present on apoptotic tubular epithelial cells (Ichimura *et al*., 2008).

It also known that an extracellular domain of the molecule KIM-1 is proteolytically processed and appears in the urine immediately after injury to the kidney especially in acute kidney injury (Hristova and Dimitrova, 2015). Studies have shown that western and northern blot analysis of KIM-1 mRNA transcripts and its protein are up regulated in *in vivo* post ischemic kidney injury (Ichimura *et al*., 1998). In situ hybridization and immunohistochemistry studies have also shown KIM-1 expression on injured cells of the proximal tubule, this is commonly seen in the medulla (Ichimura *et al*., 1998).

We report that the urinary IL-18 level in the PE group was not significantly different to the normotensive pregnant group. This result is in keeping with the findings of a similar studies (Lopez-Hernandez *et al*., 2016; Wang *et al*., 2015). The cytokine IL-18 is pro-inflammatory and the level of this molecule in the urine is considered as a marker of tubular cell injury/necrosis in a kidney exposed to ischemic injury (Adiyanti and Loho, 2012, Parikh *et al*., 2006). A study has also shown that urinary IL-18 concentration was not beneficial as a urinary biomarker in the detection of acute kidney injury (Hristova and Dimitrova, 2015). Moreover, IL-18 is known to promote or drive cellular apoptosis and has the potential of complicating kidney injury. Small amounts of IL-18 are found in the urine in physiological conditions but its level is greatly increased in cases of kidney injury (Hristova and Dimitrova, 2015).

In this study, irrespective of HIV status urinary calbindin level was significantly higher in PE compared to normotensive pregnancies. This increase corroborates a previous study by (Iida *et al*., 2014).

Studies have shown that a significant concentration of calbindin is expelled into human urine in cases of kidney injury. The urinary concentration is tied to onset of disease and the time of diagnosis (Iida *et al*., 2014), as alteration in the calbindin expression in renal cells impact the urinary calbindin levels in kidney toxicity (Iida *et al*., 2014).

Notably, urinary calbindin meets the criteria for use as an ideal biomarker for the prediction of kidney injury because it is derived from release by injured kidney distal tubular cells, where it is involved in Ca^{2+} reabsorption (Iida *et al*., 2014). Moreover, calbindin can be used to monitor the development and progression of injury to the distal convoluted tubule of the nephron (Guha *et al*., 2011).

This study does not report a significant difference in the urinary MCP-1 level in PE compared to normotensive pregnancy. These findings are similar to that of (Lopez-Hernandez *et al*., 2016). Although MCP-1 is known to mediate acute ischemic kidney injury (Munshi *et al*., 2011), it is confirmed by an *in-vitro* study that MCP-1 specifically activates cells of the tubular epithelium (Viedt and Orth, 2002). An increase in the urinary MCP-1 level correlates with the concentration of albumin and protein excretion into the urine in cases of kidney injury (Stephan *et al*., 2002). The exact cellular source for the release of MCP-1 still remains a debate as it is not clearly defined (Munshi *et al*., 2011). Studies have shown it could be released in cases of injured glomeruli, from the proximal tubules, distal segment of the kidney nephron, and from inflammatory cells, hence MCP-1 in the urine may not reflect a specific area of injury to the kidney (Zager *et al*., 2008).

Based on HIV status, we report no significant difference in urinary KIM-1, calbindin, IL-18 and MCP-1 concentration in our study population.

This lack of significant differencee may be attributed to the fact that it is a national standard of care within antenatal facilities in South Africa for HIV infected women to receive antiretroviral therapy and prevention of mother-to-child transmission of HIV (PMTCT). The use of highly active antiretroviral therapy (HAART) has helped reduce mortality and morbidity (Izzedine *et al*., 2009). However, individuals infected with HIV may develop chronic metabolic conditions associated with the use of HAART like diabetes and dyslipidaemia. These metabolic disorders may lend support to vascular and renal dysfunction (Izzedine *et al*., 2009). Acute kidney injury seen in HIV infection are mostly a product of the opportunistic infections than the toxic effect antiretroviral drugs. Studies have demonstrated that nephrotoxicity from antiretroviral therapy accounts for approximately 14% of late-onset cases of acute kidney injury (Roe *et al*., 2008). A major cause of kidney injury in HIV infection is acute interstitial nephritis which be due to HIV infection itself, or the presence of an opportunistic infection or direct drug cytotoxicity (Rho and Perazella, 2007; Izzedine *et al*., 2009).

Normal kidney function is crucial to metabolism and excretion of antiretroviral drugs, this exposes the kidney to insult from some of these therapeutic agents (Kalyesubula and Perazella, 2011). Asides nephrotoxicity associated with the use of antiretroviral drugs, the HIV can directly cause injury to the renal cells, this can be seen in cases of HIV-associated nephropathy (Campbell *et al*., 2009; Choi *et al*., 2009). Other conditions which results from HIV infection that affects kidney function include HIV immune-complex kidney disease and thrombotic microangiopathy resulting from infection with the virus (Kalyesubula and Perazella, 2011).

Though studies have shown that HIV infected patients have an increased chance developing acute kidney injury and also chronic disorder of the kidney (Campos *et al*., 2016).

The chance of HIV infected individuals suffering nephrotoxicity and nephron angiosclerosis is also increased compared to that in uninfected persons (Rosenberg *et al*., 2015; Saracho *et al*., 2015). It is estimated that approximately 30% of acute kidney injury episodes in HIV infected people is due to nephrotoxicity which may be due to treatment with anti-retroviral therapy (Randall *et al*., 2015). Drugs used in the treatment of HIV associated infections have been linked to the development of kidney conditions. Though the chance of a single or particular antiretroviral drug causing renal toxicity is low, so many factors are implicated in making HIV infected individuals susceptible in developing kidney injury (Campos *et al*., 2016). The plasma levels of MCP-1 correlates with virus load in HIV-1 infection (Weiss and others 1997; Chang and others 2004). Moreover, MCP-1 levels are diminished in HIV-1 patients after indinavir (a viral protease inhibitor) treatment (Bisset *et al*., 1997) and all infected women in our study were on ARV therapy.

3.2 Conclusion

Our results demonstrate an increase in the urinary level of kidney injury molecule-1 and calbindin in PE, implicating their value as biomarkers of kidney injury. To our knowledge this is also the first study reporting no significant difference in the levels of KIM-1, IL-18, MCP-1 and calbindin based on HIV status.

The molecules studied in this research viz., KIM-1 and calbindin.IL-18, and MCP-1 provide the source of biomarkers for non-invasive diagnosis of pre-eclampsia using urine samples.

3.3 Recommendation

We propose that since these markers are promising, studies with larger sample sizes and case control studies are required. Also, the severity of pre-eclampsia may affect the concentration of this markers in the urine, we propose that the level of these markers be investigated in the different forms of pre-eclampsia like mild to moderate pre-eclampsia and in severe pre-eclampsia or in early onset or late-onset pre-eclampsia since studies have shown these two conditions are different entities and should be treated as such.

Research in this field has demonstrated that hypertensive disorders of pregnancy may predispose the woman to developing cardiovascular complications later in life and also exposing the foetus to adverse conditions that may hinder fetal development or impair other life processes after birth. Therefore, it is appropriate and necessary to develop diagnostic procedures for the early detection of PE and for a better treatment of patients (Reckelhoff, 2012).

CHAPTER 4

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APPENDICES

APPENDIX A

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

APPENDIX B

Research approval from the KwaZulu-Natal Department of health