THE ROLE OF PRO-RENIN AND ITS RECEPTOR EXPRESSION IN PRE-ECLAMPSIA COMPLICATED BY HIV INFECTION

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 T. Naicker and Miss A. Ajith.

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DECLARATION

I, **Londiwe Nomfundo Bongiwe Silwane** 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.

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Signed: 

Date: 01.02.2016

DEDICATION

To God for his undying love

To my parents

for their continual support and their belief in me.

ACKNOWLEDGEMENTS

My Parents and Siblings

Thank you for your guidance, support and allowing me every opportunity to further my education and knowledge. I appreciate everything that you have done for me, your continuous encouragement and unwavering faith in my ability as a student and person.

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LIST OF ABBREVIATIONS

AGT	Angiotensinogen
Ang 1	Angiotensin 1
ART	Antiretroviral therapy
BP	Blood pressure
BREC	Biomedical research ethics
BSA	Bovine Serum Albumin
Cyt	Cytoplasmic domain
ELISA	Enzyme linked ImmunoSorbent Assay
ERK	Extracellular signal-regulated kinases
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
HIV+	HIV positive
HIV-	HIV negative
Kg	Kilograms
MAP	Mono Adenosine Phosphate
MmHg	millimetres mecury
NM	Normotensive
°C	Degree Celsius
Rmp	Revolutions per minute
PBS	Phosphate Buffered Saline
EOPE	Early Onset Pre-eclampsia

LOPE	Late Onset Pre-eclampsia
PR	Pro-renin
(P)RR	Pro-renin receptor
RAAS	Renin-angiotensin System
SP	Signal peptide
TM	Transmembrane domain
WHO	World Health Organisation

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ABSTRACT

Maternal mortality and morbidity is predominantly (40%) due to non-pregnancy related infections (HIV) in all South African provinces. Hypertension (14%) is the commonest direct cause of maternal deaths in pregnancy, of which 83% represents pre-eclampsia. Pre-eclampsia is unique to pregnancy and is characterised by new onset hypertension after 20 weeks of gestation in the presence of proteinuria. The renin-angiotensin-aldosterone system (RAAS) has a vital role in blood pressure development. The aim of the study is to examine the expression of circulating pro-renin (PR) and its receptor (P)RR in HIV associated normotensive pregnant and pre-eclamptic women.

Following informed consent and expedited institutional ethics approval (BE 320/14) this study was conducted at the Optics and Imaging Centre, Doris Duke Medical Research Institute, UKZN. The study population (n=90) consisted of African women attending the ante-natal clinic at Prince Mshiyeni Memorial Hospital in Umlazi, Durban Metropolitan Area. There were 6 groups (n=90 each), namely, control group of HIV positive and negative normotensive pregnant women (n=30) and an experimental group of pre-eclamptic women (n=60) stratified according to HIV positive and negative early (EOPE) and late (LOPE) onset pre-eclampsia. A quantitative sandwich enzyme immunoassay technique utilizing pro-renin Human ELISA and pro-renin receptor ELISA immunoassay kits were performed on plasma. Intensity of the colour reaction was read spectrophotometrically at the wavelength (450 nm). The pro-renin and its receptor concentration levels were calculated using a standard curve derived from known concentrations. Shapiro-Wilk test, Mann-Whitney U test and Kruskal-Wallis test were used respectively to analyse data.

The mean age ± SD of the total study population was 27±6 yrs with a significant difference in maternal age across all 6 study groups (p=0.004). There was a significant difference in maternal age (p=0.001) between HIV positive and negative groups regardless of pregnancy type. There was

no significant difference in maternal weight (p=0.153) between the HIV positive and negative groups regardless of pregnancy types. Maternal weight was higher in the pre-eclamptic groups compared to the normotensive group. In the LOPE HIV positive group the maternal weight was higher compared to the HIV negative group and also the EOPE HIV positive and negative groups. There was no significant difference in gestational age (p=0.504) between HIV positive and negative groups regardless of pregnancy type. Gestational age was higher in the normotensive and late onset pre-eclamptic groups compared to the early onset pre-eclamptic group. Also gestational age in the HIV positive EOPE group was higher than the HIV negative group. There was a significant difference in gravidity (p=0.003) between HIV positive and negative groups regardless of pregnancy type. There was a significant difference across all 6 study groups (p=0.024). Gravidity was higher in the normotensive HIV negative group compared to the EOPE and LOPE HIV negative groups.

The mean PR ± SD of the study population was 3.98±2.01ng/ml. PR was lower in the HIV positive group (3.86 ng/ml) compared to the HIV negative group (4.09 ng/ml). There was no significant difference in PR (p=0.599) between HIV positive *vs* the HIV negative group regardless of pregnancy type. There was no effect of pregnancy type (Normotensive *vs* EOPE *vs* LOPE; Normotensive *vs* PE) on PR levels (p=0.140; p=0.073) and amongst all 6 study groups (p=0.379). There was no difference in PR between the EOPE and LOPE groups (p=0.409) as well as normotensive *vs* LOPE (p=0.273). However there was a significant difference between normotensive *vs* EOPE (p=0.039).

The mean (P)RR ± SD of the total study population was 1.60±0.95ng/ml. There was no significant difference in (P)RR (p=0.541) between HIV positive and HIV negative groups regardless of the pregnancy type as well as across all pregnancy types (p=0.055). There was no difference between normotensive and pre-eclamptic (p=0.362). There was no difference between normotensive and

EOPE (p=0.074) and also between normotensive and LOPE (p=0.803). However there was a significant difference between EOPE *vs* LOPE (p=0.019).

Pearson correlation revealed no significant correlations between PR within each pregnancy type (normotensive, early onset and late onset pre-eclampsia).There was a positive correlation between diastolic blood pressure and (P)RR concentration in the late-onset pre-eclamptic group, which was statistically significant [rs=-0.380; p=0.046]. There were no significant correlation between maternal blood pressure and (P)RR in the normotensive pregnant and early onset pre-eclamptic groups. There was a positive correlation between PR concentration and the HIV negative late onset pre-eclamptic and HIV positive early onset pre-eclamptic group, which was statistically significant [rs=0.525; p=0.054]. A significant negative correlation occurred between PR concentration versus HIV positive late onset pre-eclamptic group and HIV positive early onset pre-eclamptic [rs=-0.643; p=0.018]. There were a significant positive correlation between (P)RR and diastolic blood pressure [rs = -0. 585; p=0.036].

A low (P)RR concentration will inhibit binding to the pro-renin molecule. Moreover, the pro-renin receptor represents an elegant concept to explain the existence of active pro-renin in vivo. This study provides a pathological protagonist for pro-renin receptor dysregulation in pre-eclampsia, a condition characterised with a paradoxical variation in renin/pro-renin and pro-renin receptor concentrations. Apart from the high blood pressure development, it may be associated with the ubiquitous proteinuria and the insufficient trophoblast invasion that characterises early onset pre-eclampsia. This study confirms a role for the pro-renin receptor in pre-eclampsia development.

CHAPTER ONE

CHAPTER ONE

LITERATURE REVIEW

1.1 MATERNAL MORTALITY IN SOUTH AFRICA

In South Africa, maternal mortality and morbidity for the triennium 2008-2010, is predominantly attributed to non-pregnancy related infections (40%; mainly pneumonia, TB and HIV). These non-pregnancy infections are largely prevalent in ages between 25-40 years which relates to the HIV infection spread rate or pattern (SA Department of Health Report, 2012). Other factors that contribute to maternal deaths in South Africa, include hypertension, haemorrhage, ectopic pregnancy, miscarriage, pregnancy related sepsis and patient care in the hospitals. However, the major direct cause of maternal deaths is attributed to obstetric haemorrhage (14.1%) and hypertension (14%) (SA Department of Health Report, 2012).

The two major causes of death in women of reproductive age globally is HIV and AIDS (19%) and complications (15%) related to childbearing (World Health Organisation Report, 2012). Globally, sub-Saharan Africa is the region with the highest maternal mortality rate (596 per 100000 live births) and where half of all maternal deaths occur (Hogan *et al*., 2010). In 2011, 90% of the total number of pregnant women with HIV were found to reside in sub-Saharan Africa (UNAIDS, 2012b). The region is home to 69% of the 34 million people living with HIV globally (UNAIDS, 2012a). In South Africa, maternal deaths due to non-obstetric causes predominate (40%) among women with HIV infection (SA Department of Health Report, 2012). KwaZulu-Natal is the epicentre of this HIV pandemic. The relationship between anti-retroviral treatment (ART) and pre-eclampsia is conflicting, with some studies reporting an increase in pre-eclampsia with ART and others showing no difference in rates of pre-eclampsia development between pregnant women living with HIV on ART and HIV-negative women (Kourtis *et al*., 2006; Suy *et al*., 2006; Hall *et al*., 2013). In South Africa of the 14% of

women that die from hypertension in pregnancy, 83% is attributed to a condition called pre-eclampsia (SA Saving Mothers Report, Department of Health, 2012).

1.2 NORMAL PREGNANCY

During normal pregnancy changes in the cardiovascular system occur i.e. an increase in heart rate, plasma volume, cardiac output increase and also the activation of the renin-angiotensin aldosterone system (RAAS). These changes are essential for meeting the blood and nutrient demands of the growing fetus (Tanbe and Khalil 2010; Seki, 2014). The first stage of pregnancy includes extravillous cytotrophoblast invasion of the uterine spiral arteries within the decidua and myometrium. The physiological transformation of the spiral artery includes replacement of the muscular wall of the spiral artery with a fibrinoid material. This converts the spiral artery from a narrow calibre vessel to a wide bore flaccid conduit that produces a ten-fold increase in luminal diameter hence increasing blood flow, oxygen partial pressure and nutrients required to meet the growing needs of the baby (Seki, 2014).

Normal pregnancy is allied with major vascular remodelling of both uterine and systemic circulation in order to meet the metabolic demands of the mother and the growing fetus. Vascular changes are dependent on the changes in activity of vascular mediators released from the endothelium, vascular smooth muscle and extracellular matrix (Mistry *et al*., 2013). The spiral arteries of the uterus in the gravid state and placental development are incorporated into the uteroplacental unit forming the decidual vessels, which are the afferent vessels of the maternal intervillous blood flow (Mistry *et al*., 2013). If the nutritional demands of the fetus are not met, the growth of the fetus will be inadequate with resultant intrauterine growth retardation.

1.3 PRE-ECLAMPSIA

Pre-eclampsia is a hypertensive disorder unique to pregnancy in women diagnosed as sustained systolic blood pressure of greater than or equal to 140 mmHg and a diastolic blood pressure of 90 mmHg taken on two occasions at least 6 hours apart with a new onset proteinuria (1+ on a urine dipstick analysis) or >30 mg/dl urine protein concentration at two intervals at least 4 hours apart.

Pre-eclampsia has remained a significant public health threat in both developed and developing countries contributing to maternal and perinatal morbidity and mortality (McLure *et al*., 2009; Shah *et al*., 2009; WHO., 2011b). However, the impact of the disease is felt more severely in developing countries (Igberase and Ebeigbe, 2006), where, unlike other more prevalent causes of maternal mortality (such as haemorrhage and sepsis), medical interventions are ineffective due to late presentation of cases (Onakewhor and Gharoro, 2008; Moodley, 2011).

In pre-eclampsia, the physiological transformation of the spiral arteries is believed to be limited to the decidua due to insufficient trophoblast invasion. This consequentially results in a deficient oxygen and nutrient supply to the fetus (Naicker *et al*., 2003; Berard *et al*., 2013).

Although the aetiology of pre-eclampsia is uncertain (Tanbe and Khalil, 2010; Kuśmierska-Urban *et al*. 2013), a number of theories induced by lifestyle, genetic and biological factors have been put forward (Tanbe and Khalil, 2010; Mistry *et al*., 2013). Pre-eclampsia is mainly characterised by endothelial dysfunction, elevated inflammatory response and oxidative stress and activation of thrombosis (Mistry *et al.,* 2013). Reduction of uteroplacental perfusion pressure and placental hypoxia are well recognized features in the pre-eclampsia syndrome (Verdonk *et al*., 2014).

Pre-eclampsia development emanates from the resultant hypoxic state of the placenta which is believed to release circulating bioactive factors such as hypoxia inducible growth factors and soluble fms-like tyrosine kinase that suppress pro-angiogenic factors with consequential maternal vasoconstriction of systemic arteries (Tanbe and Khalil 2010; Govender *et al*., 2013; Verdonk *et al*., 2014). Treatment of pre-eclampsia is empirical; failure of the management of hypertension can lead to seizures, a condition referred to as eclampsia. There is no known cure for pre-eclampsia other than delivery of the placenta.

1.4 RENIN-ANGIOTENSIN ALDOSTERONE SYSTEM (RAAS)

The renin-angiotensin aldosterone system is an effective medium-term regulator of systemic blood pressure, fluid and electrolyte homeostasis. It is a cascade composed of various components that via a negative feedback becomes activated and functional (Seki, 2014). Components include pro-renin (a renin precursor), renin, angiotensinogen (AGT), angiotensin (Ang) I and II, and angiotensin-converting enzyme (ACE). Renin and pro-renin are released by the juxtaglomerular cells and synthesised from intracellular granules. The kidney activates pro-renin and only 25% of activated pro-renin is secreted as renin directly into circulation (Berard *et al.*, 2013).

Plasma renin carries out the conversion of angiotensinogen released by the liver to angiotensin I which has no known bioactivity (Berard *et al*., 2013). Angiotensin I is subsequently converted to angiotensin II, by ACE found mainly in the lungs. Angiotensin II is a potent vaso-active peptide that causes blood vessels to constrict, resulting in elevated blood pressure (Verdonk *et al*., 2014).

Angiotensin II also stimulates the secretion of the hormone aldosterone from the adrenal cortex. Aldosterone causes the tubules of the kidneys to increase the reabsorption of sodium and water into the blood. This increases the volume of fluid in the body, which also results in increase in blood

pressure (Seki, 2014). This increase in blood pressure causes an increase in blood volume and cardiac contraction strength. It is the increase in cardiac output as a result of the increased blood volume and cardiac contractility which brings about the increase in blood pressure (Berard *et al*., 2013). Figure 1.1 and Table 1.1 highlight the activation and functions of the different RAAS cascade respectively.

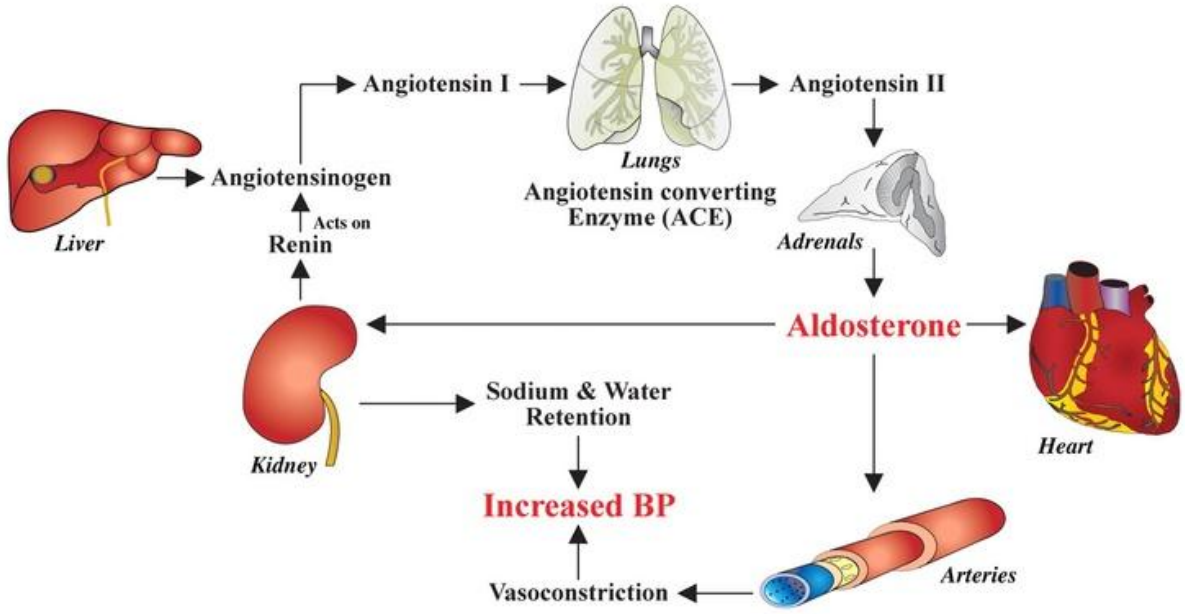

Figure 1.1: Cascade activation in renin-angiotensin aldosterone system.

http://pubs.rsna.org/journal/radiographics

Table I.I: Source and function of the components of the Renin-Angiotensin Aldosterone System.

Component	Source	Functions
Pro-renin receptor	Cleavage from furin	Activation of pro-renin and renin and acts as a V-ATPase
Pro-renin	Kidney and other extrarenal sites, constitutive release	Conversion of angiotensinogen to angiotensin I (when bound to the pro-renin receptor, functions as renin)
Renin	Kidney, activated from pro-renin via proconvertase, cathepsin B	Conversion of angiotensinogen to angiotensin I
Angiotensinogen	Liver, adipose tissue (in obesity)	Substrate for renin and pro-renin precursor of Ang I
Ang I	Angiotensinogen via renin or pro-renin.	Weak vasoconstrictor, pre-cursor of Ang II
Ang II	From angiotensin I via angiotensin converting enzyme	Potent vasoconstrictor, stimulate the secretion of aldosterone from the adrenal glands

1.4.1 Placental pro-renin

There are two different types of RAAS viz., circulatory and tissue specific RAAS. Tissue RAAS is regulated by the pro-renin receptor which also plays an important role in the assembly and functioning of H^+-ATPase (Watanabe *et al*., 2012). The placenta is a vital organ for normal healthy pregnancy and fetal growth. There are two types of placental RAAS, one occurring in the fetal placental compartment and the other in the maternal placental tissue (Kuśmierska-Urban *et al*., 2013; Yang *et al*., 2013). All the components of RAAS are found in the decidua and fetal placental tissues. In pregnancy, the high tissue RAAS activation arises from the high plasma pro-renin concentration (Jan Danser *et al*., 2007).

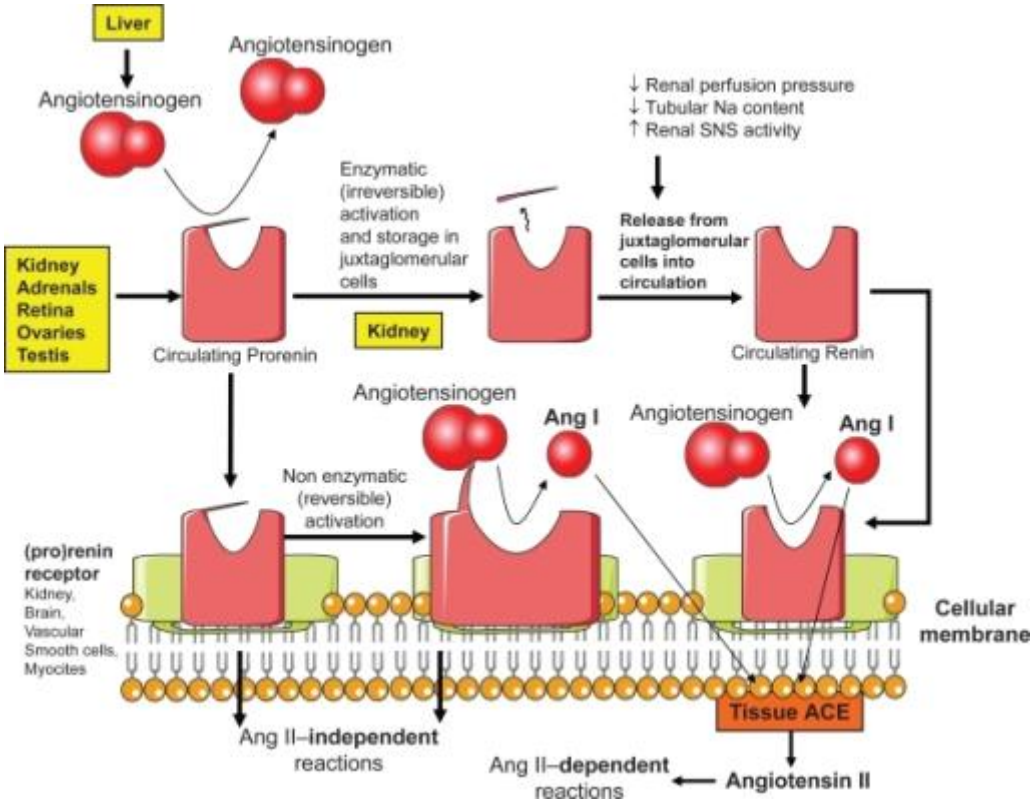

Figure 1.2: Overview of pro-renin, active renin and (pro)renin receptors. Proteolytic activation involves irreversible removal of the pro-segment by enzymes, while non-proteolytic activation is the result of reversible removal of the prosegment sequence by acidic pH or low temperature (Ichihara *et al*., 2004). (P)RR has recently been attributed for the non-proteolytic activation of pro-renin through protein-protein interactions (Verdecchia *et al*., 2008).

Pro-renin (PR) is known to be an inactive enzyme required for the production of active renin. The kidney produces both renin and pro-renin. However, a number of extrarenal tissues, including the adrenal, ovary, testis, placenta and retina can also produce pro-renin (Jan Danser *et al*., 2007; Berard *et al*., 2013). Other studies have indicated that the extrarenal sites of pro-renin production is important since the pro-renin levels in anephric subjects are approximately half the levels in normal subjects

(Morales *et al*., 2012). Pro-renin levels are normally higher than renin levels in plasma (Krop *et al*., 2013).

Activation of pro-renin occurs through the activity of cathepsin B or pro-convertase resulting in renin enzyme, via a proteolytic activation, or by a non-proteolytic activation that simply includes the binding of pro-renin to a pro-renin receptor (P)RR; (Fig 1.3; (Morales *et al*., 2012). These proteolytic activations occur exclusively in the kidney whilst the non-proteolytic activation is a reversible process which can be induced at low pH or low temperatures (Nurun *et al*., 2007; Cruciat *et al*., 2010).

Inactivated pro-renin has a low intrinsic activity of 3% compared to the activity of fully activated pro-renin (Krop *et al*., 2013). Its intrinsic activity is accredited to a slight unfolding of its pro-segment (Morales *et al*., 2012). Human renin and pro-renin are glycosylated, and a variable proportion of renin and pro-renin have mannose-6-phosphate (M6P) residues that bind to the M6P–insulin-like growth factor II receptor (Jan Danser *et al*., 2007).

Pro-renin was initially thought to be inactive; however, its secretion and possible functioning seems to be elevated in various pathologies (Berard *et al*., 2013; Seki 2014). The cause of this increase is unclear but reflects increased pro-renin gene expression and/or a decrease in pro-renin clearance (Cruciat *et al*., 2010).

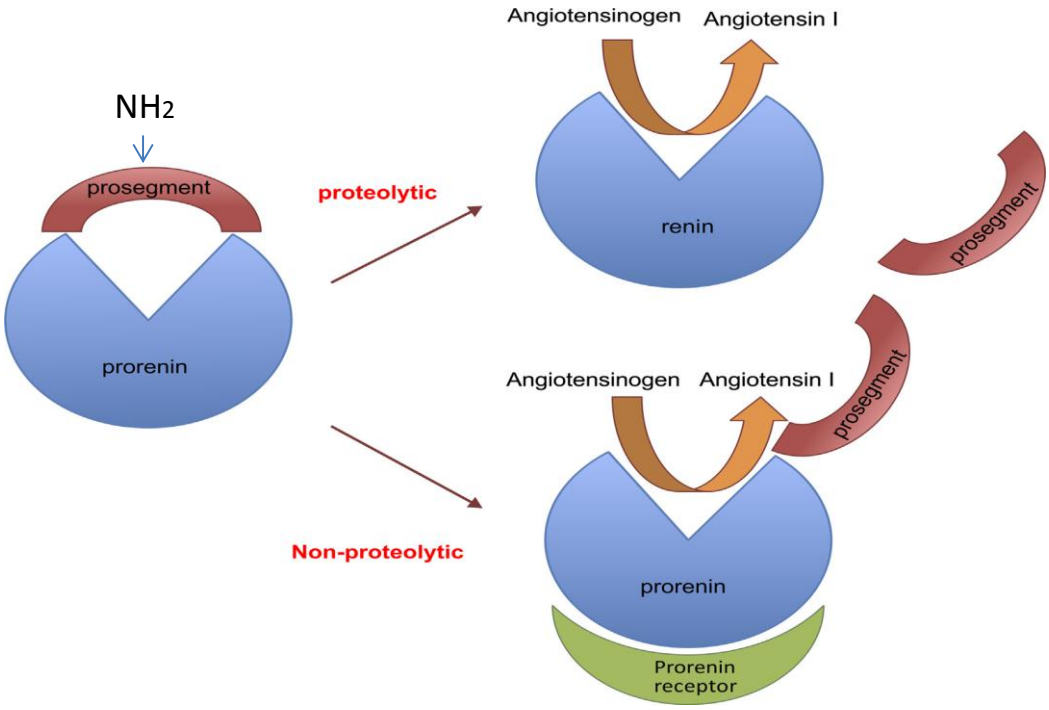

Figure 1.3: The proteolytic and non-proteolytic activation of pro-renin (Seki, 2014).

1.4.2. Pro-renin structure

Pro-renin has an amino-terminal (43 amino acid sequence) pro-sequence (Ω-shaped) that covers the cleft between the 2 lobes of the enzyme. This prevents access to the active site by its substrate, angiotensinogen (Morales *et al*., 2012). This amino acid sequence is located on the N-terminal of a renin molecule (Morales *et al*., 2012).

1.4.3 Pro-renin receptor

The pro-renin receptor (P)RR was first identified in human mesangial cells and cloned (Cousin *et al*., 2009). Pro-renin receptor is a multifunctional protein that is involved in the control of intracellular and extracellular pH via its interaction with the V-ATPase/MAP kinase signalling and Wnt/beta catenin signalling pathways (Cruciat *et al*., 2010; Nguyen and Muller, 2010). Pro-renin receptor also

has an ability to activate pro-renin to form renin and then Ang I from AGT and enhances the affinity of renin for AGT (Cruciat *et al*., 2010). Oxidized AGT forms a sulfhydryl bridge (cys-18-cys 138) that has more than a 10-fold increase in affinity for (P)RR bound renin (Nguyen and Muller, 2010).

The pro-renin receptor is a newly identified regulator of RAAS that is essential for blood pressure and electrolyte balance. It is a cell surface receptor for pro-renin and renin. (P)RR binds renin and its inactive pro-renin which also triggers the phosphorylation of extracellular signal related protein kinase. This binding causes an increase in the catalytic activity of renin and causes the non-proteolytic activation of pro-renin that will lead to the generation of angiotensin I (Krop *et al*., 2013).

Pro-renin receptor is largely located intra-cellularly with an intracellular vascular H-ATPase function. Pro-renin receptor is generated via the cleavage of the full length form by the enzyme furin or by ADAM19 in the golgi apparatus (Cruciat *et al*., 2010; Krop *et al*., 2013). Furin has an ubiquitous expression in adults, unlike other pro-convertases, however, it cannot be excluded that other pro-convertases may also be involved in (P)RR processing (Cousin *et al*., 2009). Pro-renin receptor is detected within the Golgi apparatus, and is probably cleaved in the trans-Golgi to generate a soluble form (Cousin *et al*., 2009).

The molecular forms of (P)RR have been identified as a full-length form, a truncated transmembrane form that has the C terminal region (M8-9 fragment) and the most recently identified truncated soluble form that has the N-terminal region (Krop *et al*., 2013; Suzuki-Nakagawa *et al*., 2014). The C-terminus region is said to be capable of the highly conserved cellular function, which might be related to the V-ATPase (Cousin *et al*., 2009). The extracellular domain is responsible for binding with renin and pro-renin, whereas the transmembrane and cytosolic domains are associated with V-ATPase (Cousin *et al*., 2009).

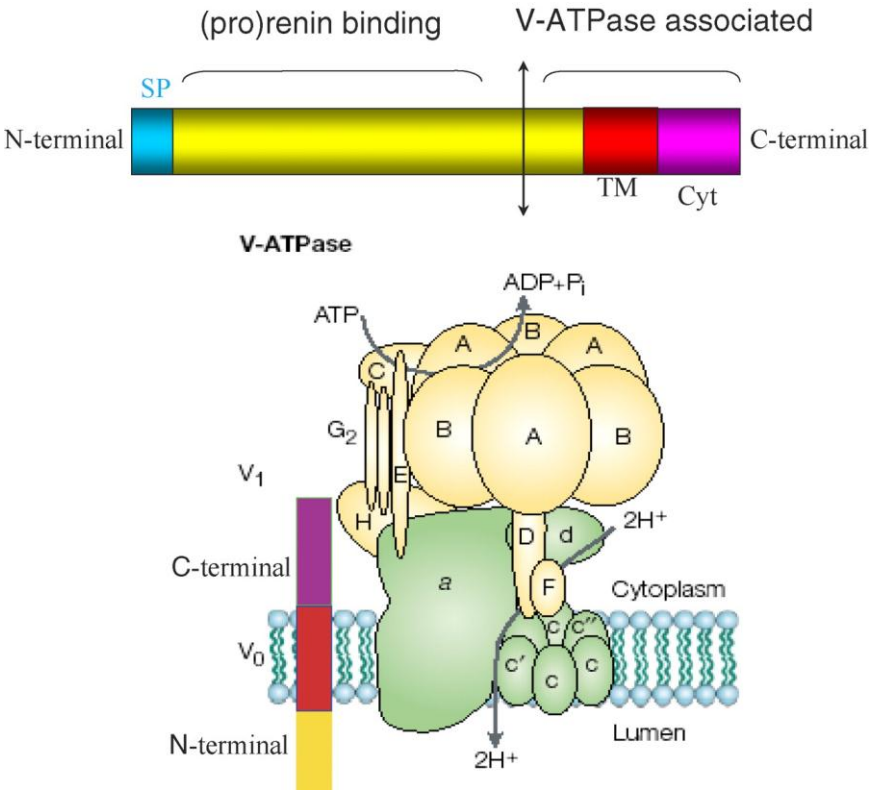

Figure 1.4: The diagram showing the structure of (P)RR (top) and its association with V-ATPase (bottom). Abbreviation: SP, signal peptide; TM, transmembrane domain; and Cyt, cytoplasmic domain. Adapted from Nishi and Forgac (2002).

The pro-renin receptor is highly expressed in the brain, kidney, heart and placenta (Reudelhuber, 2010). Pro-renin receptor is also expressed in mesangial cells, distal and collecting duct cells of the kidney and at a lower level in the liver, pancreas and human retina (Pringle *et al*., 2011). Experimental models suggest that the levels and activation of (P)RR increase in disease, especially in patients with high blood pressure, cardiac fibrosis, and diabetic nephropathy (Nguyen, 2011; Berard *et al*., 2013).

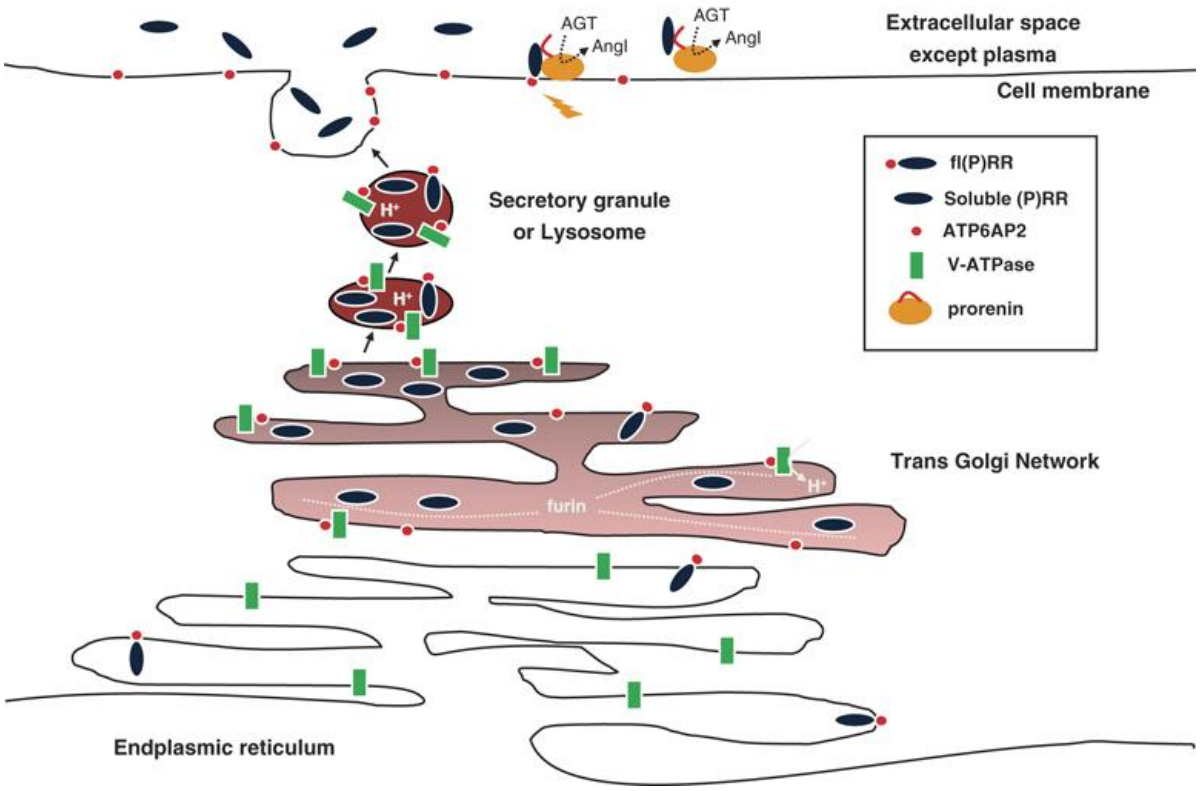

Figure 1.5: Schematic diagram showing the processing of full length pro-renin receptor to soluble pro-renin receptor and ATPase 6 associated protein (Ichihara *et al*., 2010).

These different types of (P)RR can bind to renin and pro-renin and stimulate the activation of tissue RAAS. Pro-renin receptor over expression is associated with slow progression of hypertension and activation of tissue RAAS whilst the full length and truncated (P)RR forms are said to also pose a potential effect in causing the dysfunction of V-ATPase (Watanabe *et al*., 2012).

The (P)RR gene is a highly conserved protein amongst a number of species. Its multi-species protein sequence comparison has revealed the existence of homologues to the human receptor in a variety of species, such as rat, mouse, chicken, frog, zebra fish, mosquito, drosophila, or even in species as remotely related to humans as *Caenorhabditis elegans* (Cousin *et al*., 2009).

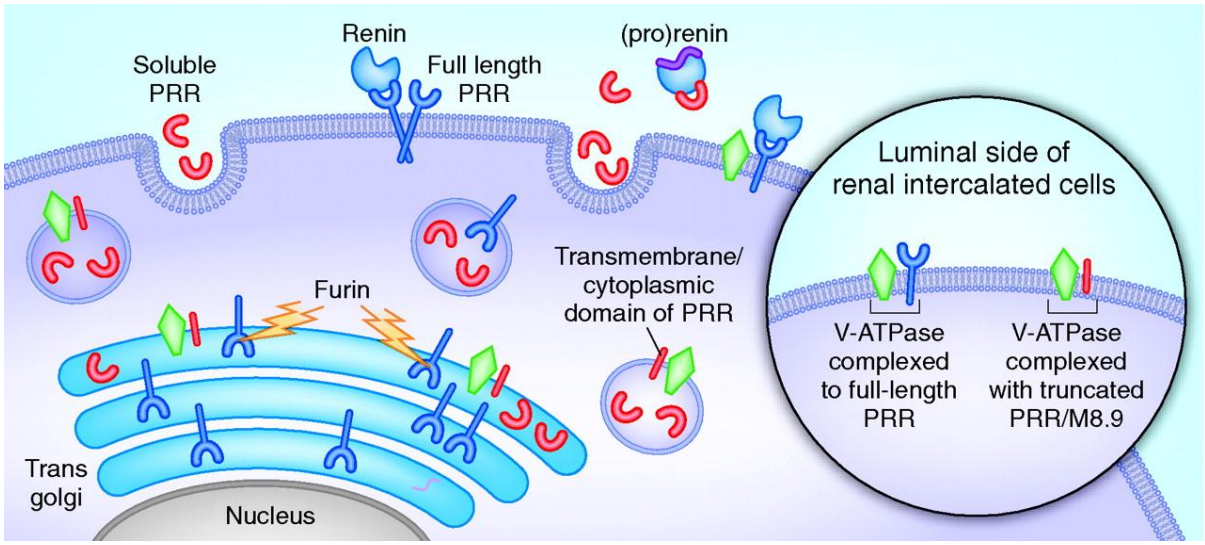

Figure 1.6: The intracellular processing of (P)RR. Part of the receptor is cleaved in the trans-golgi to produce soluble (P)RR and the other part associates with V-ATPase and remains in the plasma membrane (Nguyen and Muller, 2010).

In humans, the (P)RR gene is located on the X chromosome at locus *p11.4* (Nguyen and Muller, 2010). Pro-renin receptor messenger RNA is 2034 base pairs long and there is no alternative splicing product present (Cruciat *et al*., 2010; Nguyen and Muller, 2010). The sequence alignment shows that the highest degree of homology encompasses the transmembrane and cytoplasmic regions of the protein corresponding to ATP6AP2, discovered in chromaffin granules and the M8.9, the fragment of (P)RR that was reported to co- precipitate with the membrane sector V-ATPase (Cousin *et al*., 2009).

In addition, the extracellular domain displays high amino acid sequence identity exclusively in invertebrates. Because RAAS emerged relatively late in evolution, the ATP6AP2 protein may have acquired renin and pro-renin binding properties.

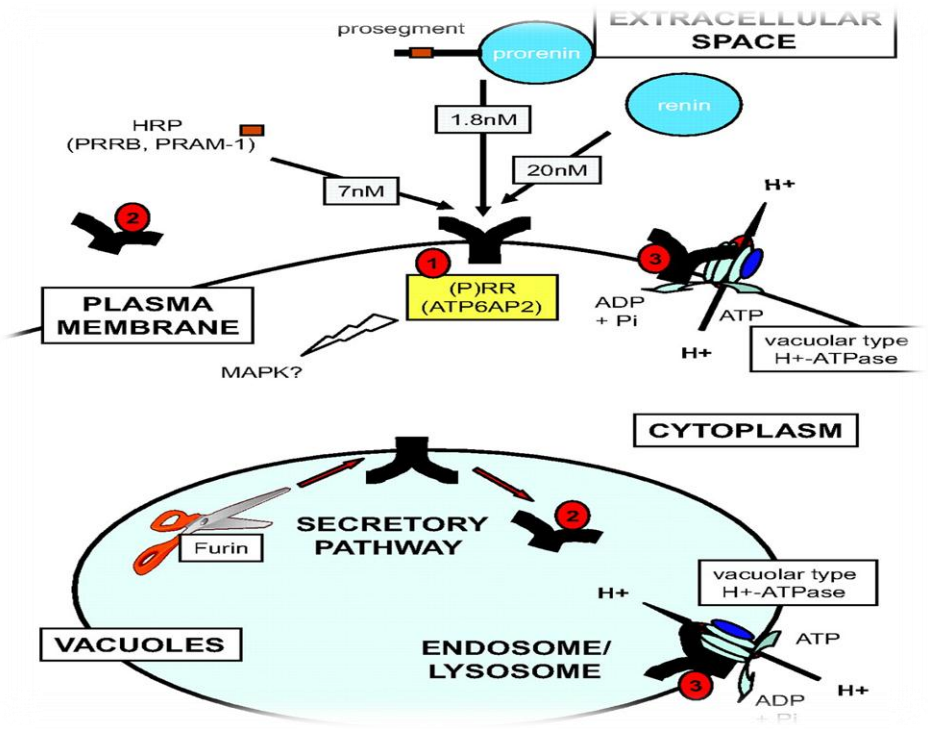

Figure 1.7: Binding of pro-renin receptor with renin and pro-renin with the post binding pathways (Reudelhuber, 2010).

1.4.4 Pro-renin receptor structure

Several studies have indicated that plasma membrane (P)RR occurs in a dimer form (Cousin *et al*., 2009). However, the fact that (P)RR is detected as a 35-kDa band in Western blots indicates that (P)RR dimer on the cell membrane is not covalently bound.

Pro-renin is a single peptide that is composed of a large extracellular domain, a single transmembrane domain and a short cytoplasmic domain from the N terminal to the C terminal (Cruciat *et al*., 2010; Krop *et al*., 2013).

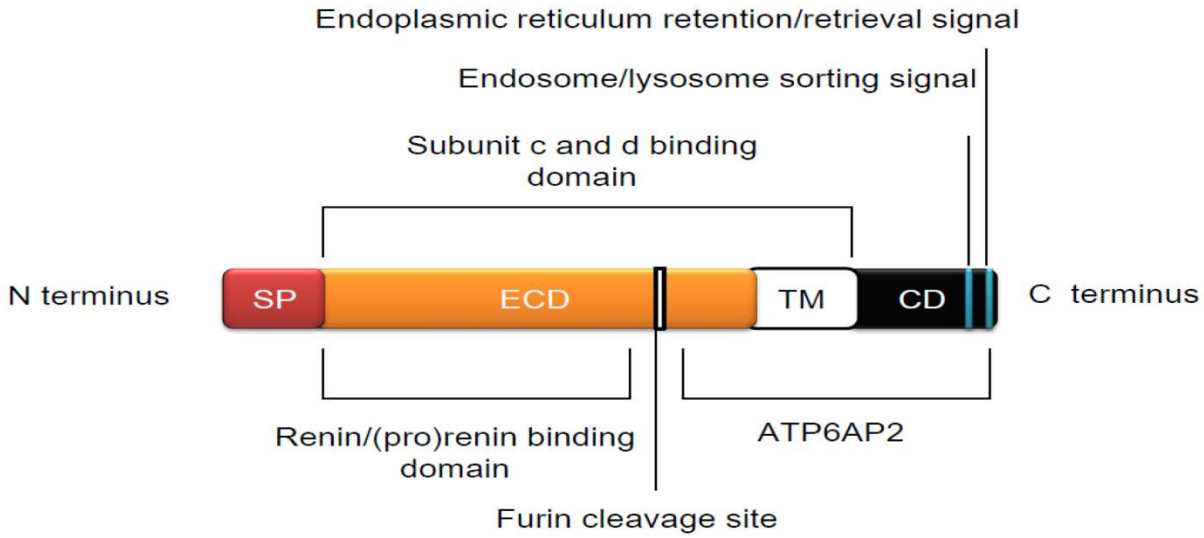

Figure 1.8: Structure of the pro-renin receptor (Ichihara and Keio, 2012).

1.5 ASSOCIATION BETWEEN PRE-ECLAMPSIA, PRO-RENIN AND ITS RECEPTOR

The role of pro-renin and its receptor in cells and in biological fluids include the activation of vacuolar proton-ATPase (intracellular pH enzyme) and a mitogen-activated protein kinase p44/42 (that induces hypertension and glomerulosclerosis) (Berard *et al*., 2013). Pro-renin has pathological effect both inside and outside the renin-angiotensin system. Despite the cleavage of the pro-segment, pro-renin can gain the functional ability of renin and cause the production of angiotensin I which generates angiotensin II (Cruciat *et al*., 2010). This production reinforces the pro-fibrotic and pro-inflammatory effect of angiotensin II (Pringle *et al*., 2011).

Evidence that PR, (P)RR and renin is believed to play a direct role in vascular pathologies are:

- elevations of circulating pro-renin is associated with diabetic microvascular disease (Luetscher *et al.*, 1985).

- transgenic rats engineered to have a 400-fold increase in circulating pro-renin develop severe cardiac remodelling and renal lesions in the absence of hypertension (Veniant *et al.*, 1996) raising the possibility that pro-renin is not only associated with tissue damage but that it may even be responsible for the damage by a mechanism that did not require Ang II generation.
- (pro)renin receptor binds both pro-renin and renin with nanomolar affinity (Nabi *et al*., 2006), causing an unfolding of pro-renin rendering it capable of contributing to local Ang II generation, and triggers several mitogen-activated protein kinase (MAPK) signalling pathways (Huang *et al*., 2006; Saris *et al*., 2006), on pro-renin and renin binding. Because this MAPK stimulation occurs in the presence of RAAS inhibitors, the triggered signalling appears to be independent of the angiotensin-generating enzymatic activity of pro-renin and renin (Feldt *et al*., 2008).
- inhibiting the binding of renin and pro-renin to (P)RR with a competing peptide called handle region peptide (HRP; later also called pro-renin receptor blocker or (P)RRB; reduces cardiac hypertrophy and fibrosis in hypertensive rats without reducing blood pressure, diabetic glomerulosclerosis and proteinuria in rats and in mice in which the Ang II type 1 (AT1)α receptor had been inactivated (Ichihara *et al*., 2006a and b).

This data has led to a model in which renin and pro-renin, through their interaction with (P)RR, stimulate a signal other than Ang II that promotes microvascular damage. Therefore, PR and (P)RR levels in pre-eclampsia requires evaluation. New and more effective treatment developments such as blocking their action will prevent the characteristic vasoconstriction and endotheliosis that characterises pre-eclampsia.

1.6 AIMS/HYPOTHESIS

Aim: To examine the expression of circulating pro-renin (PR) and its receptor (P)RR in HIV associated normotensive pregnant and pre-eclamptic women.

Objectives:

- To compare the plasma expression of pro-renin and its receptor between:
  - normotensive and pre-eclamptic patients based on HIV infection
  - early-onset and late-onset HIV associated pre-eclamptic women

Hypothesis:

- The level of pro-renin and its receptor will remain the same across:
  - normotensive and pre-eclamptic pregnancies irrespective of the HIV status.
  - the early-onset and the late-onset pre-eclamptic pregnancy groups.

CHAPTER TWO

CHAPTER TWO

MATERIALS AND METHODS

2.1 ETHICAL APPROVAL

This study utilized plasma obtained retrospectively (BE 027/13), stored at the Optics and Imaging Centre, Doris Duke Medical Institute, College of Health Sciences, University of KwaZulu-Natal (UKZN) which received institutional ethical clearance (BE 320/14). Informed consent was obtained from all patients participating in the study.

2.2 STUDY POPULATION

The study population consisted of pregnant normotensive and pre-eclamptic African women attending the ante-natal clinic at Prince Mshiyeni Memorial Hospital in the Umlazi, Durban metropolitan area. This is a district hospital servicing at least sixteen clinics in the Durban South region of KwaZulu-Natal, South Africa. These patients were recruited by a research nurse who informed them about the procedures and their role in the study. Demographic profile of the patients was obtained from patient clinical/medical charts.

2.2.1 Selection criteria

Each group and subgroup was matched for gestational age, HIV status. All participants in our setting were on full HAART from 28 weeks of gestation with a single dose of nevirapine in labour if their CD4 count were less than 350 units.

Inclusion criteria were:

- Women who were 18 years of age and older.

- Pre-eclampsia was described as the new onset hypertension (BP>140/90 mmHg) and proteinuria (1+ on a dipstick) after 20 weeks of gestation.
- HIV tests were conducted as a standard of care in the ante-natal clinic.

Exclusion criteria for the pre-eclamptic group were:

- Chorioamnionitis, chronic hypertension, eclampsia and placental related complications e.g., abruption placentae;
- Intrauterine death, pre-gestational diabetes, gestational diabetes and chronic renal disease;
- Systemic lupus erythematous, sickle cell disease and anti-phospholipid antibody syndrome;
- Thyroid disease, cardiac disease and active asthma requiring medication during pregnancy;
- Pre-existing seizure disorder and infection with human immunodeficiency virus.

2.2.2 Sample size

A sample size of 90 participants, determined by the institutional biostatistician, was considered sufficient to determine a statistical significance between the different variables being measured. The sample size for each group was decided from examination of previous studies.

There were 6 groups, 15 samples per group.

The sample was divided into

a) control group (normotensive pregnant women; n=30);
b) experimental group (early- and late-onset pre-eclamptic women; n=60).

Both groups were further stratified according to HIV status. These groups are schematically depicted in Figure 2.1.

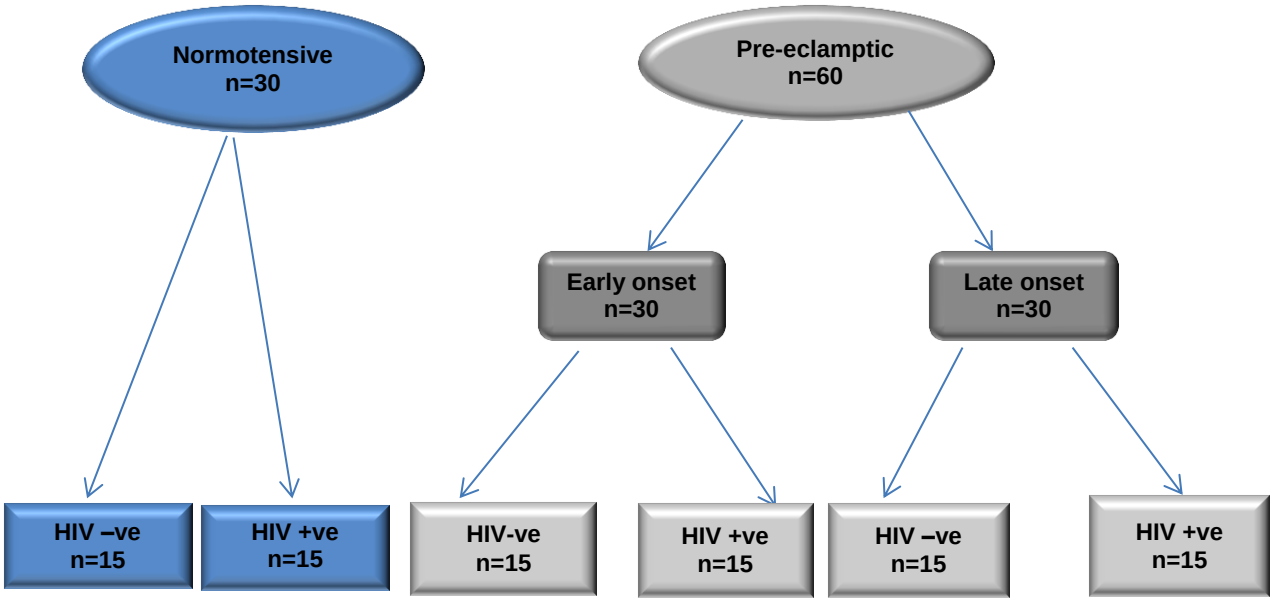

Figure 2.1: Schematic representation of study groups based on pregnancy type and HIV status

2.3 METHODS

2.3.1 Plasma separation from whole blood

Prior to archival, plasma was separated from blood samples and stored at -80°C until required.

2.3.2 The principle of enzyme-linked immunosorbent assay (ELISA) techniques

The ELISA experiment was carried out according to the manufacturer's instructions (Figure 2.2). The levels of pro-renin and its receptor expression were determined by use of the following commercial kits:

- pro-renin human ELISA immunoassay kit (Abcam, Biocom Biotech, Johannesburg)
- pro-renin receptor ELISA kit (Humor Diagnostic, Johannesburg).

Both kits were based on a quantitative sandwich enzyme immunoassay technique. Antigens from the sample are attached to a surface. Then, a specific antibody is applied over the surface so it can bind to the antigen. This antibody is linked to an enzyme, and, in the final step, a substance containing the enzyme's substrate is added. The subsequent reaction produces a detectable signal, most commonly a color change in the substrate.

Performing an ELISA involves at least one antibody with specificity for a particular antigen. The sample with an unknown amount of antigen is immobilized on a solid microtiter plate specifically via capture by another antibody specific to the same antigen, in a "sandwich" ELISA. After the antigen is immobilized, the detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody that is linked to an enzyme through bioconjugation. Between each step, the plate is typically washed with a mild detergent solution to remove any proteins or antibodies that are non-specifically bound. After the final wash step, the plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen in the sample.

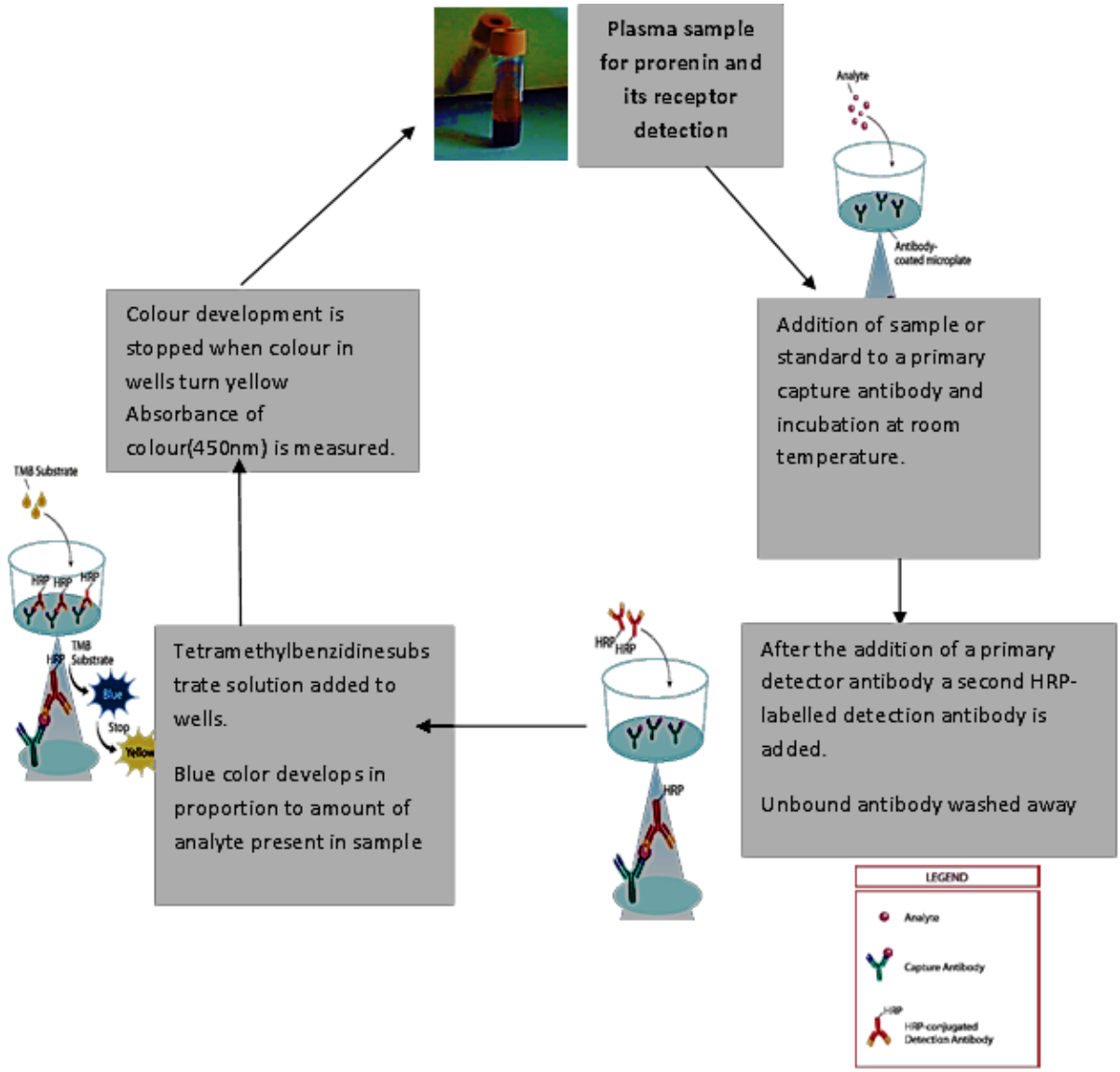

Figure 2.2: Schematic representation of ELISA technique www.rndsystems.com.

2.3.3 Procedure for the estimation of Pro-renin

The standards, plasma samples and blanks, 100 µl each were added in duplicate to the wells and the plate was incubated on a shaker at 300 rpm for 30 minutes at room temperature. All wells were washed thrice with 300 µl of wash buffer. Pro-renin primary antibody, 100 µl was added to each well and the plate incubated on a shaker at 300 rpm for 30 minutes at room temperature. All wells were washed three times with 300 µl of 1x wash buffer and 100 µl of 1x HRP antibody was added to each

well. The plate incubated on a shaker at 300 rpm for 30 minutes at room temperature .and all wells were washed three times with 300 µl of 1x wash buffer. TMB substrate solution, 100 µl was added to each well and the plate was incubated at 300 rpm for 10 min at room temperature. All wells changed from colourless to different shades of blue, thereafter wells turned yellow when 1N HCL stop solution was added. The plate was measured at 450 nm within 30 minutes of adding the stop solution with the VersaMax™ ELISA Microplate Reader (Sunnyvale, CA 94089 USA).

2.3.4 Procedure for the estimation of Pro-renin receptor

Plasma samples were diluted (1:20) with EIA buffer. The standards, samples and reagent blank (EIA buffer), 100 µl each were added in duplicate to the wells and the plate was covered and incubated overnight at 4°C. All wells were washed four times with wash buffer, thereafter 100 µl of labelled antibody solution was added to each well. The plate was incubated for 60 min at 4°C and then washed five times with wash buffer. The chromogen (100 µl) was added to each well and the plate incubated for 30 min at room temperature in the dark. All wells turned blue and then yellow when 100 µl of stop solution was added to each well. The plate was measured at 450 nm within 30 minutes of adding the stop solution with the ELx800 Absorbance Reader (Winooski, VT 05404, USA).

2.4 ANALYSES

Absorbance was read at 450 nm. The mean of duplicate standards and samples were calculated and a standard curve was plotted. The concentration of pro-renin and its receptor in the samples under study were extracted from the standard curve. The data was imported into an Excel spreadsheet and analysed.

2.5 STATISTICAL ANALYSIS

IBM SPSS Statistics Version 22 was used to analyze the data. Descriptive statistics for continuous data is presented as mean ± standard deviation. Both parametric and non-parametric tests were used to analyze clinical data. To determine the statistical differences between HIV positive and negative groups, pregnancy groups (normotensive versus early-onset pre-eclampsia versus late-onset pre-eclampsia) and across all 6 study groups (normotensive HIV negative versus normotensive HIV positive versus early-onset pre-eclamptic HIV positive versus early-onset pre-eclamptic HIV negative versus late-onset HIV positive versus late-onset HIV negative) the Mann Whitney U and Kruskal-Wallis tests were used. Spearman rank correlation was used to determine the correlation between prorenin and its precursor and specific maternal demographics. Differences at $p<0.05$ were considered statistically significant.

CHAPTER THREE

CHAPTER 3

RESULTS

A total of 87 patients which comprised normotensive pregnant women (n=30; 34%) and pre-eclamptic (n=57; 66%) were studied. The pre-eclamptic group was further stratified into early-onset pre-eclampsia (EOPE; n=29; 51%) and late-onset pre-eclampsia (LOPE; n=28; 49%). Each group included HIV positive (HIV+) and negative (HIV-) women.

3.1 CLINICAL DEMOGRAPHICS ACROSS ALL STUDY COHORTS

The clinical characteristics of the study participants are shown in Table 3.1.

Table 3.1 Clinical characteristics across study groups

CLINICAL DATA	Normotensive HIV-ve n=15	Normotensive HIV+ve n=15	EOPE HIV-ve n=15	EOPE HIV+ve n=15	LOPE HIV-ve n=15	LOPE HIV+ve n=15
Maternal age (yrs)	25.7±6.4	27.1±5.5	26.7±5.7	31.4±5.2	22.9±5.5	29.5±6
Systolic (mmHg)	107.7±18.1	109.3±12.1	155.9±9.6	160.9±9.1	152.9±10.8	156.7±16.6
Diastolic (mmHg)	64.2±10.3	66.5±9.7	102.7±8.1	107.4±11.5	102.2±7.2	97.3±5.2
Maternal weight (Kg)	62.5±9.2	65.2±10.9	75.8±13.7	73.6±14.6	70.0±19.1	87.6±23.9
Gestational age (weeks)	38.0±2.4	37.3±1.3	28.4±3.7	25.1±4.1	36.5±2.2	36.6±2.0
Gravidity	1.9±3.7	2.4±1.5	1.9±1.3	2.8±1.7	1.5±0.8	2.5±0.9

3.1.1 Maternal age

The mean age ± SD of the total study population was 27±6 yrs (Table 3.1). There was a significant difference in maternal age (U=560, p=0.001; Mann-Whitney U test) between HIV positive and negative groups regardless of pregnancy type (Figure 3.1). Maternal age in the HIV positive group (mean rank=53.18) was higher than the HIV negative group (mean rank=35.43).

The distribution of maternal age was the same across pregnancy types [$\chi^2(2)=3.946$, p=0.139; Kruskal-Wallis test]. However, there was a significant difference in maternal age across all 6 study groups [$\chi^2(5)=17.527$, p=0.004]. Maternal age was higher in the HIV positive early- and late-onset pre-eclamptic groups compared to the HIV positive normotensive group and all HIV negative groups (Figure 3.1).

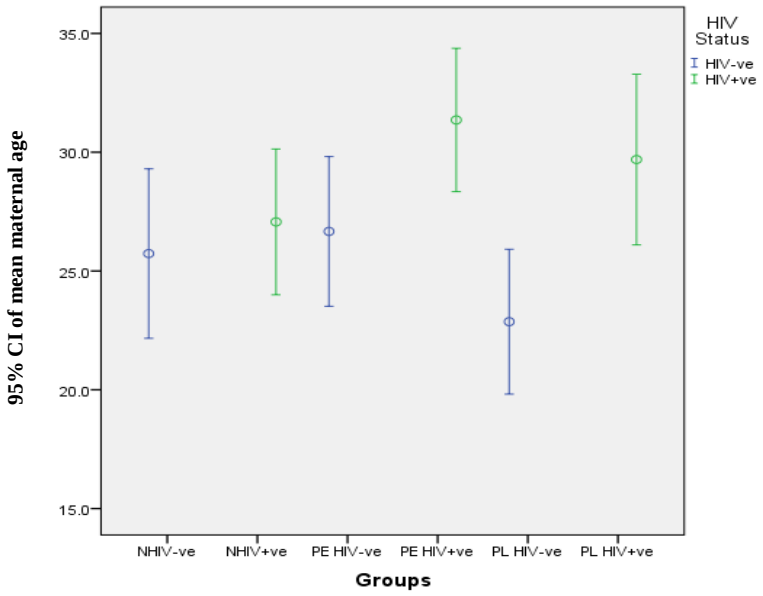

Figure 3.1: Maternal age across all 6 study groups

3.1.2 Maternal blood pressure

The mean systolic and diastolic blood pressure ± SD for each group is outlined in Table 3.1. The mean systolic and diastolic blood pressure ± SD of the total study population was 139.94 ± 26.34 and 89.69 ± 20.00 mmHg. Based on HIV status there was no significant difference in systolic (U=874, p=0.546) and diastolic blood pressure (U=917.5, p=0.815).The systolic [$\chi^2(2)=59.37$, $p.<0.001$] and diastolic blood pressures [$\chi^2(2)=59.74$, $p<0.001$] were significantly different in all pregnancy types and all 6 study groups [$\chi^2(5)=60.18$, $p=<0.001$; $\chi^2(5)=62.08$, $p<0.001$; Kruskal-Wallis test respectively]. The diastolic and systolic blood pressures were higher in the early onset (mean rank=62.36 and mean rank=63.24 respectively) and late onset (mean rank=55.52 and mean rank=54.52 respectively) pre-eclamptic groups compared to the normotensive group (mean ranks=15.50 and 15.58 respectively).

3.1.3 Maternal weight

The mean maternal weight ± SD for each group is outlined in Table 3.1. The mean maternal weight ± SD of the total study population was 72.09 ± 17.31 kg. There was no significant difference in maternal weight (U=777, p=0.153; Mann-Whitney U test) between the HIV positive and negative groups regardless of pregnancy types. Maternal weight was significantly different across pregnancy types [$\chi^2(2)=11.386$, p=0.003] and amongst all 6 study groups [$\chi^2(2)=18.075$, p=0.003; Mann-Whitney U test; Table 3.1]. Maternal weight was higher in the pre-eclamptic groups (early onset mean rank=51.02 and late onset mean rank=50.21) compared to the normotensive group (mean rank=31.42). In the late onset pre-eclamptic HIV positive group the maternal weight was higher (mean rank=62.54) compared to the HIV negative group (mean rank=39.53) and also to the early onset HIV positive (mean rank=48.39) and negative groups (mean rank=53.47) (Figure 3.2).

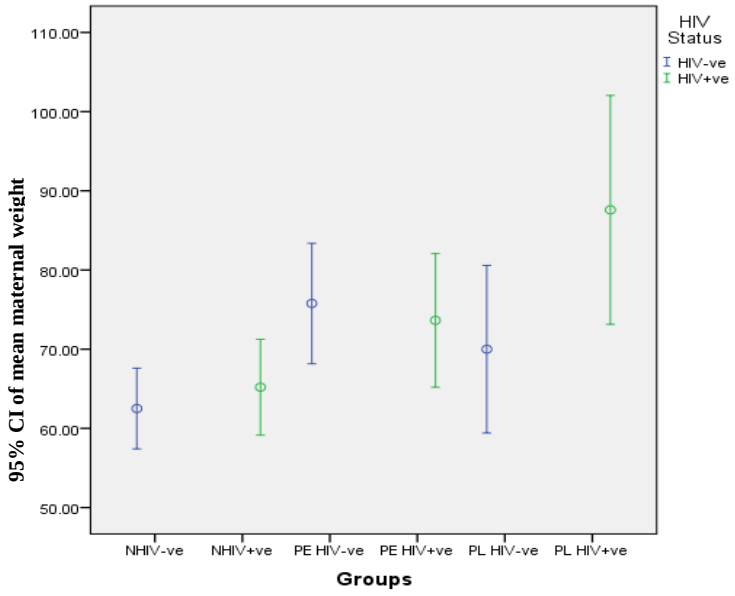

Figure 3.2: Maternal weight across all 6 study groups

3.1.4 Gestational age

The mean gestational age ± SD for each group is outlined in Table 3.1. The mean gestational age ± SD of the total study population was 33.68 ± 5.68 weeks. There was no significant difference in gestational age (U=866.5, p=0.504) between HIV positive and negative groups regardless of pregnancy type. The distribution of gestational age was different across all pregnancy types [$\chi^2(2)=59.482$, $p<0.001$] and all 6 study groups [$\chi^2(2)=60.438$, $p<0.001$; Kruskal-Wallis test]. Gestational age was higher in the normotensive (mean rank=62.90) and late onset pre-eclamptic (mean rank=53.75) groups compared to the early onset pre-eclamptic group (mean rank=15.03). Also gestational age in the HIV positive early onset pre-eclamptic group (mean rank=11.43) was higher than the HIV negative group (mean rank=18.40; Figure 3.3).

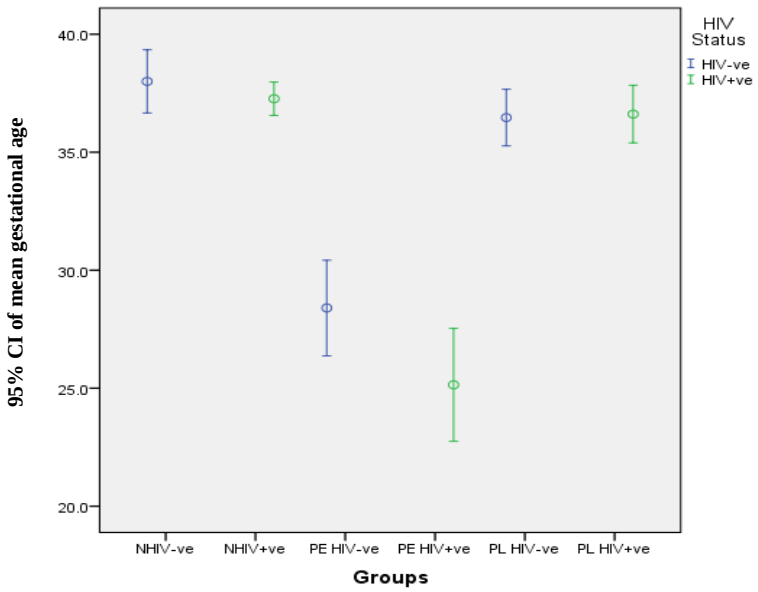

Figure 3.3: Gestational age across all 6 study groups

3.1.5 Gravidity

The mean gravidity ± SD for each group is outlined in Table 3.1. The mean gravidity ± SD of the total study population was 2±1. The Mann-Whitney U test showed that there was a significant difference in gravidity (U=606, p=0.003) between HIV positive and negative groups regardless of pregnancy type. The Kruskal-Wallis test showed that the distribution of gravidity was the same across all pregnancy types [$\chi^2(2)=0.801$, p=0.670], however there was a significant difference across all 6 study groups [$\chi^2(2)=12.98$, p=0.024]. Gravidity was higher in the normotensive HIV negative group (mean rank=42.50) compared to the early (mean rank=38.13) and late onset pre-eclamptic HIV negative groups (mean ranks=28.77; Figure 3.4).

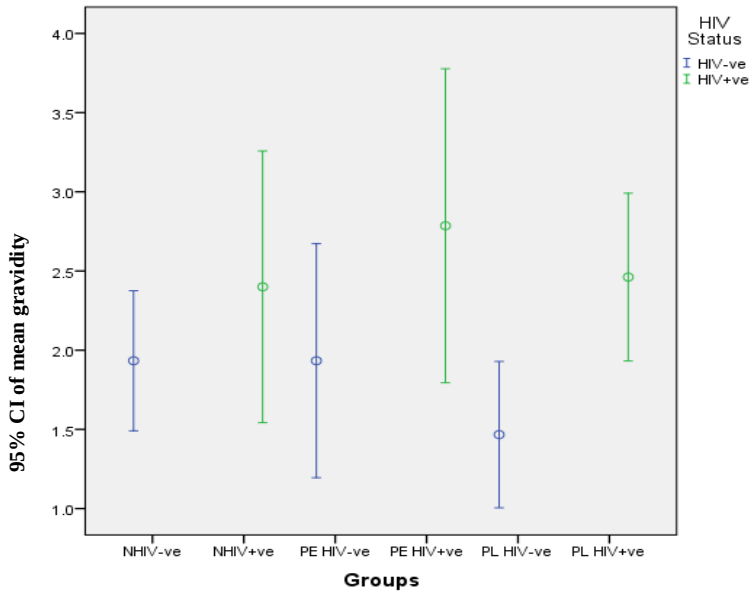

Figure 3.4: Gravidity across all 6 study groups

3.2 PRO-RENIN LEVELS

The mean PR ± SD for each group is outlined in Table 3.1. The mean PR±SD of the study population was 3.98±2.01ng/ml.

PR was lower in the HIV positive group (3.86 ng/ml) compared to the HIV negative group (4.09 ng/ml). There was no significant difference in PR [t(83)=0.527, *p*=0.599] between HIV positive *versus* the HIV negative group regardless of pregnancy type. There was no effect of pregnancy type (Normotensive *vs* EOPE *vs* LOPE; Normotensive *vs* PE) on PR levels [F(2)=2.01, *p*=0.140; *p*=0.073] and amongst all 6 study groups [F(5)=1.08, *p*=0.379; Fig 3.5]. There was no difference in PR between the EOPE and LOPE groups (*p*=0.409) as well as normotensive vs LOPE (*p*=0.273; Fig 3.6). However there was a significant difference between normotensive vs EOPE (*p*=0.039; Fig 3.6).

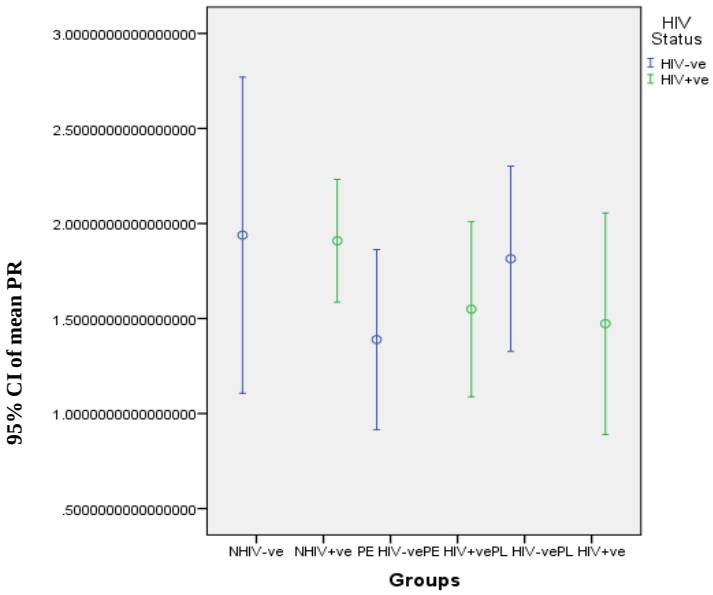

Figure 3.5 Pro-renin concentrations (ng/ml) across all 6 study groups

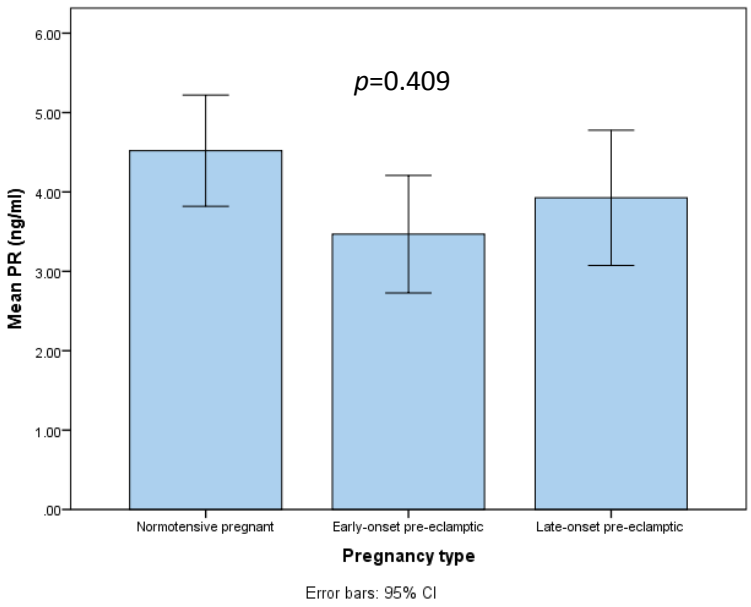

Figure 3.6 Pro-renin concentrations (ng/ml) across all pregnancy types

3.3 PRO-RENIN RECEPTOR LEVELS

The mean (P)RR ± SD for each group is outlined in Table 3.1. The mean (P)RR ± SD of the total study population was 1.60±0.95ng/ml.

The Mann-Whitney U test showed that there was no significant difference in (P)RR (U=927873, p=0.541) between HIV positive (mean rank=42.29) and HIV negative (mean rank=46.60) groups regardless of the pregnancy type as well as across all pregnancy types [$\chi^2(2)=5.803$, p=0.055; Kruskal-Wallis test]. The levels were lower in the early onset pre-eclamptic group (mean rank=34.88) compared to the normotensive (mean rank=47.40) and late onset pre-eclamptic group (mean rank=49.80). However, there was no significant difference in (P)RR across all 6 study groups [$\chi^2(5)=7.251$, p=0.203; Fig 3.7]. There was no difference between normotensive and pre-eclamptic (p=0.362). There was no difference between normotensive and EOPE (p=0.074) and also between normotensive and LOPE (p=0.803). However there was significant difference between EOPE *vs* LOPE (p=0.019; Fig 3.8).

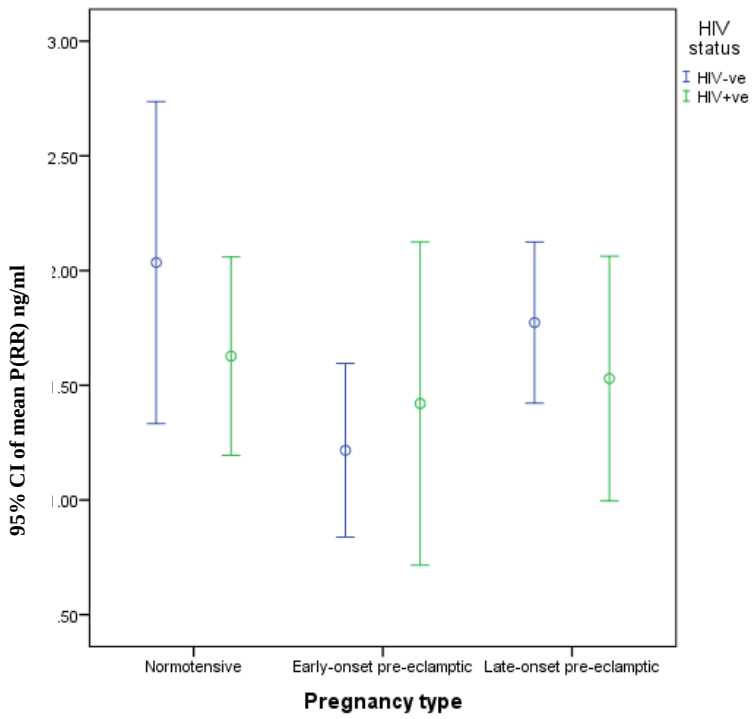

Figure 3.7 Pro-renin receptor concentrations (ng/ml) across all 6 study groups

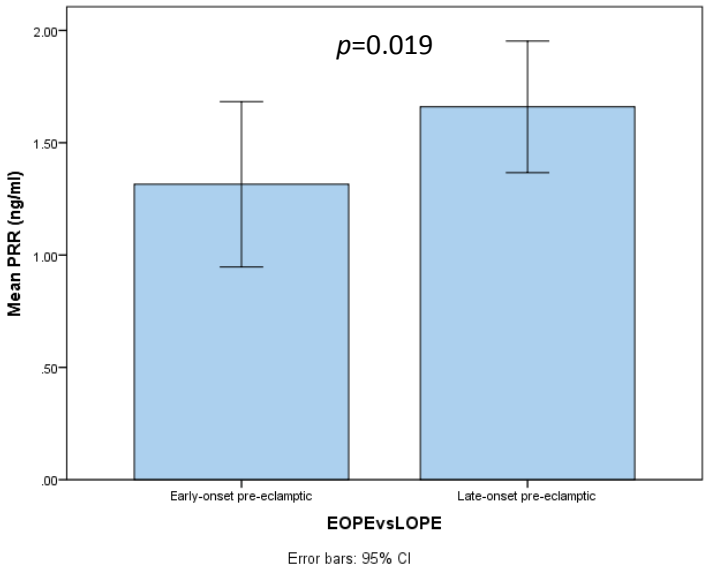

Figure 3.8 Pro-renin receptor concentrations (ng/ml) for the early- versus late-onset pre-eclamptic groups

Table 3.2 Pro-renin and pro-renin receptor levels across study groups

	N HIV-ve n=15	**N HIV+ve** n=15	**EOPE HIV-ve** n=15	**EOPE HIV+ve** n=15	**LOPE HIV-ve** n=15	**LOPE HIV+ve** n=15
PR ng/ml	1.9±1.4	1.9±0.6	1.4±0.8	1.5±0.8	1.8±0.8	1.5±0.9
(P)RR µg/ml	1.83±0.95	1.83±1.18	1.51±0.77	1.06±1.11	1.66±0.88	1.65±0.62

Summary statistics shown as mean±SD

3.4 CORRELATION BETWEEN BLOOD PRESSURE AND PRO-RENIN WITHIN EACH PREGNANCY TYPE

Pearson correlation revealed no significant correlations between PR within each pregnancy type (normotensive, early onset and late onset pre-eclampsia; Table 3.2).

3.5 CORRELATION BETWEEN BLOOD PRESSURE AND PRO-RENIN RECEPTOR

There was a positive correlation between diastolic blood pressure and (P)RR concentration in the late-onset pre-eclamptic group, which was statistically significant [rs=-0.380; *p*=0.046; Spearman's rank order correlation; Table 3.2]. There were no significant correlation between maternal blood pressure and (P)RR in the normotensive pregnant and early onset pre-eclamptic groups.

3.6 CORRELATION OF PR BETWEEN EACH STUDY GROUP

There was a positive correlation between PR concentration and the HIV negative late onset pre-eclamptic and HIV positive early onset pre-eclamptic group, which was statistically significant [rs=0.525; *p*=0.054 Spearman's rank-order correlation]. A significant negative correlation occurred between PR concentration versus HIV positive late onset pre-eclamptic group and HIV positive early onset pre-eclamptic [rs=-0.643; *p*=0.018; Spearman's rank-order correlation].

3.7 CORRELATION OF (P)RR WITHIN STUDY GROUP

There were a significant positive correlation between (P)RR and diastolic blood pressure [rs = 0. 585; *p*=0.036; Spearman’s rank order correlation; Table 3.3].

Table 3. 3 Correlation of maternal blood pressure with PR and (P)RR concentration

Study Group	PR		(P)RR	
	Systolic blood pressure	**Diastolic blood pressure**	**Systolic blood pressure**	**Diastolic blood pressure**
Normotensive	$r_s(29) = -0.092$, $p=0.636$	$r_s(29) = -0.067$, $p=0.732$	$r_s(30) = -0.202$, $p=0.285$	$r_s(30) = -0.184$, $p=0.330$
EOPE	$r_s(28) = 0.172$, $p=0.382$	$r_s(28) = 0.145$, $p=0.462$	$r_s(29) = -0.099$, $p=0.609$	$r_s(29) = -0.050$, $p=0.798$
LOPE	$r_s(28) = -0.304$, $p = 0.116$	$r_s(28) = 0.050$, $p = 0.802$	$r_s(28) = 0.303$, $p = 0.117$	$r_s(28) = 0.380$, $p = 0.046$
Normotensive HIV-ve	$r_s(14) = -0.161$, $p = 0.582$	$r_s(14) = -0.226$, $p = 0.437$	$r_s(15) = -0.344$, $p = 0.209$	$r_s(15) = -0.259$, $p = 0.351$
Normotensive HIV+ve	$r_s(15) = 0.018$, $p = 0.950$	$r_s(15) = 0.116$, $p = 0.681$	$r_s(15) = 0.261$, $p = 0.348$	$r_s(15) = 0.023$, $p = 0.934$
EOPE HIV-ve	$r_s(14) = 0.258$, $p = 0.371$	$r_s(14) = 0.485$, $p = 0.079$	$r_s(15) = -0.266$, $p = 0.338$	$r_s(15) = -0.474$, $p = 0.074$
EOPE HIV+ve	$r_s(14) = 0.041$, $p = 0.889$	$r_s(14) = -0.061$ $p = 0.836$	$r_s(14) = -0.029$, $p = 0.922$	$r_s(14) = 0.236$, $p = 0.416$
LOPE HIV-ve	$r_s(15) = -0.393$, $p = 0.147$	$r_s(15) = 0.015$, $p = 0.957$	$r_s(15) = -0.503$, $p = 0.056$	$r_s(15) = 0.104$, $p = 0.711$
LOPE HIV+ve	$r_s(13) = -0.217$, $p = 0.477$	$r_s(13) = -0.080$, $p = 0.794$	$r_s(13) = 0.122$, $p = 0.692$	$r_s(13) = 0.585$, $p = 0.036$

significance $p< 0.05$

CHAPTER FOUR

CHAPTER 4

DISCUSSION

The foremost source of women dying in pregnancy in Sub-Saharan Africa is HIV related. Pre-eclampsia, is the commonest direct cause of maternal and neonatal morbidity and mortality in South Africa. The prevalence of HIV in our geographical area deems it a severe obstetric predicament. The co-location of both pathological conditions in pregnant women is frequent especially since KwaZulu-Natal is the epicentre of the HIV pandemic. Hence study, into the HIV associated pre-eclampsia in our province is warranted.

The aetiology of pre-eclampsia is enigmatic but it is fundamentally a pathology associated with a shallow trophoblast invasion compared to normal pregnancies (Naicker *et al*., 2003; Naicker *et al*., 2013). Of the multifactorial aetiological agents underlying pre-eclampsia, the renin–angiotensin–aldosterone system (RAAS) serves as the groundwork for the elevated blood pressure in pre-eclampsia with a resultant series of events causing the resultant hypoxia. The relationship between pre-eclampsia, pro-renin and its receptor is poorly understood.

Pre-eclampsia is characterised physiologically by plasma volume reduction, intravascular coagulation and potent vasoconstriction. Furthermore, compared with the surge in RAAS components in normotensive pregnancy, the circulating levels of renin, ANG I, ANG II, and aldosterone are much lower, yet with little erraticism of ACE levels (Shojaati *et al*., 2004). The hypothesis preferred by present researchers is that a gestational increase in plasma volume, viewed as a compensatory adaptation to support placental perfusion and fetal delivery, is vulnerable in pre-eclampsia due to a decrease in aldosterone, resulting in fluid insufficiency and ensuing placental ischemia (Gallery and Brown, 1987; Roberts and Cooper, 2001; Takeda *et al*., 2002). Therefore, the reduced circulating volume delimits placental blood supply, finally prompting a raised blood pressure during pre-eclampsia. Due to our limited understanding of the underlying pathophysiology of pre-eclampsia, the

portrayal of the renin-aldosterone system in hypertensive pregnancy remains problematic. Furthermore, the renin-aldosterone system in both normotensive and hypertensive pregnancy may respond to physiologic signals such as changes in blood pressure and volume status, as well as to alterations in placental hormones such as estradiol and progesterone.

(P)RR binds both renin and its inactive precursor pro-renin. This binding initiates intracellular signalling that up-regulates the expression of profibrotic genes. Simultaneously, binding of renin to the (P)RR increases its angiotensin I-generating activity, whereas binding of PR allows the 'inactive' renin precursor to become fully enzymatically active. Therefore, the (P)RR system reflects two functions, an angiotensin-independent function related to (P)RR induced intracellular signalling and its downstream effects and an angiotensin-dependent function related to the increased catalytic activity of receptor-bound PR (Nguyen and Danser, 2008; Nguyen, 2011; Cao and Feng, 2013).

In this study, there was a significant difference in maternal age across all study groups ($p<0.004$). The overall mean age of the study population was 27± 6 yrs, however one must be cognisant that an exclusion factor for this study was maternal age below 18 yrs. Nevertheless, the maternal age of our study population is in keeping with the global trend to delay first pregnancy (Johnson and Tough, 2012). Progressive maternal age is a risk factor for low birth weight (Jolly *et al*., 2000; www.sciencedaily.com), preterm delivery (Aldous and Edmonson, 1993), placenta previa (Williams and Mittendorf, 1993), and admission of infants to special care facilities (Berkowitz and LaSala, 1990). In this study, we also observed a significant difference in maternal age between the HIV positive and HIV negative cohorts ($p<0.001$). This may be incidental or linked to the fact that HIV is endemic to KwaZulu-Natal, as it is the global epicentre of the HIV pandemic. However our data is consistent with that of Mitqitti *et al*., (2008) who showed that maternal HIV positive status, young maternal age and gestational age were significant factors after adjusting for potential confounders.

In our study, as expected (inclusion criteria) both the diastolic and the systolic blood pressure in the early and late onset pre-eclamptic groups were higher compared to the normotensive groups (p=0001

respectively). Additionally, based on HIV status, there were no significant difference in systolic (p<0.546) and diastolic (p<0.815) pressure. A previous study has indicated lower blood pressure (both systolic and diastolic) in HIV negative patients (Govender *et al*., 2014). In contrast, studies by Gazzaruso *et al.,* (2003) have linked the development of high blood pressure with HIV infection.

In this study, maternal weight was higher in the pre-eclamptic group compared to the normotensive groups. We also observed no significant difference in maternal weight between the HIV infected and uninfected groups (p=0.153). The amount of weight gained during pregnancy can affect the immediate and future health of a woman and her infant. Evidence indicates relationships between extreme gestational weight gain and increased birth weight and postpartum weight retention but also between insufficient weight gain and decreased birth weight (Siega-Riz *et al*., 2009).

In our study, gestational age was higher in the normotensive group compared to the pre-eclamptic groups (p=0.001), with gestational age been the lowest in the early onset group. Studies have shown that the risk of pre-eclampsia re-occurrence increases with earlier gestational age at the first delivery complicated by pre-eclampsia and with increasing maternal body mass index (Mostello *et al*., 2008). Additionally, we report no significant difference in gestational age *(*p=0.504) between HIV infected and non-infected groups in our study. However the latter finding is directly in contrast with that of Mitgitti *et al*., (2008) who showed that gestational age correlated with maternal HIV positive status after adjusting for potential confounders.

Pre-eclampsia is also considered a significant higher risk for low birth weight babies (Brand and Milder, 2013; Society for Maternal-Fetal Medicine, 2015). In our study gestational age was higher in the normotensive group compared to the pre-eclamptic groups (p=0.000) and this may be correlated with the low birth weight of infants. Supporting our data are numerous studies from Africa that show HIV-infected pregnant women are at increased risk of delivering low birth weight infants, of preterm delivery, and of intrauterine growth retardation (Dreyfuss *et al*., 2001).

Different types of hypertension in pregnancy have different profiles of plasma renin and pro-renin concentration in relation to development of pre-eclampsia (de León *et al*., 2001). In our study there were no difference in PR across all study groups (p=0.379) and between HIV positive versus the HIV negative group (p=0.599) regardless of pregnancy type. There was a positive correlation between PR concentration and the HIV negative late onset pre-eclamptic and HIV positive early onset pre-eclamptic group, which was statistically significant (p=0.054). A significant negative correlation occurred between PR concentration versus HIV positive late onset pre-eclamptic group and HIV positive early onset pre-eclamptic [r=-0.643; p=0.018].

Additionally, with regards to PR versus pregnancy type in this study there was no difference in PR between the EOPE and LOPE groups (p=0.409) as well as normotensive *vs* LOPE (p=0.273). However there was a significant difference between normotensive *vs* EOPE (p=0.039).

In this study we report decreased PR levels in EOPE. These results are in contrast to that of Itskovitz *et al*., (1992b) however, they correspond to the gestational period. Early onset pre-eclampsia is preceded by trophoblast invasion and irregular placentation and poor angiogenesis occurring early in pregnancy. Strongly increased angiotensin II type 1 (AT1) receptor levels have been found in pre-eclampsia (Herse *et al*., 2007). Likewise, microalbuminuria is known to be related with the pre-eclampsia development. We speculate that the alteration in the renin–angiotensin system observed in the current study reflect maternal susceptibility emanating early in gestation from defective placentation.

In pre-eclampsia, plasma renin activity and concentration and plasma angiotensin II and aldosterone concentrations are reduced compared to normal pregnancy. Plasma renin concentration is (two fold) greater in pre-eclampsia than normal pregnancy. Renin levels, remains stable throughout gestation

even during the third trimester of hypertensive pregnancy (Elsheikh *et al*., 2001). Plasma renin is said to increase 10 fold during pregnancy (Itskovitz *et al*., 1992a and b) whilst high plasma pro-renin levels, are derived from the ovaries (Derkx *et al*., 1987). It must be noted however, that our study reflects the precursor form viz., pro-renin levels obtained at term. These results albeit displaying a trend are in contrast with the observation of higher PR levels in pre-eclamptic patients compared to normal pregnancies, the values being significantly low in the first and the third trimester (de Leon *et al*., 2001; Pringle *et al*., 2011; Pringle *et al*., 2015). Notably our results for PR stratified according to gestational age reflects a difference between normotensive and EOPE, which is characterised by inadequate placentation.

In our study, (P)RR concentration was not significantly different between HIV positive and HIV negative groups (p=0.541) regardless of the pregnancy type as well as across all pregnancy types (p=0.055). There was no difference between normotensive and pre-eclamptic (p=0.362), specifically, there was no difference between normotensive and EOPE (p=0.074) and also between normotensive and LOPE (p=0.803). However there was significant difference between EOPE *vs* LOPE (p=0.019).

An increase in (P)RR and PR is usually associated with an increase in blood pressure (Batenburg and Jan Danser, 2012). (P)RR binds renin and the inactive pro-enzyme form of renin, pro-renin and this binding triggers the activation of the mitogen-activated protein kinase p42/p44 followed by up-regulation of the expression of pro-fibrotic genes. In addition, pro-renin bound to (P)RR undergoes a conformational change and becomes catalytically active (Nguyen and Jan Danser *et al*., 2008). Additionally, binding to (P)RR increases the catalytic efficiency of renin 4-fold (Nguyen *et al*., 2002). The PR-(P)RR binding occurs under physiological conditions (37°C, pH=7.4) and only 1-2% of pro-renin displays catalytic activity (Cousin *et al*., 2009). The binding of (P)RR and PR has been shown to be associated with enhanced Ang I generation resulting in the pre-eclamptic state (Cousin *et al*., 2009).

(P)RR has been put forward as a potential candidate/ or as a therapeutic target to optimize RAAS blockade in hypertension and end organ damage. (P)RR activation plays a significant role in the development of end-organ damage in hypertension (Ichihara and Keio, 2012). However studies by Nguyen *et al*., (2011) refute this suggestion as their results in animal studies were disappointing and was not compelling enough to launch (P)RR as key components in hypertension or in organ damage, although human studies have suggested a link between a polymorphism in the (P)RR gene and blood pressure (Nguyen, 2011). More specifically, the stimulation of (P)RR initiates intracellular pathways allied to fibrosis (Gonzalez and Prieto, 2008; Gonzalez *et al*., 2014).

In type II diabetes, the increase in PR correlates with the severity of proteinuria and retinopathy (Luetscher *et al*., 1985). Renin inhibitors may however block pro-renin receptors which modulate vaso-constriction, hypertrophy, atherosclerosis and fibrosis in target organs including kidney, heart and blood vessels (Jan Danser *et al*., 1997; Nguyen *et al*., 2002; Jan Danser, 2010).

Angiotensin receptor blockers inhibit the interaction between angiotensin II and angiotensin AT1 receptors, which modulate all the potential adverse effects of this peptide. Renin inhibition has potential for therapeutic benefits in conditions associated with high PRA (Laragh, 1992; Lin and Frishman, 1996). Aliskiren is the prototype of an oral renin inhibitor *in vitro* and *in vivo* (Wood *et al*., 2003). PRA, angiotensin I and angiotensin II are depressed in a dose-dependent manner (Webb *et al*., 1985; Nussberger *et al*., 2002). Blood pressure reduction is also dose-dependent and duration of action is more than 24 h (Webb *et al*., 1985; Oh *et al*. 2007). Aliskiren is the only orally active renin inhibitor that has progressed to phase III clinical trials. Although much more work needs to be done, optimal suppression of the RAS medicated by renin inhibition may unlock the potential for RAS blockade to improve the control of hypertension and end organ protection.

Another archetypal role of (P)RR has been put forward. Whilst it is a contributor to the pathogenesis of blood pressure via the amplification of renin- or pro-renin-induced angiotensin (Ang) formation, a recent paradigm shift suggests a new role for (P)RR, separate from the renin-angiotensin system (RAAS), in contributing to cellular homeostasis. (P)RR binds renin and pro-renin with affinity in the nanomolar range (Nguyen *et al*., 2002; Nabi *et al*., 2006). Depending on the model, (P)RR activation may initiate diverse intracellular signaling pathways. In most types of cells, (P)RR activation generates the mitogen activated protein kinases ERK1/2 phosphorylation, with resultant up regulation of the expression of pro-fibrotic genes such as transforming growth factor, plasminogen activator inhibitor type 1, collagens and fibronectin (Huang *et al*., 2006; Sakoda *et al*., 2007; Feldt *et al*., 2008). These pro-fibrotic enhancing cytokines would decrease trophoblast cell migration with consequential deficient trophoblast invasion that characterises pre-eclampsia. Both (P)RR and PR have been found in the extravillous trophoblast cells, suggesting that they play a key role in trophoblast migration (Pringle *et al*., 2011).

Specifically, (P)RR is thought to be crucial for vacuolar H+ -ATPase (V-ATPase) activity and acts as a connector between the V-ATPase and the Wnt signalling conduit (Müller *et al*., 2012; Nguyen., 2012). The Wnt proteins are growth factors that signal via a complex formed of their primary receptor comprising seven transmembrane regions, and low-density lipoprotein receptor–related protein (LRP), a single-pass transmembrane protein (Nguyen *et al*., 2012). Wnt signaling is essential for normal embryo development. In adults, Wnt signaling is involved in cell proliferation, migration, polarity, and tissue repair and abnormal Wnt signaling is known to stimulate human degenerative diseases (Nguyen *et al*., 2012). It is therefore plausible to hypothesize that a decrease in (P)RR levels would decrease Wnt signalling hence affect trophoblast cell proliferation and migration as is characteristic of pre-eclampsia.

It must be noted that although the kidney is the foremost source of renin in the body, there are other tissues discharging pro-renin into the circulation (Jan Danser, 1998; Krop and Jan Danser, 2008). For instance, pregnant women have high plasma pro-renin levels, derived from the ovaries (Derkx *et al*.,

1987). Pro-renin was discovered first in amniotic fluid. The reproductive organs, together with the adrenal, eye and submandibular gland are now well-accepted sites of extrarenal renin gene expression (Krop and Jan Danser, 2008). Our future investigation will include the immunostaining of (P)RR and PR in the placenta and placental bed of HIV associated normotensive and hypertensive pre-eclamptic women in an attempt to elucidate paracrine and/autocrine modes of production.

The role of (P)RR in pre-eclampsia pathology requires the use of an antagonist of the receptor; and studies in knock-out mice to-date has not expressed the gene encoding for the receptor. The antagonist, is not yet ideal, and the total knock-out of the (P)RR is surprisingly for a component of the renin–angiotensin system, not possible (Zaman *et al*., 2002; Burcklé and Bader, 2006). The generation of (P)RR conditional knock-out mice is thus mandatory to further establish the role of (P)RR in disease.

With the introduction of highly active antiretroviral therapy (HAART), the survival benefit from ARV therapy is indisputable. Yet anti-retroviral treatment regimens are associated widely with HIV associated nephropathy (Szczech and Winston, 2004). All HIV positive patients in this study were on anti-retroviral treatment as deemed by the Department of Health, South Africa hence they may have confounded the PR and (P)RR levels in our study.

CONCLUSION

This novel study demonstrates no significant difference in PR between HIV positive *versus* the HIV negative pregnancies regardless of pregnancy type. There was no difference in PR between the EOPE and LOPE groups as well as normotensive *vs* LOPE, however there was a significant difference between normotensive *vs* EOPE. This study also reports significant difference in (P)RR between HIV positive and HIV negative groups regardless of the pregnancy type as well as across all pregnancy types, however, there was a significant difference between EOPE *vs* LOPE groups.. Both (P)RR and

PR probably play key roles in trophoblast migration in pregnancy and that the low levels shown in this study correlates with the defective invasion that occurs in EOPE.

A low (P)RR concentration will inhibit binding to the pro-renin molecule. Moreover, the pro-renin receptor represents an elegant concept to explain the existence of active pro-renin *in vivo*. Additionally, this study provides a pathological protagonist for pro-renin receptor dysregulation in pre-eclampsia, a condition characterised with a paradoxical variation in renin/pro-renin and pro-renin receptor concentrations. Apart from the high blood pressure development, it may be associated with the ubiquitous proteinuria and the insufficient trophoblast invasion that characterises early onset pre-eclampsia. This study confirms a role for the pro-renin receptor in pre-eclampsia development.

FUTURE PERSPECTIVE

RAAS enquiries have been proficient for more than a century. Yet, new constituents of the RAAS, in disease disorders such as pre-eclampsia are increasing its complexity. Understanding the binding of pro-renin to its receptor, and to mature renin is vital. Nonetheless, bearing in mind its pathophysiological contribution in hypertension, a more potent inhibitor that can preclude pro-renin binding to pro-renin receptor would be of immense clinical value.

CHAPTER FIVE

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CHAPTER SIX

CHAPTER 6

APPENDICES