NEPHROTOXICITY ASSOCIATED WITH PREECLAMPSIA IN AN ARGININE VASOPRESSIN INDUCED RAT PREGNANCY MODEL

BY

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ABSTRACT

Introduction: Globally, preeclampsia (PE) complicates an estimated 2-8% of pregnancies and is a leading cause of maternal and fetal morbidity and mortality. Renal injury is closely involved in the pathophysiology of PE and is associated with future risk of kidney disease. Identifying an early biomarker of renal dysfunction is essential for the diagnosis and treatment of PE. Given the clinical and ethical complexities associated with pregnancy studies in humans, animal models provide a more feasible alternative to pregnancy research.

Aim: In view of this, this study aimed to determine the physiological and biochemical features of the arginine vasopressin (AVP) induced pregnancy model in the Sprague Dawley rat and to demonstrate nephrotoxicity associated with this model.

Methodology: Urine, blood and kidney samples (n = 6 per study group) were collected from female Sprague Dawley rats, based on four study groups, viz., pregnant AVP, pregnant saline, non-pregnant AVP, and non-pregnant saline groups. The AVP rat model was physiologically characterized by evaluating the clinical, biochemical, haematological and fetal parameters across all study groups. Renal injury in AVP-treated rats was histologically determined by haematoxylin and eosin staining, as well as immunolocalizing kidney injury molecule-1 (KIM-1) and podocalyxin in both AVP-treated and untreated kidneys using immunohistochemistry. Ultrastructural changes in AVP-treated rats were determined by transmission electron microscopy. The Multiplex kidney toxicity immunoassay panels were used to determine the urinary concentration of albumin, vascular endothelial growth factor-A, clusterin, cystatin C, beta-2-microglobulin, KIM-1, neutrophil gelatinase-associated lipocalin-2, osteopontin and tissue inhibitor of metalloproteinases-1 in AVP-treated rats.

Key findings: Chronic infusion of AVP throughout gestation reproduced the phenotypes viz., increased blood pressure, elevated urinary protein levels and fetal growth restriction, characteristic of human PE development. Immunohistochemical analysis confirm KIM-1 immunolocalization in the proximal convoluted tubules of AVP-treated *vs.* untreated

groups. Comparatively, a mild immunolocalization of podocalyxin was observed in the glomeruli of pregnant AVP-treated *vs.* pregnant untreated rats. Histological and ultrastructural evaluation of the AVP-treated pregnant rats demonstrated several abnormalities including, reduced Bowman's space, necrosis of tubules and blood vessels, along with podocyte effacement, glomerular basement membrane abnormalities, podocyte nuclear crenations, mitochondrial dysfunction and cytoplasmic lysis consistent with renal injury in PE. Our findings indicate that AVP significantly reduces the urinary levels of vascular endothelial growth factor A and concomitantly up-regulates the urinary expression of clusterin, cystatin C, beta-2-microglobulin, KIM-1, neutrophil gelatinase-associated lipocalin-2, osteopontin and tissue inhibitor of metalloproteinases-1.

Conclusion: This is the first study to demonstrate that AVP induces glomerular and tubular injury, as well as endothelial dysfunction in the pregnant Sprague Dawley rat model. These features are characteristic of renal injury observed in PE. Furthermore, AVP successfully elevated the urinary levels of most glomerular and tubular injury biomarkers as well as produced histological and ultrastructural renal abnormalities associated with human PE. Our data demonstrates the importance of kidney injury as early detection biomarkers for PE development. The findings support the use of the AVP rat model in future studies investigating the pathogenic processes involved in PE development.

PREFACE

The experimental work described in this thesis was carried out at the Durban University of Technology, Durban, South Africa and the Optics & Imaging Centre, Doris Duke Medical Research Institute, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa from January 2020 to December 2022, under the supervision of Professor Thajasvarie Naicker and Dr Nalini Govender.

This study represents original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text

05 December 2022

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Supervisor: Professor Thajasvarie Naicker — Date

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Co-supervisor: Dr Nalini Govender — Date

DECLARATION

I, Sapna Ramdin declare that:

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
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5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed:  Date: 05 December 2022

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CONTENTS

LIST OF FIGURES

ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
AKT	Protein kinase B pathway
ALT	Serum alanine transaminase
AST	Aspartate aminotransferase
AT1	Type-1 angiotensin II receptor
AT1-AA	Angiotensin II type 1 receptor autoantibody
AVP	Arginine vasopressin
BPH	Blood pressure high
Ca^{2+}	Calcium
CD2AP	CD-2 associated protein
ELISA	Enzyme-linked immunosorbent assay
EOPE	Early onset preeclampsia
FAT1	FAT cadherin 1
GBM	Glomerular basement membrane,
GD	Gestational day
GFA	Glomerular filtration apparatus
GFB	Glomerular filtration barrier
GFR	Glomerular filtration rate
H/E	Haematoxylin and Eosin
HDL	High density lipoprotein
HDP	Hypertensive disorders of pregnancy
HELLP	Haemolysis, elevated liver enzymes, low platelet syndrome

HIV	Human immunodeficiency virus
IDO	Indoleamine 2,3-dioxygenase
KIM-1	Kidney injury molecule 1
LDH	Lactate dehydrogenase
L-NAME	Nitro-L-arginine methyl ester
LOPE	Late onset preeclampsia
MMR	Maternal mortality ratio
mPTP	Mitochondrial permeability transition pore
NAVP	Non-pregnant AVP
NGAL/ lipocalin 2	Neutrophil gelatinase-associated lipocalin-2
NHERF2	Na^+/H^+ exchanger regulatory factor 2;
NOS	Nitric oxide synthase
NS	Non-pregnant saline
OPN	Osteopontin
PAVP	Pregnant AVP
PE	Preeclampsia
PlGF	Placental growth factor
PS	Pregnant saline
RAAS	Renin-angiotensin aldosterone system
RUPP	Reduced uterine perfusion pressure
SA	South Africa
SD	Slit diaphragm
SEng	Soluble endoglin

sFlt-1	Soluble fms-like tyrosine kinase-1
STOX1	Storkhead box 1
TIMP-1	Tissue inhibitor of metalloproteinases-1
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
TRPC6	External short transient receptor potential channel 6
VEGF	Vascular endothelial growth factor
VEGF-A	Vascular endothelial growth factor A

PUBLICATIONS

Journal Article

Ramdin, S., Naicker, T, Pillay, V., Singh, S.D., Baijnath, S., Mkhwanazi, B.N. and Govender, N. 2022. Physiological characterization of an arginine vasopressin rat model of preeclampsia. *Systems Biology in Reproductive Medicine*, 68:1, 55-69, DOI: https://doi.org/10.1080/19396368.2021.1981486

Ramdin, S., Baijnath, S., Naicker, T. and Govender, N. 2023. The Clinical Value of Rodent Models in Understanding Preeclampsia Development and Progression. *Current Hypertension Reports* 25, 77–89, DOI: https://doi.org/10.1007/s11906-023-01233-9

Ramdin, S., Naicker, T., Baijnath, S. and Govender, N., 2023. Kidney injury molecule-1 and podocalyxin dysregulation in an arginine vasopressin induced rodent model of preeclampsia. *European Journal of Obstetrics and Gynecology and Reproductive Biology*, 284, 58-65, DOI: https://doi.org/10.1016/j.ejogrb.2023.03.012

Conference presentations and abstracts

Ramdin, S., Baijnath, S., Naicker, T., Singh, S.D. and Govender, N., 2021. Does arginine vasopressin induce kidney injury in pregnant Sprague Dawley rats? *Keystone Symposia eSymposia meeting Maternal-Fetal Newborn Immunity 22EK1*. Virtual attendance, 28-29th October 2021.

Ramdin, S., Baijnath, S., Naicker, T. and Govender, N., 2022. Renal injury associated with arginine vasopressin induction in a rat model of preeclampsia. *The 29th Scientific Meeting of the International Society of Hypertension*. Kyoto, Japan, 12-16th October 2022.

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THESIS LAYOUT

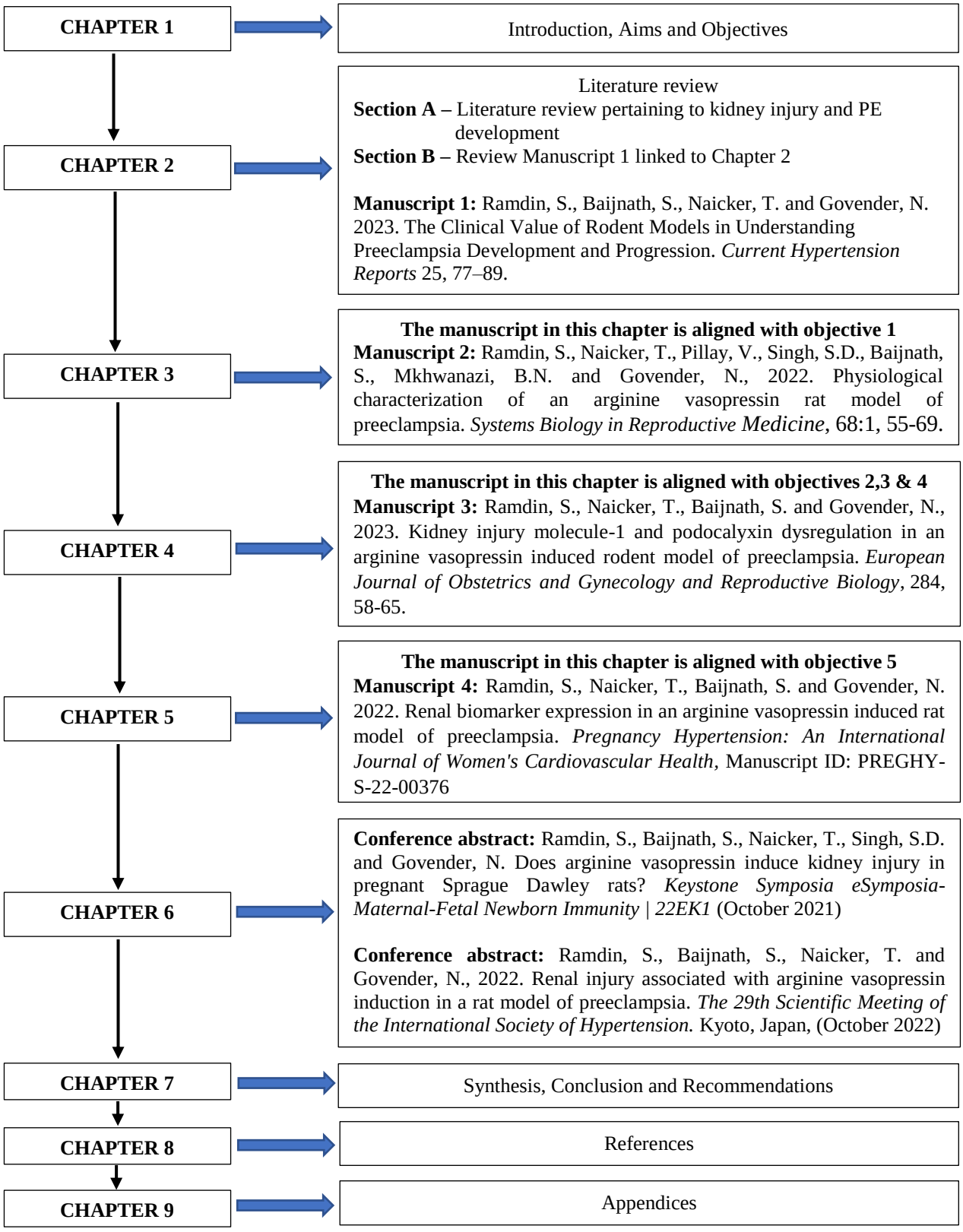

Figure 1. Schematic diagram showing layout of thesis

CHAPTER 1

INTRODUCTION

1.1. PROBLEM IDENTIFICATION

Worldwide, improving maternal health remains a public health challenge. One of the Sustainable Development Goals of the World Health Organization is to lower the global maternal mortality ratio to less than 70 maternal deaths/100 000 live births by 2030 (World Health Organization, 2017). In fact, women in low- and middle-income countries are more susceptible to severe morbidity and mortality during pregnancy and childbirth; where 99% of global maternal deaths occur (World Health Organization, 2019a). Globally, approximately 295 000 maternal deaths occur per annum with 66% (196 000 maternal deaths) occurring in sub-Saharan Africa (World Health Organization, 2019b). South Africa has a projected maternal mortality ratio (MMR) of 119 per 100 000 live births (World Health Organization, 2019b).

Worldwide, hypertensive disorders of pregnancy (HDP) are the most prevalent direct cause of maternal and fetal morbidity and mortality (Garovic *et al.*, 2020). Chronic hypertension, gestational hypertension, preeclampsia (PE) with or without severe features, eclampsia and chronic hypertension with superimposed PE are major HDPs affecting maternal health (ACOG, 2019). In low-income countries, 75% of maternal deaths emanate from severe bleeding, infections, pregnancy-specific hypertension (PE and eclampsia), complications from delivery and unsafe abortion (Say *et al.*, 2014).

Sub-Saharan Africa also has the largest prevalence of human immunodeficiency virus (HIV) infection globally with women of childbearing age making up a significant percentage of this population (Vandormael *et al.*, 2019; Joint United Nations Programme on HIV/AIDS, 2019). Of note, an estimated 13.7% of the South African population were HIV infected in 2021, with women aged 15-49 years accounting for 23.92% of the HIV-infected population (Statistics SA, 2021). Approximately 40.9% of women attending antenatal care within the province of KwaZulu-Natal, South Africa (SA), are HIV-infected women (Woldesenbet *et al.*, 2021). Therefore, maternal mortality stemming from the duality of HIV infection and HDP remains a dilemma in sub-Saharan Africa.

Given the clinical and ethical complexities associated with pregnancy studies in humans, animal models provide a more feasible alternative to pregnancy research. The reduced uterine perfusion pressure (RUPP) rat model was developed to investigate placental ischemia (Alexander *et al.*, 2001), while abnormal placentation has been studied using the storkhead box 1 (STOX1) overexpression mouse model (Doridot *et al.*, 2012). The mechanism of angiogenic imbalance has been previously evaluated using the adenoviral soluble fms-like tyrosine kinase-1 (sFlt-1) infusion rat model (Maynard *et al.*, 2003) whereas endothelial dysfunction associated with PE has been studied using the nitro-L-arginine methyl ester (L-NAME) rat model (Ramesar *et al.*, 2011). Arginine vasopressin (AVP) has also garnered attention as a prospective biomarker in PE pathogenesis. Santillan and co-workers have previously highlighted the role of AVP in PE development in pregnant mice (Santillan *et al.*, 2014). In human clinical research, the use of animal models that mimic a disease is crucial for the identification and advancement of novel treatment options and strategies. These models have contributed significantly to our understanding of pathophysiological pathways associated with PE development. In view of this, this study focused on determining the physiological and biochemical features of the AVP-induced pregnancy model in the Sprague Dawley rat.

1.2 AIM

This study aims to provide a full physiological and biochemical characterization of the arginine vasopressin-induced pregnancy model in the Sprague Dawley rat and to demonstrate nephrotoxicity associated with this model.

1.3 HYPOTHESIS

Arginine vasopressin infusion reproduces human preeclampsia phenotypes and induces renal injury associated with preeclampsia development in pregnant Sprague Dawley rats.

1.4 OBJECTIVES

- To determine the biochemical, haematological and fetal parameters in the AVP treated pregnant rats and provide a complete physiological characterization of the model

- To determine the morphological changes in the kidney of AVP-treated rats using Haematoxylin and Eosin **(**H/E) staining.

- To determine the ultrastructural features of the glomerular filtration apparatus in rats infused with AVP, using transmission electron microscopy.

- To immunolocalize the structural proteins of the glomerular filtration apparatus in the kidney and kidney injury markers viz., podocalyxin and kidney injury molecule-1 in the AVP-treated rat kidney using immunohistochemistry.

- To determine the urinary expression of albumin, vascular endothelial growth factor-A, clusterin, cystatin C, beta-2-microglobulin, kidney injury molecule-1, neutrophil gelatinase-associated lipocalin-2, osteopontin and tissue inhibitor of metalloproteinases-1 in the AVP-induced rat kidney using the Multiplex ELISA immunoassay procedure.

CHAPTER 2

LITERATURE REVIEW

This chapter provides a detailed literature review on PE and kidney injury associated with its development as well as the clinical value of rodent models in understanding PE development and progression.

SECTION A

2.1 PREECLAMPSIA

Preeclampsia (PE) and eclampsia, are the most common HDP and feature as the leading direct cause of maternal deaths in SA with an institutional MMR of 24 per 100 000 live births in the 2014–2016 triennium (Saving Mothers, 2018; Moodley *et al.*, 2019). In addition to HIV infection and the Acquired Immunodeficiency Syndrome (AIDS), PE is responsible for an estimated 18% of all deaths in SA (South African National Department of Health, 2017; Moodley, 2018; Moodley *et al.*, 2016; Sidley, 2000). Preeclampsia is a multisystem disorder that is characterised by new-onset hypertension (≥140/90 mmHg), manifesting after 20 weeks of gestation, with or without proteinuria (>300 mg/dL) or, maternal target organ dysfunction (Magee *et al.*, 2021).

2.1.1 Pathogenesis of PE

Impaired placentation emanating from deficient trophoblast invasion and a lack of physiological transformation of myometrial spiral arteries initiates PE development (Pankiewicz *et al.*, 2019). Preeclampsia is characterised as a two-stage disorder, with the first stage involving placental ischemia as a consequence of reduced luminal diameter and high resistance vasculature (Roberts and Hubel, 2009). Placentas from preeclamptic women exhibit several structural and anatomical changes, including infarctions, arteriole constriction, fibrin deposition, and thrombosis (Redman and Sargent, 2005; Ducray *et al.*, 2011).

The ischemic placenta up-regulates the production of soluble fms-like tyrosine kinase-1 (sFlt-1), which then binds to the pro-angiogenic vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) (Chaiworapongsa *et al.*, 2014; Govender *et*

al., 2013; Govender *et al.*, 2014). This action alters the balance between pro- and anti-angiogenic factors, causing widespread systemic endotheliosis, leading to the second stage of PE, *viz.*, the acute maternal syndrome with systemic multi-organ dysfunction (Chaiworapongsa *et al.*, 2014). This shift in angiogenic balance is also linked with systemic vasoconstriction, reduced levels of nitric oxide and other vasodilatory factors, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (Granger *et al.*, 2001; Maynard *et al.*, 2003; LaMarca *et al.*, 2007).

While PE has been established as a two-stage disorder, a five-stage model has been suggested recently (Staff, 2019). This study expanded the two-step model originally recommended by Redmann (1991) to encompass additional aspects (Redman, 1991; Staff *et al.*, 2014). This new model proposes that impaired local maternal immune tolerance to allogenic trophoblasts and resulting inadequate placentation be separated into Stages 1 and 2 respectively (Staff *et al.*, 2014). Stage 3 involves reduced uteroplacental perfusion resulting from poor placental development caused by dysfunctional immune tolerance. Subsequent to these events, Stage 4 comprises the placental secretion of inflammatory factors into the maternal circulation which induces widespread maternal inflammation and overt PE. Lastly, Stage 5 is associated with the manifestation of decidual acute atherosis potentially arising from decidual inflammation in late gestation (Staff *et al.*, 2013; Staff *et al.*, 2014; Alnaes-Katjavivi *et al.*, 2016).

Additionally, several other pathways are involved in PE pathogenesis such as oxidative stress (Vaughan and Walsh, 2002), type-1 angiotensin II receptor (AT1) autoantibodies (Wallukat *et al.*, 1999), platelet and thrombin activation (Chaiworapongsa *et al.*, 2002; Kenny *et al.*, 2009), intravascular inflammation (Lau *et al.*, 2013), protein kinase B (AKT) pathway (Wang *et al.*, 2019), mitogen-activated protein kinase/extracellular-signal-regulated kinases (MAP/ERK) pathway (D'Oria *et al.*, 2017).

2.2 THE KIDNEYS AS TARGET ORGANS IN PE DEVELOPMENT

The functional unit of the kidneys is the nephron, which function essentially as regulators of homeostasis, by controlling blood pressure, acid-base balance, electrolyte reabsorption, and the filtration and excretion of metabolic toxins and waste products (Madsen *et al.*, 2007; Kriz and Kaissling, 2008; Agarwal *et al.*, 2021). The glomerulus is responsible for filtration and enables retention of important plasma proteins in the blood, while the primary urine produced is passed from the Bowman's capsule to the tubules (Zhuo and Li, 2013; Pietilä and Vainio, 2014). The tubules ensure that electrolytes and water filtered by the glomerulus are reabsorbed and restored to the circulation (Thomson and Blantz, 2008; McDonough, 2010).

The glomerular filtration apparatus (GFA) is composed of a layer of highly differentiated podocytes, the glomerular basement membrane (GBM) and a fenestrated endothelium (Figure 2.1) (Haraldsson *et al.*, 2008; Miner, 2011). The adjacent foot processes of podocytes are linked together by a slit diaphragm (SD), which is a zipper-like scaffold that is created by the cross-linking of proteins *viz*., nephrin, podocin, Neph1 and FAT cadherin 1 (FAT1) (Grahammer *et al.*, 2016; Perico *et al.*, 2016). These proteins create pliable protein bridges which prohibit the movement of macromolecules across this bridge (Grahammer *et al.*, 2016). Additionally, the CD2-associated protein (CD2AP) aids in the maintenance of slit diaphragm proteins and a3ß1 integrins assist in anchoring the podocyte to the GBM (Daehn and Duffield, 2021). Short transient receptor potential channel 6 (TRPC6), located on the surface of the primary foot process, open due to various stimuli such as stretch and oxidative stress to release Ca^{2+} in order to regulate cellular responses (Greka and Mundel, 2012). Podocalyxin is another crucial structural component responsible for regulating morphogenesis and differentiation of developing podocytes via tight and adherens junctions (Figure 2.1) (Freedman *et al.*, 2015; Freedman and Steinman, 2015; Kim *et al.*, 2017).

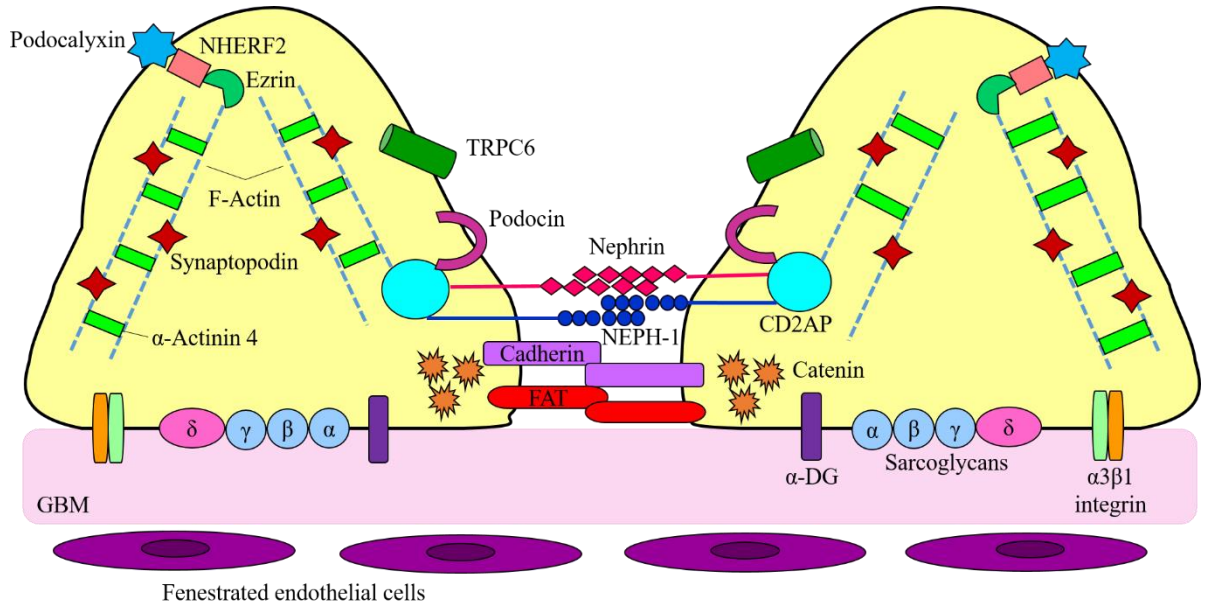

Figure 2.1 Schematic representation of the structural proteins involved in the podocyte foot process and formation of the glomerular slit diaphragm in healthy podocytes. Podocyte foot processes are affixed to the GBM by matrix tethering receptors such as dystroglycans and integrins. These receptors then aid in maintaining the foot process by modulating the actin–myosin contractile apparatus. Adjacent foot processes are linked by the slit diaphragm containing NEPH1/2, P-cadherin, FAT1 and nephrin. This protein scaffold forms part of the GFB and connects with the actin–myosin contractile apparatus via CD2AP and podocin. Once stimulated, external short transient receptor potential channels (TRPCs) open to release Ca^{2+} and regulate cellular responses. α-DG: α–dystroglycan; Ca^{2+}: calcium; CD2AP: CD-2 associated protein; FAT1: FAT cadherin 1; GBM: glomerular basement membrane, GFB: glomerular filtration barrier; NHERF2: Na^{+}/H^{+} exchanger regulatory factor 2; TRPC6: external short transient receptor potential channel 6. Adapted from (Mundel and Shankland, 2002; Gigante *et al.*, 2011).

2.2.1 The Kidney and Normal pregnancy

Various adaptive changes within the kidneys facilitate a successful pregnancy. They increase in size by approximately 30% and lengthen by 1-1.5 cm to accommodate higher renal blood flow (Hussein and Lafayette, 2014; Armaly *et al.*, 2018). Furthermore an up-regulation in vasodilatory hormones reduces systemic vascular resistance and mean arterial pressure despite a rise in cardiac output (Cornelis *et al.*, 2011). Additional hemodynamic modifications include the placental release of vasodilatory factors and a reduced sensitivity to angiotensin II by the peripheral vasculature (Noris *et al.*, 2004). These changes results in vasodilation, elevations in renal plasma flow and glomerular

filtration rate (GFR), as well as reduced plasma osmolality and mild hyponatremia (Cornelis *et al.*, 2011).

2.2.2 The kidney and PE

The kidney is a significant target organ in the pathological processes involved in PE development. Glomerular dysfunction characteristic of PE development, is mainly attributed to endothelial cell damage and subsequent occlusion of capillary lumens, which consequently determines the severity of PE (Naicker *et al.*, 1997; Alladin and Harrison, 2012). In contrast to normal pregnancy, renal blood flow and GFR are reduced in PE (Mustafa *et al.*, 2012). A loss of anionic charge of the GFA leads clinically to endotheliosis, proteinuria, and podocyturia (Sani *et al.*, 2019). Renal biopsies taken from preeclamptic patients reveal diffuse fibrin deposits, enlarged endothelial cells, podocyturia, and loss of capillary space (Ramsuran *et al.*, 2012; Sircar *et al.*, 2015; Pankiewicz *et al.*, 2019), indicative of reduced kidney function (Alladin and Harrison, 2012).

Normal functioning of the GFA is inhibited by increased sFlt-1, which impedes VEGF from binding to its receptor sites on endothelial cells and podocytes leading to glomerular damage (Sani *et al.*, 2019). Increased sFlt-1 also down-regulates several structural components of the glomerular SD such as nephrin and podocin, as well as the podocyte foot processes (e.g. synaptopodin and podocalyxin) (Garovic *et al.*, 2007a). Of note, higher urinary levels of kidney injury molecule-1 (KIM-1), a marker of proximal tubular cell damage, are observed in preeclamptic women in comparison to normotensive pregnant women (Wang *et al.*, 2015).

2.3 ANIMAL MODELS OF PE

As PE is a placental disorder, its study in humans proves difficult. Human studies pose a number of ethical challenges, given the potential harm to the mother and developing fetus (Cushen and Goulopoulou, 2017). In this regard, animal models offer a suitable substitute for PE study. A number of different methodologies have been implemented to produce a suitable animal model of PE (Maynard *et al.*, 2003; Santillan *et al.*, 2014; Gillis *et al.*,

2015; Santillan *et al.*, 2015; LaMarca *et al.*, 2016). Such models have made significant contributions towards understanding the mechanisms involved in PE pathogenesis and the development of novel forms of treatment. However, a single model that fully embodies all clinical features of PE such as abnormal placentation, fetal growth restriction, pregnancy-specific hypertension, elevated urinary protein levels, endothelial impairment and an imbalance in angiogenic factors, remains elusive. A comprehensive interrogation of several commonly used animal models of PE is provided in Section B of this chapter.

2.4 ARGININE VASOPRESSIN

2.4.1 Physiology

Arginine vasopressin (AVP) is a polypeptide vasoconstrictor and an antidiuretic hormone which acts as a homeostatic regulator, by controlling tubular water absorption in the kidneys (Oh, 2008). It is also involved in regulating pituitary function, stress, immune response and behaviour (Richter, 1988; Berczi *et al.*, 2009; Berczi *et al.*, 2012). Arginine vasopressin is made up of nine-amino acids, where two cysteine amino acids are connected by a disulphide bridge and is synthesized and secreted by the supraoptic and paraventricular nuclei of the hypothalamus (Figure 2.2) (Kounin and Bashir, 2000).

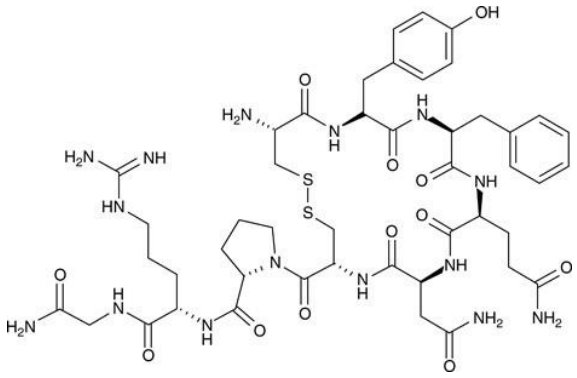

Figure 2.2 Chemical structure of AVP. This hormone constitutes nine amino acids with two cysteine amino acids linked by a disulphide bridge. It is produced by the hypothalamic supraoptic nuclei and secreted by the posterior pituitary in response to elevated plasma osmolality, low blood pressure and reduced renal perfusion. Arginine vasopressin exerts its actions though V2 receptors in the kidney to stimulate water reabsorption, and induces vasoconstriction via its V1 receptors located in vascular smooth muscle in order to maintain blood pressure homeostasis. Adapted from (Thomson and Napier, 2010).

The synthesis of AVP is encoded by the AVP gene located in chromosome region 20p13 (Rao *et al.*, 1992; Burbach *et al.*, 2001). The AVP gene also encodes the proteins, neurophysin 2 and copeptin (Rao *et al.*, 1992; Burbach *et al.*, 2001). This generates precursor peptides of AVP, prepro-vasopressin and pro-vasopressin, which undergo enzymatic cleavage to produce the individual peptides of AVP, neurophysin 2 and copeptin (Burbach *et al.*, 2001). These peptides are secreted into systemic circulation in equimolar ratios.

Since direct quantification of circulating AVP levels are inhibited due to its short biological half-life, copeptin is used as a surrogate biomarker to measure circulating AVP concentrations (Dobša and Edozien, 2013; Santillan *et al.*, 2014). Changes in plasma osmolality, blood volume and blood pressure influences AVP secretion. The elevations in plasma osmolality are detected by hypothalamic osmoreceptors, stimulating increased AVP secretion into the circulation, thereby maintaining blood pressure homeostasis (Robertson *et al.*, 1976).

2.4.2 Role of AVP in the kidney

Within the renal system, the principal role of AVP is to minimize water loss and to increase urine osmolarity (Dunn *et al.*, 1973; Kondo *et al.*, 2004). Signalling occurs via V2 receptors to mediate an increase in water permeability thus regulating extracellular fluid volume (Kondo *et al.*, 2004; Boone and Deen, 2008; Bankir *et al.*, 2010). Elevations in AVP concentration may produce increased blood volume, cardiac output and arterial pressure as the water reabsorption is increased while urine formation is simultaneously down-regulated (Dunn *et al.*, 1973; Sharabi and Schmid, 1983; Kondo *et al.*, 2004). Arginine vasopressin also regulates electrolytic balance by controlling water retention in the kidneys and is thus critical in preserving serum osmolarity (Dunn *et al.*, 1973).

2.4.3 AVP Receptors

The receptors that enable the physiological effects of AVP include V1a, V1b and V2 (Thibonnier *et al.*, 2002). The V1a receptors are primarily localized on vascular smooth muscle cells where they facilitate vasoconstriction (Koshimizu *et al.*, 2012). Additionally, their expression in the myometrium mediates uterine contraction whilst in

the hepatocytes and platelets, they play a role in glycogenolysis and platelet accumulation. These receptors are also found in the brain, adrenal cortex and adipose tissues (Koshimizu *et al.*, 2012). In the central nervous system, the V1a receptors modulate circadian rhythm (Li *et al.*, 2009), body temperature (Xu *et al.*, 2014), social behaviour, social cognition and emotion (Albers, 2015), as well as angiogenic regulators in the hypothalamus (Alonso *et al.*, 2008; Alonso, 2009). In the kidney, the specific arrangements of the V1a receptors ensures that their activation results only in efferent rather than afferent arteriolar constriction (Maybauer *et al.*, 2008).

In contrast, the V1b receptors are located mainly in corticotrophs of the anterior pituitary where they up-regulate the release of adrenocorticotropic hormone via corticotrophin-releasing hormone (Maybauer *et al.*, 2008). This receptor is also implicated in the hormonal release of prolactin, growth hormone, insulin, angiotensin, endothelin, and atrial natriuretic peptide (Balakrishnan *et al.*, 1997; Cogan *et al.*, 1986; Katoh *et al.*, 2005; Meller *et al.*, 1991). Notably, AVP also influences the hypothalamic-pituitary-adrenal axis via the V1b receptors, which indirectly influences the immune, vascular, and renal functioning (Koshimizu *et al.*, 2012).

In spite of the presence of V1a receptors in the kidney, the V2 receptor subtype is the most abundantly expressed AVP receptor in the kidney, with large numbers located on the cells of the distal convoluted tubule and collecting duct (Holt and Haspel, 2010). Activation of V2 receptors enables the insertion of aquaporin-2 water channels into the apical membrane of the distal and collecting duct cells, as well as the production of aquaporin-2 (Rotondo *et al.*, 2016). This allows for the physiological response of AVP, which increases water permeability and reabsorption, thereby restoring the intravascular compartment (Rotondo *et al.*, 2016). In the loop of Henle, the V2 receptors are involved in sodium reabsorption. Outside of the kidneys, V2 receptors are expressed in the vascular endothelium (Koshimizu *et al.*, 2012). They are stimulated by elevations in desmopressin and endogenous circulating AVP levels which results in the secretion of von Willebrand factor and Factor VIII from Weibel–Palade bodies, which are indicators of vascular injury (Kaufmann *et al.*, 2000).

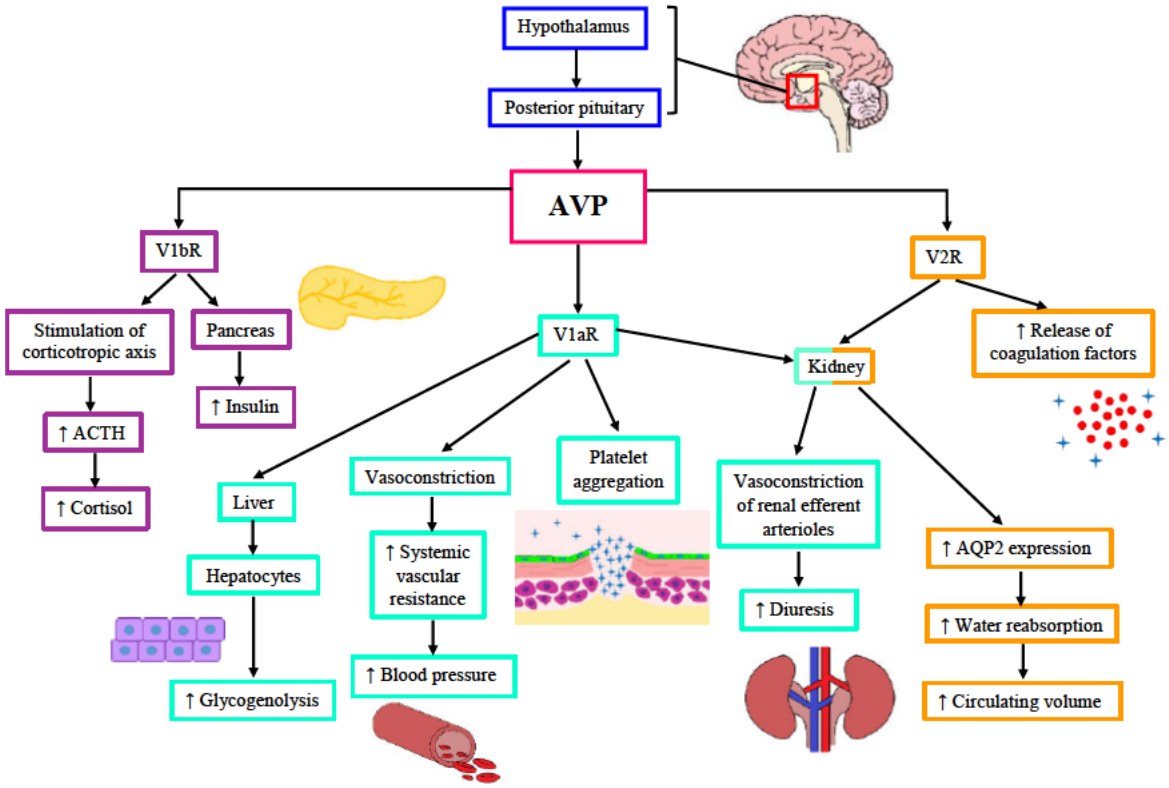

Figure 2.3 Physiological functioning of AVP via its receptor subtypes. Arginine vasopressin is synthesized in the hypothalamus and is released by the post-pituitary gland following stimulation. AVP exerts its physiological effects via three receptor subtypes (V1aR, V1bR and V2R). Binding of AVP on V1a receptors induces peripheral vascular smooth cell contraction, renal efferent arteriole vasoconstriction and platelet aggregation. Via its V2 receptors, AVP up-regulates aquaporin 2 expression, resulting in increased water reabsorption and stimulates the release of coagulation factors through extra-renal V2 receptors. Binding on V1b receptors stimulates corticotropic axis release of cortisol and insulin secretion from the pancreas. ACTH: adrenocorticotropic hormone. Adapted from (Demiselle *et al.*, 2020).

2.4.4 AVP and its role in PE development

Arginine vasopressin and its receptors are implicated in several immune mechanisms that are essential to the primary pathogenic stages of PE, including T helper 1 cell predominance, dendritic cell activation, and T helper 17 cell activation (Germer *et al.*, 1996; Elenkov and Chrousos, 2006; Russell and Walley, 2010). The normal physiological actions of AVP in the non-pregnant state closely relates to vascular, immune, renal, and angiogenic impairments linked with PE development.

2.4.5 AVP studies linked to PE

To-date, several studies report on the possible usefulness of copeptin in the early prediction of PE development and in evaluating the severity of the disorder (Zulfikaroglu *et al.*, 2011; Yeung *et al.*, 2014; Santillan *et al.*, 2014; Thadhani *et al.*, 2001). The first human study to highlight the role of copeptin in PE development and its potential use as an early predictor biomarker for the early identification of PE was conducted by Zulfikaroglu et al. (2011). These investigators demonstrated elevations in circulating copeptin levels during late second trimester and early third trimester PE compared to normotensive pregnancies, and suggested its utility as a biomarker for PE identification (Zulfikaroglu *et al.*, 2011). The association between circulating copeptin levels and the development of PE, gestational hypertension and preterm birth in pregnant women was subsequently investigated (Yeung *et al.*, 2014). Copeptin levels were positively associated with gestational age in both PE and normotensive pregnant groups but were not associated with other pregnancy-related disorders (Yeung *et al.*, 2014). Circulating AVP levels were significantly increased as early as the sixth week of gestation in PE, which is much earlier than other PE biomarkers previously investigated (Santillan *et al.*, 2014; Sandgren *et al.*, 2015b; Sandgren *et al.*, 2018a; Sandgren *et al.*, 2018b; Scroggins *et al.*, 2018). The data from these studies suggests that high levels of AVP are released early in gestation and maintained across all trimesters. Moreover, this group further demonstrates that AVP administration to pregnant mice throughout gestation successfully mimics human PE clinical symptoms such as increased systolic blood pressure, proteinuria and intrauterine growth restriction (Sandgren *et al.*, 2018a).

2.4.6 Implementation of the AVP Sprague Dawley rat model

Despite the successful replication of the characteristic features of PE in the mouse model developed by Santillan et al. (2014), the composite AVP effects on the mouse kidney has not been fully established. Hence, the physiological role of AVP in the kidney requires a comprehensive morphological exploration as the AVP model is yet to be replicated in a rodent model, whose placental vasculature with its spiral arteries closely resembles human placentation.

SECTION B

This section provides the review manuscript associated with the clinical value of rodent models in understanding preeclampsia development and progression.

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PREECLAMPSIA (VD GAROVIC, SECTION EDITOR)

The Clinical Value of Rodent Models in Understanding Preeclampsia Development and Progression

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Abstract

Purpose of Review Preeclampsia (PE) is a leading global cause of maternal and fetal morbidity and mortality. The heterogeneity of this disorder contributes to its elusive etiology. Due to the ethical constraints surrounding human studies, animal models provide a suitable alternative for investigation into PE pathogenesis and novel therapeutic strategies. The purpose of this review is to compare and contrast the various rodent models used to study PE, in order to demonstrate their value in investigating and identifying different characteristics of this disorder.

Recent Findings Several approaches have been employed to create an appropriate animal model of PE, including surgical, genetic manipulation, and pharmacological methods in an attempt to mimic the maternal syndrome. Despite the absence of a model to completely model PE, these models have provided valuable information concerning various aspects of PE pathogenesis and novel therapeutic strategies and have led to the discovery of potential predictive markers of PE.

Summary Rodent and murine models have contributed significantly to the study of the pathology associated with specific aspects of the disorder. As a single fully encompassing animal model of PE remains absent, the use of a combination of models has potential value in understanding its etiology as well as in new treatment and management strategies.

Keywords Animal model · Blood pressure · Placenta · Preeclampsia · Pregnancy

Introduction

Preeclampsia (PE) is a hypertensive disorder of pregnancy and a principal cause of maternal and fetal morbidity and mortality worldwide, resulting in approximately 46,000 maternal and 500,000 neonatal deaths per annum [1, 2]. Clinically, PE manifests as new-onset hypertension developing after 20 weeks of gestation (defined as blood pressure ≥ 140/90 mm Hg) together with one or more of the following: proteinuria (≥ 300 mg/day), maternal organ dysfunction, or uteroplacental dysfunction [3, 4••]. Thus far the only effective treatment is premature delivery and consequent early placental delivery, which is associated with the risk of neonatal morbidities [5].

The development of PE is a complex process that involves a number of dysfunctional physiological processes. A key event is abnormal placentation [6], characterized by reduced trophoblast invasion, inadequate remodeling of the maternal spiral arteries, and consequent placental ischemia [7]. This reduction in placental perfusion leads to placental hypoxia and oxidative stress [6]. The hypoxic placenta consequently secretes increased levels of anti-angiogenic markers, including soluble fms-like tyrosine kinase (sFlt-1) and soluble endoglin (sEng), into the maternal circulation, thereby inhibiting the bioavailability of proangiogenic vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) [8, 9]. This angiogenic imbalance precedes the maternal syndrome of new-onset hypertension with or without the presence of proteinuria and with systemic endothelial dysfunction [10]. Preeclampsia is further categorized into early-onset PE, which can be diagnosed prior to 34 weeks of gestation and late-onset PE that is diagnosed from 34 weeks [11]. Early-onset PE is associated with abnormal

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placentation and fetal growth restriction, while late-onset PE is linked with maternal endothelial dysfunction [12].

While the precise etiology remains unclear, poor maternal and fetal outcomes continue to be exacerbated by inaccuracies in the identification and early diagnosis of women at high risk of PE development, especially in low- and middle-income countries. Therefore, identifying an appropriate animal model that mimics aspects of PE etiology will advance the current understanding of the conceptual framework underlying its development. However, since PE is a disorder of extreme heterogeneity, developing a gold standard model in this field remains challenging.

Current therapeutic interventions include anticoagulants such as aspirin, antihypertensive drugs such as labetalol, methyldopa, and nifedipine, as well as magnesium sulfate to prevent seizures [13]. Low-dose aspirin is reported to exert a prophylactic effect, by lowering the risk of early-onset PE development if administered before the 16th week of gestation [14]. However, it is ineffective in decreasing the risk of late-onset PE development [15]. Moreover, PE is identified by the American Heart Association, as a risk factor for impending circulatory disorders [16] and stroke [17]. Thus far, treatment strategies are only effective in managing the symptoms associated with PE and cannot be used to cure this disorder. With the absence of definitive treatment options and ethical limitations associated with the research in pregnancy, the development of animal models that cover the pathological aspects of PE is essential to increase our understanding of the disorder. Moreover, these models will enable the evaluation of novel treatment strategies in determining the safety and effectiveness of treatment interventions prior to clinical trial testing [18••].

The progress made towards understanding the development and progression of PE has seen a greater demand for the identification of novel agents for the treatment or prevention of PE [5]. Effective therapeutic interventions apart from delivery of the placenta would significantly improve maternal and neonatal health and pregnancy outcomes [5]. Despite the availability of extensive literature surrounding various models of PE development, this review will examine and provide a summary of the most extensively studied, as well as novel rodent models that have been used to study hypertension, proteinuria, maternal organ dysfunction, and fetal growth restriction in PE development.

Animal Models of PE

Animal models to study PE development have been implemented using various methods. Preeclampsia may be induced via surgical and [19–21], pharmacological intervention [22, 23••], genetic, and immunological modification [24, 25••, 26, 27] and via the use of animals with pre-existing hypertension that develop superimposed PE [28, 29]. These models exhibit aspects of PE such as pregnancy-induced hypertension, elevated urinary protein levels, renal dysfunction, placental ischemia, and fetal growth restriction [18••]. However, the primary focus of several of these PE models lie in reproducing the maternal syndrome, and therefore, only a limited number of studies address initiating factors and primary stages of PE development [30].

Examining the initiation and progression of fetoplacental disorders in the first trimester in humans remains a challenge due to the potential danger associated with both maternal and fetal wellbeing [30]. Moreover, human clinical studies are associated with constraints that hinder a comprehensive investigation of the time-dependent processes that occur in PE development [31••]. In contrast, rodent models of PE support the study of the mechanisms that initiate development and progression of this disorder [23••, 25••, 32], since they have short gestations relative to humans. This permits the investigation of specific aspects of this multifactorial disorder, forming the preclinical basis for experimental testing and supporting the development of predictive tests and therapeutic strategies [6]. Moreover, in vivo, in vitro, and molecular methods may be explored to examine the underlying processes elicited by novel therapeutic interventions as well as effecting proof of concept experimental studies [18••, 31••].

Animal models have provided valuable insights into our knowledge of PE, including the mechanisms of deficient trophoblast migration [24, 33, 34] and placental ischemia [19, 35]. They have added to our understanding of endothelial dysfunction arising from the release of various factors from the hypoxic placenta [31••, 36]. Moreover, they have contributed significantly to the development of novel diagnostic strategies such as angiogenic screening platforms and immunoassays to aid in PE diagnosis [25••]. The use of Triage PlGF and the Elecsys immunoassay sFlt-1/PlGF ratio tests in conjunction with standard clinical assessment and subsequent clinical follow-up have been fully endorsed by the National Institute for Health and Care Excellence (NICE) [37]. Moreover, their clinical potential in helping to diagnose or rule out PE in women presenting with suspected symptoms between 20 and 34 weeks is undeniable. Albeit, the lack of sufficient evidence regarding their accuracy prevents their use as standard procedure for PE diagnosis [37].

Additionally, the accuracy of the BRAHMS Kryptor sFlt-1 and PlGF assays has been investigated, and the results have been found to be comparable to the Elecsys assays for sFlt1 and PlGF [38, 39•]. When used in combination with standard clinical methods of evaluation, the KRYPTOR assays have also displayed their utility in predicting the risk for PE-associated short-term adverse maternal and perinatal outcomes occurring within 2 weeks of presentation in

women with suspected PE [38]. A recent study has demonstrated the KRYPTOR assays ability to rule in or rule out PE within a week and also proposes the clinical implementation of a simpler single decision sFlt1/PlGF ratio threshold of 66 as opposed to the currently used gestation-specific dual thresholds [39•].

Rodent Models of PE

Rodent models have substantially contributed to the conceptual framework underlying the pathogenic mechanisms enabling a better understanding of the clinical manifestation of PE (Fig. 1). A brief overview of these models is displayed in Table 1. Since PE is a placental disorder, it is crucial for PE models to share common anatomical and functional features of human placentation [30]. Humans, rats, and mice are among the species that share hemochorial placentation. In rodents, placental development commences with the invasion of trophoblast cells into the maternal decidua which is followed by the remodeling of the maternal spiral arteries [40]. Humans and rodents share a similar profile of immune cells, including uterine natural killer cells in the maternal decidua [40]. While mice have enabled the study of placentation [41, 42], they exhibit shallow intrauterine trophoblast invasion, and placentation is superficial [43]. This is in direct contrast to human and rat placentation which is characterized by extensive trophoblast invasion and uterine arterial remodeling [44]. Furthermore, humans and rats share a similar discoid placental shape and deep invasiveness of the placenta but do differ in histological structure, as humans have a hemomonochorial structure, while rats display a hemotrichorial placenta [44, 45].

Additionally, both mice and rats mimic human pregnancy-induced cardiovascular changes such as hypotension in early pregnancy, reduced pressor responses to angiotensin II, and decreased hematocrit, as well as elevations in cardiac output, stroke volume, and plasma volume [46, 47].

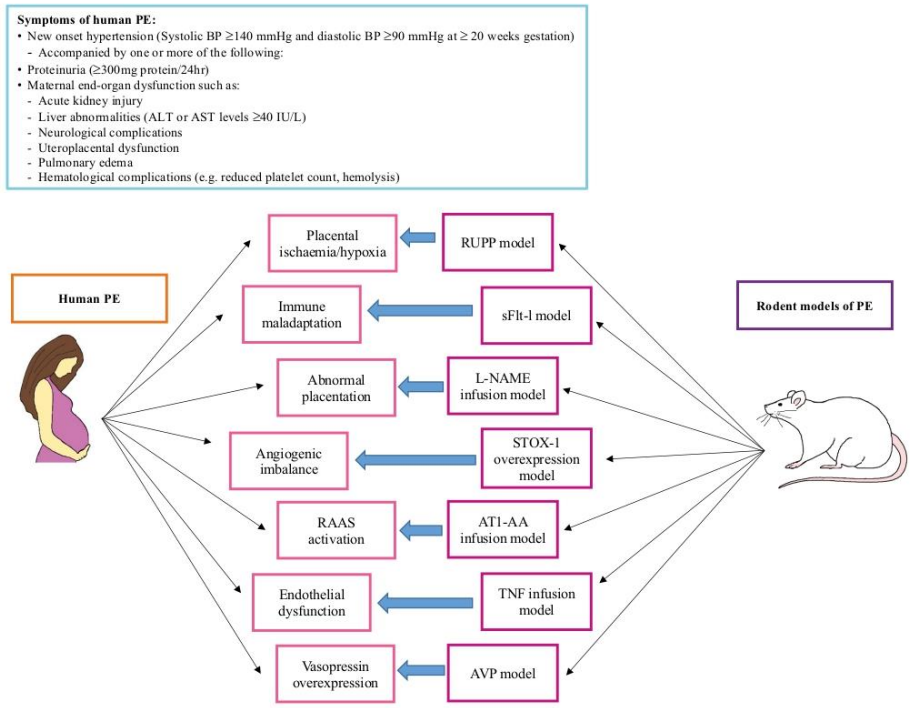

Fig. 1 Various rodent models employed to study the pathogenic pathways associated with PE development

Table 1 A brief overview of current rodent and murine models of PE

Model	Phenotypic induction	Potential mechanism	Species	Clinical features	References
Reduced uterine perfusion pressure	Surgical	Placental ischemia	Rat	↑blood pressure, ↑urinary protein, ↑sFlt-1, ↑sEng, ↓PlGF, ↓VEGF, ↑ROS, ↑AT1-AA, ↑TNF-a, ↑regulatory T cells, ↑natural killer cells, ↑endothelin-1, ↓nitric oxide, ↓renal plasma flow, ↓GFR, ↓placental weight, fetal growth restriction	[19–21]
BPH/5	Spontaneous	Pre-existing hypertension	Mouse	↑blood pressure, ↑ urinary protein, ↑endothelial dysfunction, ↑glomerulosclerosis, ↓placental weight ↑fetal mortality, fetal growth restriction, ↓VEGF, ↓PlGF	[6, 29, 64]
Ad-sFlt-1 (vector)	Genetic	Angiogenic imbalance	Rat	↑blood pressure, ↑urinary protein, glomerular endotheliosis	[25••]
L-NAME	pharmacological	Endothelial dysfunction	Rat	↑blood pressure, ↑ urinary protein, ↑sFlt-1, ↓placental weight, fetal growth restriction	[22]
TNF-α	Pharmacological	Immune activation	Rat	↑blood pressure, ↑prepro-endothelin-1, ↓nitric oxide synthase, ↑AT1-AA	[93, 94]
Indoleamine 2,3-Dioxygenase knockout	Genetic	Immune activation	Mouse	↑blood pressure, ↑glomerular endotheliosis, fetal growth restriction, ↑urinary protein	[26, 27]
AT1-AA	Pharmacological	RAAS activation	Rat	↑blood pressure, ↑NK cells, ↑sFlt-1, ↑sEng, ↑prepro-ET-1, ↑AT1-AA	[50, 102, 104]
TLR3	Pharmacological	Immune activation	Rat	↑blood pressure, ↑systemic inflammation, ↑urinary protein, endothelial dysfunction	[105]
TLR7/8	Pharmacological	Immune activation	Mouse	↑blood pressure, endothelial dysfunction, splenomegaly, ↑placental inflammation	[108, 109]
TLR9	Pharmacological	Immune activation	Rat	↑blood pressure, ↑vasoconstriction, ↑vascular oxidative stress, ↑inflammation	[110]
STOX1	Genetic	Abnormal placentation	Mouse	↑systolic pressure, ↑ urinary protein, ↑renal capillary swelling, ↑sFlt-1, ↑sEng, fetal growth restriction, ↑cardiac hypertrophy, ↑renal artery resistance	[24, 33, 118, 119]
Dahl salt-sensitive rat	Spontaneous	Pre-existing hypertension	Rat	↑blood pressure, ↑ urinary protein, ↑sFlt-1, glomerulomegaly, fetal growth restriction	[28, 121]
Regulatory T cell depletion	Immunological	Immune activation	Mouse	blood pressure (no change), ↑TNF-a, ↑IFN, ↑IL-6, ↑IL-17, ↑MCP-1, ↑CXCL1, ↑fetal mortality	[130]
Arginine vasopressin	Pharmacological	Vasopressin (CNS)	Rat	↑blood pressure, ↑glomerular endotheliosis, ↑urinary protein, fetal growth restriction	[23••]

Ad-sFlt-1 adenovirus expressing soluble fms-like tyrosine kinase-1, *AT1-AA* angiotensin II type 1 receptor autoantibody, *BPH* blood pressure high, *CNS* central nervous system, *CXCL1* C-X-C motif chemokine ligand 1, *GFR* glomerular filtration rate, *IFN* interferon, *IL* interleukin, *L-NAME* L-NG-nitroarginine methyl ester, *MCP-1* macrophage chemoattractant protein-1, *NO* nitric oxide, *PlGF* placental growth factor, *ROS* reactive oxygen species, *sEng* soluble endoglin, *sFlt-1* soluble fms-like tyrosine kinase, *STOX1* storkhead box-1, *TLR* toll-like receptor, *TNF-a* tumor necrosis factor-a, *VEGF* vascular endothelial growth factor

Thus, the extensive use of mice and rats to study hypertensive disorders of pregnancy such as PE is not surprising [45, 48]. Numerous experimental models of PE are also associated with elevated tissue levels of prepro-endothelin-1 mRNA. These models have been used to investigate whether the inhibition of the endothelin pathway could improve

hypertension. Additionally, the reduced uterine perfusion pressure model, soluble fms-like tyrosine kinase rat model, BPH5 mouse model, and the nitric synthase inhibition (L-NAME) model have been explored as models to mimic PE development. The mean arterial pressure is reduced in the RUPP, sFlt-1 infusion, TNF infusion, and AT1-AA infusion models by the administration of the endothelin type A receptor antagonist [19, 49–51]. This implies that endothelin-1 is a potential common pathway in which placental factors exert their effects on the maternal vasculature to induce vasoconstriction and hypertension [31••]. These rodent models are also used to test vitamins (D and B) and drug (e.g., statins) interventions. Vitamin D administration in the RUPP model lowers blood pressure, endothelin-1, sFlt-1, and AT1-AA levels; however, fetal outcomes were not improved [52–54]. In contrast, the L-NAME model demonstrated reduced levels of sFlt-1 and TNF in response to vitamin D treatment [55].

Established Rodent Models of PE

Reduced Uterine Perfusion Pressure Model

The reduced uterine artery perfusion (RUPP) rat model is the most widely characterized experimental model of placental ischemia. It reproduces the PE phenotype of endothelial dysfunction, glomerular endotheliosis, hemodynamic changes, and higher circulating levels of sFlt-1 and sEng [21]. This model is produced by clipping the aorta and uterine ovarian arteries on gestational day 14, resulting in a 40% decrease in uteroplacental perfusion along with a 20–30 mmHg increase in maternal mean arterial pressure on gestational day 19 in comparison to control groups [21]. The RUPP model has made substantial inroads in elucidating the role of the adaptive immune system in PE development [30]. Zenclussen and co-workers studied the role of inflammatory T cells in PE development and showed that the transference of activated T helper-1-like cells into healthy mice leads to the development of PE-like symptoms [56]. The RUPP model demonstrates elevated levels of inflammatory CD4 + T cells [57], along with reduced anti-inflammatory regulatory T cell levels [58]. This model also found that the adoptive transfer of CD 4 + T cells from RUPP rats to control rats induced hypertension, proteinuria, glomerular endotheliosis with concomitant elevated cytokines, and anti-angiogenic expression in circulation [59]. Albeit a major limitation of this model is its inability to replicate the immune mechanisms, deficient trophoblast invasion, and abnormal remodeling of the spiral arteries since the clipping of the lower abdominal aorta and uterine arteries is conducted mid-pregnancy (gestational day 14) [21]. Moreover, liver dysfunction and intrauterine growth restriction associated with human PE development are not replicated.

Moreover in the RUPP rat model, pravastatin treatment downregulates blood pressure and reactive oxygen species and improves angiogenic balance [60]; however, this was not observed in early human clinical trials [61, 62]. Pravastatin administration in early-onset PE women reduces the incidence of poor fetal outcomes but does not influence the concentration of plasma sFlt-1 levels or the sFlt-1:PlGF ratio in comparison to the placebo group [61, 62]. However, the focus still remains on angiogenic factors sFlt-1, PlGF, and VEGF due to the importance of angiogenic imbalance in PE pathogenesis. The administration of PlGF in RUPP rats has been reported to lower sFlt-1 levels, blood pressure, and proteinuria [63].

BPH/5 Mouse Model

This is a spontaneous or superimposed model of PE since an existing mild hypertension is present in the non-pregnant mice. Hypertension increases with pregnancy together with endothelial dysfunction, elevated uterine vascular resistance, placental dysfunction, and reduced litter size [29, 64]. This model was created through the continued mating of inbred BPH (blood pressure high)/2 mice leading to the development of the BPH/5 mouse strain [65]. A study using the BPH/5 model found that the maternal phenotype of PE may be initiated by the increased decidual expression of cyclooxygenase-2 (COX-2) and interleukin-15 [6]. This model has also been used to determine the effectiveness of therapeutic intervention of proangiogenic factor, where adenoviral delivery of $VEGF_{121}$ inhibited the development of superimposed PE [66]. These findings highlight the therapeutic potential of early proangiogenic intervention in pregnancy-associated hypertensive disorders. Despite the pre-existing hypertension displayed by non-pregnant mice, the usefulness of this model in providing information of how pre-existing hypertension influences the pathogenesis of superimposed PE is valuable [30].

sFlt-1 Sprague Dawley Rat Model

The clinical importance of the sFlt-1/PlGF ratio encouraged research that evaluated its clinical value in the diagnosis and prediction of PE. This ratio has contributed substantially to the creation of automated angiogenic biomarker platforms (sFlt-1 and PlGF) to aid the diagnosis and prognosis of PE in high-income countries. Maynard and co-workers developed the novel experimental rat model which replicated clinical characteristics of PE [25••]. Their findings indicate that exogenous administration of sFlt-1 to pregnant rats produces hypertension, proteinuria, and glomerular endotheliosis, which are characteristic of PE [25••]. In contrast Thadhani and co-workers reported that sFlt-1 clearance by apheresis

improve angiogenic balance in preeclamptic women and demonstrated lowered mean arterial pressure and extended gestation by up to 15 days [67, 68].

Maynard's breakthrough study thus endorsed the development of the immunoassays, Alere Triage PlGF test and the Elecsys sFlt-1/PlGF from Roche, which have the potential to exclude PE diagnosis in women with suspected PE between 20 and 34 weeks [69]. The BRAHMS Kryptor sFlt-1 and PlGF assays have also demonstrated comparable accuracy to the Elecsys assays for sFlt1 and PlGF when ruling in or ruling out PE [38, 39•].

Additionally, this model was used to study the long-term effects of pregnancy-induced hypertension on maternal and fetal outcomes. The offspring of sFlt-1 treated mice exhibited elevated blood pressure levels and also reported that baseline maternal cardiovascular function was not adversely affected postpartum [70]. Off note, a shortcoming of this model is its inability to reproduce the liver dysfunction and intrauterine growth restriction associated with human PE development.

Nitric Oxide Synthase Inhibition (L-NAME)

The nitro-L-arginine methyl ester (L-NAME) model is a model of endothelial dysfunction associated with PE development. In PE, the nitric oxide pathway is defective and polymorphisms in nitric oxide synthase (NOS) exist [71, 72]. Using a rodent model, the administration of L-NAME inhibits NOS and produces PE-like characteristics such as high blood pressure, proteinuria, decreased glomerular filtration rate, and intrauterine growth restriction [73–76]. Additionally, early-onset PE (EOPE) and late-onset PE (LOPE) phenotypes may also be produced by altering the timing of L-NAME administration in pregnant Sprague Dawley rats [32]. The L-NAME rat and mouse models of PE have been utilized in testing potential treatment and biomarker predictor tests for PE development. Administration of sildenafil improves hypertension, proteinuria, and fetal outcomes in early- and late-onset cases of PE [36, 74, 77, 78] and downregulates plasma sFlt-1 and sEng levels [22]. Furthermore, urinary nephrin and podocin mRNA levels are significantly higher in both EOPE and LOPE L-NAME-treated rats in comparison to pregnant control rats, suggestive of the presence of podocyturia. A significant reduction in urinary mRNA levels of podocin in EOPE rats and nephrin levels in LOPE rats was demonstrated following treatment with sildenafil citrate [79].

In contrast, sildenafil treatment administered late in gestation to rats and pregnant women with PE is associated with poor outcome [80, 81]. The uncertainty of NOS regulation in PE development raises concerns regarding the validity of the L-NAME model in PE investigations [18••, 30, 65, 80, 82, 83•]. Despite the polymorphism in the NOS gene displayed in pregnant women with severe PE [71, 72], inconsistencies are reported in studies involving the genetic deletion of NOS in pregnant mice [84–86]. Reduced blood pressure has been reported in pregnant NOS knockout mice [85], in contrast to higher blood pressure observed in non-pregnant NOS knockout mice [84]. Non-pregnant mice administered with L-NAME also show elevations in blood pressure with aortic vascular contraction, suggestive that the PE-like symptoms produced by this model may not be pregnancy-specific [86].

Tumor Necrosis Factor Alpha Model

Tumor necrosis factor alpha (TNF) is a pro-inflammatory cytokine involved in physiological processes such as cellular proliferation and differentiation, apoptosis, cell proliferation, differentiation, apoptosis, and inflammation [87]. Higher levels of TNF are reported in preeclamptic compared to normotensive pregnancies and hypertensive pregnancies uncomplicated by PE [88–91]. Women with pregnancies complicated by early-onset PE have significantly elevated levels of serum TNF in contrast to women with late-onset PE [92]. Normotensive pregnant rats administered with TNF (50 ng/d) between gestational days 14–19 develop hypertension and express elevated levels of renal, placental, and aortic prepro-endothelin-1 [93]. The administration of an endothelin type A receptor antagonist in pregnant rats ameliorates hypertension induced by TNF [51]. Additionally this model proposes that TNF-induced hypertension may emanate from the decline in renal NOS expression [93] and increase in AT1-AA production [94]. A major limitation observed in this model is that the PE phenotypes produced in response to chronic administration of TNF may be an exaggeration of the pro-inflammatory state of pregnancy [83•].

Indoleamine 2,3-Dioxygenase Knockout Model

Indoleamine 2,3-dioxygenase (IDO) is a cytosolic heme-protein which catalyzes the rate-limiting step in tryptophan breakdown [83•] and is essential in the T cell-mediated immune response [95]. The IDO mouse model is produced via IDO inhibition using 1-methyl-tryptophan [27]. These mice develop high blood pressure or proteinuria, and their placentae express edematous changes and fibrin deposits, and there is no fetal growth restriction [27]. Santillan and co-workers reported glomerular endotheliosis, intrauterine growth restriction, and proteinuria in IDO-knockout mice; no changes in placental morphology and blood pressure were observed [26]. Preeclamptic women display downregulated placental levels of IDO [96–98]; interestingly, this is not observed in placentae from pregnancies with fetal growth restriction but without hypertension [97–99]. Late-onset PE patients demonstrate significantly lower IDO expression on endothelial cells in comparison to women with early-onset

PE [100]. A shortfall of this model is its inability to fully mimic the clinical characteristics of hypertension and placental abnormalities associated with human PE development; however, it supports investigations surrounding placental inadequacy and renal dysfunction in pregnancy.

Angiotensin II Type 1 Receptor Autoantibody Model

The angiotensin II type 1 receptor autoantibody (AT1-AA) mouse model was generated by administering AT1-AAs obtained from preeclamptic women into mice on day 13 of gestation [101]. These mice displayed significantly higher blood pressure, proteinuria, glomerular endotheliosis, elevated sFlt-1 and sEng levels, smaller placentae, and fetal growth restriction compared to the control group [101]. Normal pregnancy and PE are significantly affected by the renin-angiotensin system (RAS). Angiotensin II type 1 receptor autoantibodies (AT1-AA) are reported to be higher in some women with PE and are linked to other disorders such as systemic sclerosis, tissue fibrosis, hypertension, and reno-vascular disease [102]. These autoantibodies influence vasoconstriction and increase blood pressure through the stimulation of angiotensin II type 1 (AT1) receptors with transduction of signals via the MAPK/ERK pathway [102, 103]. LaMarca and co-workers have reported that pregnant rats infused with purified AT1-AA between gestational days 12–19 demonstrated high blood pressure and high serum AT1-AA levels as well as dysregulated angiogenic factor levels and increased tissue levels of prepro-endothelin-1 in comparison to normotensive pregnant control rats [50]. The endothelin system may play a role in the elevation of blood pressure induced by AT1-AA administration; this theory is corroborated by the report of endothelin type A receptors inhibiting the blood pressure response in AT1-AA-infused rats [104]. These studies display a significant interaction between inflammatory and angiogenic markers that are released in response to placental ischemia. A disadvantage of the AT1-AA mouse model is that some aspects observed are not specific to pregnancy. The study by Zhou and co-workers also assessed the involvement of AT1-AAs independently of excess sFlt1; they reported elevations in blood pressure in non-pregnant mice following treatment with IgG isolated from preeclamptic women [101]. In these animals, renal injury and elevations in urinary protein and sFlt-1 levels were absent, suggesting that AT1-AA in non-pregnant women would chronically induce high blood pressure in an autoimmune manner. Furthermore, in the case of pregnancy, PE symptoms may not be resolved post-delivery [83•].

Toll-Like Receptor Rat Model

The Toll-like receptor (TLR) rat model was induced by the activation of TLR3 in pregnant rats with a viral mimetic called polyinosinic:polycytidylic acid [105]. This results in elevated maternal blood pressure, systemic inflammation, proteinuria, and endothelial dysfunction [105]. This study was the first to demonstrate that activation of TLR signaling during pregnancy adversely effects maternal cardiovascular function. These receptors are a class of pattern recognition receptors which induce signaling cascades to elicit appropriate inflammatory responses to pathogen- and damage-associated molecular patterns [106]. These receptors are present at the maternal–fetal interface and function to ensure a successful pregnancy outcome; conversely, excessive TLR signaling may induce maternal systemic inflammation with adverse pregnancy outcomes [107]. This rat model has since been replicated in pregnant mice [108, 109], and mice treated with synthetic ligands for TLR7/8 have produced similar outcomes as earlier reports [109]. Low-dose of unmethylated CpG DNA administered to female rats have been shown to stimulate TLR9 signaling in late gestation and increases blood pressure, vasoconstriction, vascular oxidative stress, and inflammation [110]. Earlier studies in mice showed that activation of TLR9 with fetal DNA or high doses of CpG DNA resulted in poor fetal outcome, including increased fetal resorption and malformations [111–113]. The findings of these studies imply that the TLR-induced initiation of the innate immune system plays a role in the development of hypertension in pregnancy.

STOX1 Mouse Model

Storkhead box-1 (STOX1) is a transcription factor found in column extravillous trophoblast cell populations and facilitates cytotrophoblast invasion during normal placentation by regulating α-T-catenin expression [114, 115]. Alterations in STOX1 expression have been implicated in PE development [116, 117]. Overexpression of STOX1 in pregnant mice elevates systolic blood pressure in early pregnancy, proteinuria, occlusion of renal capillaries, fibrin deposition, and increases sFlt-1 and sEng expression [24, 33]. The increase in systolic blood pressure precedes placental development and indicates that abnormal placental development may not be responsible for the hypertension observed in this model. This finding also suggests that the placenta may not be the initiating organ of PE development [83•]. This model also reports increased renal artery resistance, cardiac hypertrophy, fetal growth restriction, and greater umbilical resistance [118, 119] and demonstrates left ventricular hypertrophy, cardiac fibrosis, and markers of inflammation and cellular stress up to 8 months postpartum [120]. A benefit of this model is its ability to replicate the early pathogenic aspects of PE as well as the later systemic features. However, a limitation of the STOX1 mouse model is the observation of increase in blood pressure in early gestation

and non-comparable to what is observed in preeclampsia. Therefore, the hypertension observed cannot be attributed to abnormal placentation.

Dahl Salt-Sensitive Rat Model

The Dahl salt-sensitive rat has pre-existing hypertension and during the course of gestation develops further PE-like symptoms such as increase in blood pressure, proteinuria, glomerulomegaly, placental hypoxia, fetal growth restriction, and higher circulating levels of TNF and sFlt-1 [28]. This model represents a spontaneous or superimposed rat model of PE. Gillis and co-workers used this model to validate the usefulness of sildenafil citrate treatment in PE [121]. Their data confirm that administration of sildenafil citrate between gestational day 10 and 20 alleviated further increase in blood pressure and proteinuria, decreased uterine artery resistance, and aided fetal growth [121]. Nonetheless, a disadvantage of this model is the pre-existing hypertension exhibited by pregnant rats [30].

Arginine Vasopressin Mouse Model

Hypertension induced by arginine vasopressin (AVP) is characterized by low circulating renin-angiotensin system activity, which is also found in PE compared to normotensive pregnant women [23••]. AVP exerts its physiological functions via V1a and V2 receptors [122]; the activation of these receptors has been implicated in proteinuria, renal glomerular endotheliosis, and intrauterine growth restriction, respectively. Additionally, the V1b receptor is a regulator of adrenocorticotropic hormone secretion, which can exert its effects on the immune system and blood pressure [123]; cullin-5 plays a role in angiogenesis [124], while the oxytocin receptor is involved in pregnancy and labor [125].

Studies by Santillan and co-workers highlight the role of AVP in PE development; however, chronic infusion of AVP does not reproduce placental hypoxia in mice which is a characteristic of human PE [122]. This model demonstrates the potential of AVP as both a predictive biomarker for PE development as well as an initiator of this disorder. Plasma copeptin levels, a biomarker of AVP, was reported to be significantly higher at 6 weeks of gestation in PE cases compared to normotensive pregnancy [23••]. The chronic administration of AVP in pregnant mice replicated pregnancy-specific hypertension, glomerular endotheliosis, proteinuria, and intrauterine growth restriction, thus supporting the role of AVP in PE progression and copeptin as an early biomarker for PE prediction [23••].

Santillan and co-workers have further expanded this work, in that AVP infusion into pregnant C57BL/6 J mice reduced the levels of placental expression of placental growth factor, altered placental morphology, placental oxidative stress, and placental gene expression consistent with the characteristic features of human PE [122]. They have also demonstrated that AVP infusion throughout gestation in mice promoted pro-inflammatory T_H1-associated interferon gamma in maternal plasma [126]. The effectiveness of AVP in inducing PE-like symptoms in a mouse model was therefore successfully demonstrated. This model proposes the concept that regulators of blood pressure are activated in the early stages of pregnancy,and could therefore be a potential new model for studying the origins of PE [127].

In addition, our laboratory has extrapolated the AVP mouse model of Santillan and co-workers to a Sprague Dawley rat model [128••]. Our findings demonstrate that chronic AVP infusion (150 ng/h) in pregnant rats over 18 days successfully reproduced the PE phenotype of elevated blood pressure (≥ 140/90 mmHg) [10], increased urinary protein levels, and fetal growth restriction. Albeit, our study models a mild case of PE development, and future studies should explore higher AVP dosages to induce more severe features of PE development. Additionally, our study did not confirm the levels of angiogenic markers such as PlGF and sFlt-1 which are commonly associated with PE.

Biochemical analysis revealed significantly upregulated serum alanine transaminase and triglyceride levels along with downregulated high-density lipoprotein levels in pregnant AVP-treated rats. We further demonstrate alterations in kidney morphology including a mild increase in mesangium, mild glomerular crescents, and reduced Bowman's space in AVP-treated rats. An earlier study performed by our lab assessed liver injury in the AVP rat model and found that serum expression of the liver injury enzymes arginase and 5′-nucleotidase, as well as transforming growth factor-2, was significantly higher in pregnant rats treated with AVP [129••]. Our findings are indicative of acute pregnancy-initiated liver dysfunction and support the utility of this model in the study of PE development.

The novel AVP mouse model highlights the potential use of AVP as a predictive biomarker for PE development. This model recapitulates phenotypes consistent with human PE, most notably pregnancy-specific hypertension. The activation of V1a and V2 receptors has been implicated in proteinuria and renal glomerular endotheliosis and intrauterine growth restriction, respectively, in this model. Future studies using the AVP model should explore the roles of the V1b, the oxytocin receptor, and cullin-5 (VACM-1) in the pathogenesis of this disorder.

Conclusion

Despite the advances made in understanding PE development, it continues to be a leading cause of maternal and fetal mortality and morbidity worldwide. This review provides

a brief overview of various rodent and murine models that mimic PE development while also highlighting the associated limitations. We report that there is no ideal rodent model to date that fully epitomizes the phenotype of PE such as abnormal placentation, fetal growth restriction, pregnancy-specific hypertension, proteinuria, endothelial dysfunction, and an imbalance in angiogenic factors. Despite the invaluable contribution of the different models, they do not unravel the early events in PE development that precede abnormal placentation. We advocate that the combined use of different models is still required to enable novel developments regarding PE pathogenesis and treatment. However, as the advances made in this field of research continue to grow, the refinement of these models will undoubtedly occur, leading to the discovery of new aspects of this disorder.

Author Contribution All authors contributed to the conception and design of the manuscript. The first draft of the manuscript was written by Sapna Ramdin, and the work was critically revised and edited by Sooraj Baijnath, Thajasvarie Naicker, and Nalini Govender. All authors read and approved the final manuscript.

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Compliance with Ethical Standards

Ethics Approval Ethical approval was not required as our study did not involve humans or animals.

Conflict of Interest The authors have no competing interests to declare that are relevant to the content of this article.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- • Of importance
- •• Of major importance

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CHAPTER 3

Research article: Physiological characterization of an arginine vasopressin rat model of preeclampsia

This chapter consists of a research article that provides a comprehensive physiological characterization of the arginine vasopressin-induced rat model of preeclampsia to determine its value in studying the pathophysiology of preeclampsia.

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RESEARCH COMMUNICATION

Physiological characterization of an arginine vasopressin rat model of preeclampsia

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ABSTRACT

Rodent models have contributed greatly to our understanding of preeclampsia (PE) progression in humans, however to-date no model has been able to effectively replicate the clinical presentation of the disease. This study aimed to provide a thorough physiological characterization of the arginine vasopressin (AVP)-induced rat model of PE to determine its applicability in studying the pathophysiology of PE. Female Sprague Dawley rats (n = 24) were separated into four groups (n = 6 per group) viz., pregnant AVP, pregnant saline, non-pregnant AVP, and non-pregnant saline. All animals received a continuous dose of either AVP (150 ng/h) or saline via subcutaneous mini osmotic pumps for 18 days. Full physiological characterization of the model included measuring systolic and diastolic blood pressure, and collecting urine and blood samples for biochemical analysis. AVP infusion significantly increased blood pressure and urinary protein levels in the pregnant rats ($p < 0.05$). Biochemical markers measured, differed significantly in the AVP-treated *vs* the pregnant saline groups ($p < 0.05$). Placental and individual pup weight decreased significantly in the pregnant AVP *vs* pregnant saline group ($p < 0.05$). The physiological and hematological data confirm the usefulness of this rat model in the study of PE, since AVP-induced vasoconstriction increases peripheral resistance and successfully mimics the pathological changes associated with PE development in humans.

Abbreviations: PE: preeclampsia; AVP: arginine vasopressin; ISSHP: International Society for the Study of Hypertension in Pregnancy; ACOG: American College of Obstetricians and Gynecologists; RUPP: reduced uterine perfusion pressure; sFlt-1: soluble fms-like tyrosine kinase; VEGF: vascular endothelial growth factor; PlGF: placental growth factor; AVP: arginine vasopressin; PAVP: pregnant AVP-treated; PS: pregnant saline; GD: gestational day; ALT: alanine transaminase; NAVP: non-pregnant AVP-treated; NS: non-pregnant saline; AST: aspartate aminotransferase; HDL: high-density lipoprotein; RBC: red blood cell; RAAS: renin-angiotensin aldosterone system; HELLP: hemolysis, elevated liver enzymes, low platelet.

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Introduction

Preeclampsia (PE) is a multifactorial, multi-organ hypertensive disorder manifesting after 20 weeks of gestation, impacting 5–8% of all pregnancies, making it the leading cause of maternal and fetal morbidity and mortality globally (Marshall et al. 2018). Despite advancements in understanding the pathophysiology of PE, its early diagnosis remains a challenge. Initially, PE was characterized by *de novo* hypertension coupled with proteinuria (Brown et al. 2018) however, the International Society for the Study of Hypertension in Pregnancy (ISSHP) and the American College of Obstetricians and Gynecologists (ACOG) have redefined PE, stating that proteinuria is not mandatory for PE diagnosis and allowing for the inclusion of cases that exhibit signs of renal, hepatic, and/or hematological pathologies (Brown et al. 2018).

Treatment and management of PE is hindered by late clinical presentation often warranting premature fetal delivery. Ethical consideration and large enough sample numbers make it difficult to study PE in humans (Sones and Davisson 2016), which has resulted in a shift toward the use of animal models. Various animal models have contributed greatly to our understanding of PE. These including the reduced uterine perfusion pressure (RUPP) (Li et al. 2012), soluble fms-

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like tyrosine kinase (sFlt-1) rodent model (Maynard et al. 2003) and BPH/5 mouse models (Davisson et al. 2002) have been widely used to study the disorder. However, these models are limited in that the RUPP and sFlt-1 models are unable to reproduce liver dysfunction and intrauterine growth restriction associated with PE development (Maynard et al. 2003; Li et al. 2012), while the BPH/5 model indicates a preexisting hypertension in non-pregnant mice (Davisson et al. 2002). The recent data demonstrate the effectiveness of some models in treating PE symptoms, for example; aspirin improved maternal and fetal symptoms in a preclinical model of PE (Li et al. 2018; Zhang et al. 2018). Moreover, vasodilators such as sildenafil ameliorates maternal phenotype in PE models, however, its fetal and placental effects are inconsistent (Motta et al. 2015; Gillis et al. 2016). Also, proangiogenic vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), improve PE phenotypes in soluble fms-like tyrosine kinase-1 (sFlt1)-induced preeclampsia (PE) rodent models (Spradley et al. 2016; Zhu et al. 2016). Despite the plethora of evidence, not all aspects of PE are fully understood. Therefore, it is essential that alternative animal models are available to further develop the study of PE pathogenesis.

Arginine vasopressin (AVP), a vasoconstrictor and antidiuretic hormone was recently used in the development of a murine PE model, which displayed all of the characteristic phenotypes of PE (Santillan et al. 2014). AVP plays a central role in the mechanisms related to PE development viz., vascular function, angiogenesis, renal dysregulation, and immunological abnormalities (Santillan et al. 2014). In non-pregnant states, AVP exerts its physiological effects via V1a receptors, inducing vasoconstriction and regulating neuronal and immune cell function (Koshimizu et al. 2012), as well as modulating angiogenesis in the hypothalamus (Alonso et al. 2008). It promotes water reabsorption in the kidney via V2 receptors (Guelinckx et al. 2016), thereby regulating blood pressure and blood volume (Koshimizu et al. 2012; Santillan et al. 2014). It also supports the regulation of the hypothalamic-pituitary-adrenal axis via V1b receptors, which indirectly alters immune, vascular, and renal functions (Buchwalter et al. 2008; Koshimizu et al. 2012). AVP is also involved in T helper 1 cell predominance and activation of dendritic and T helper 17 cells associated with PE development (Elenkov and Chrousos 2006; Russell and Walley 2010). Hence, the normal physiological role of AVP is similar to the mechanisms underlying the vascular, immune, renal, and angiogenic dysfunction associated with PE (Sandgren et al. 2015), warranting its investigation in PE development.

Despite the murine AVP model of PE producing symptoms similar to those observed clinically, this model has yet to be replicated in rats, a prototype which is preferred for pregnancy-specific disorders due to a similar placental development observed in humans (Soares et al. 2014). In a previous study conducted by our lab, we showed significantly increased serum levels of transforming growth factor beta-2 and a significant elevation in the activity of the liver injury enzymes arginase and 5′-nucleotidase suggestive of AVP-induced injury (Govender et al. 2021). Therefore, this study aimed to characterize the physiological, hematological, and biochemical changes associated with an AVP-induced rat model of PE, in order to determine its applicability in studying the pathophysiology of human PE.

Results

Preliminary dose-response study

The physiological dose-response outcomes supported the use of an AVP dose of 150 ng/h (Figure 1). Lower AVP dosages (50 ng/h and 100 ng/h) were unable to significantly elevate blood pressure and proteinuria nor did it reduce placental and pup weight. Pregnant rats treated with AVP (150 ng/h) showed significantly increased systolic blood pressure and urinary protein levels and reduced individual pup and placental weights, respectively (Figure 1).

Experimental study

Statistically significant elevations were noted for both systolic [143.00 (139.67–144.00) mmHg *vs* 125.00 (-124.00–127.00) mmHg; $p < 0.05$] and diastolic [103.50 (100.33–106.88) mmHg *vs* 81.00 (80.00–87.00) mmHg; $p < 0.001$] blood pressure throughout pregnancy in the pregnant AVP-treated (PAVP) vs the pregnant saline (PS) group (Figure 2A-B). The urine protein: creatinine ratio was increased in the PAVP compared to the pregnant saline group on gestational day (GD) 8 [PAVP: 0.79 (0.73–0.88) g/day *vs* PS: 0.49 (0.43–0.49) g/day; $p = 0.006$] and GD 14 [PAVP: 0.84 (0.74–0.94) g/day *vs* PS: 0.41 (0.39–0.44) g/day; $p = 0.0002$; Figure 2E]. Water intake decreased in the PAVP in comparison to the PS group on GD 8 and GD 14 (Figure 2C), whereas urinary output decreased significantly in PAVP rats [17.00 (12.00–20.00) ml] compared to the PS rats [25.50 (25.00–29.00) ml; $p < 0.05$; Figure 2D].

The hematology results show significantly higher serum alanine transaminase (ALT) levels in the PAVP

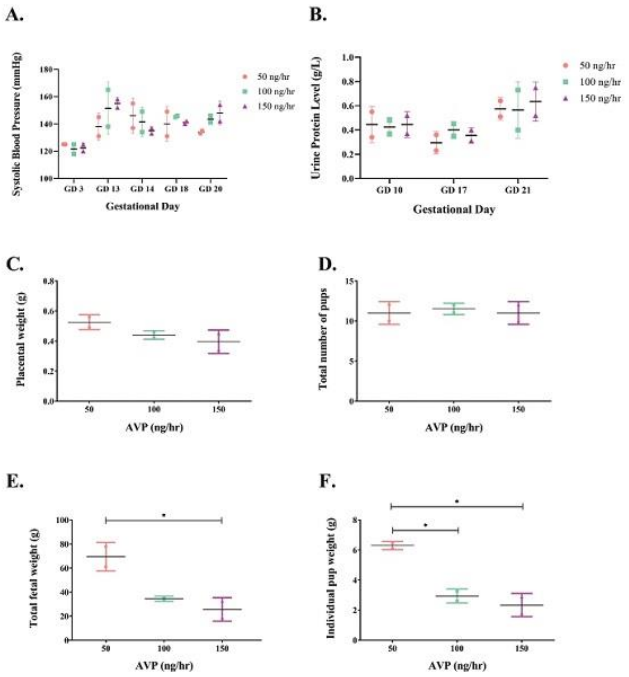

Figure 1. Effect of chronic AVP infusion (50, 100 and 150 ng/h) on (A) systolic blood pressure, (B) urinary protein concentration and (C-F) fetal and placental parameters in pregnant *Sprague-Dawley* rats. (A) Infusion of 150 ng/h AVP produced a larger increase in systolic blood pressure in pregnant rats than lower doses (50 and 100 ng/h). (B) Rats infused with 150 ng/h AVP showed a marked increase in urine protein levels in comparison to lower doses (50 and 100 ng/h). (C) Individual placental weight, AVP-infusion reduces placental weight. (D) Total number of pups, AVP-infusion does not induce a great change in the total number of pups produced. (E) 150 ng/h AVP infusion induced a significant reduction in total fetal weight in comparison to 50 ng/h. (**f**) Individual pup weight was significantly reduced pups produced from rats infused with 150 ng/h AVP in comparison to rats infused with 50 ng/h AVP. One-way ANOVA analyses followed by Tukey's multiple comparison analyses. Data is shown as mean ± SD, *$p < 0.05$.

[58.50 (57.00–60.00) IU/L] compared to the non-pregnant AVP-treated (NAVP) [40.00 (38.00–44.00) IU/L; $p < 0.001$] and saline-treated groups [non-pregnant saline (NS): 51.50 (46.00–54.00) IU/L; PS: 48.00 (45.00–49.00) IU/L; $p < 0.05$], with liver weight also significantly higher in the former group [12.39 (11.99–13.40) g; $p < 0.001$; Figure 3B & G]. Conversely, aspartate aminotransferase (AST) levels were significantly down-regulated in the PAVP [131.00 (130.00–135.00) IU/L] compared to both the NS [149.00 (143.50–150.00) IU/L] and NAVP [143.50 (140.00–148.00) IU/L; $p < 0.05$; Figure 3H] groups. A non-significant increase in AST levels was noted in the PAVP group in comparison to the PS group [129.50 (127.00–133.00) IU/L; $p > 0.05$; Figure 3H]. Despite down-regulated lactate dehydrogenase (LDH) levels in the PAVP [1300.00 (1176.50–1359.50) IU/L] *vs* the NAVP group [1431.00 (1400.00–1738.00) IU/L; $p < 0.05$; Figure 3I], both AVP-treated groups displayed significantly higher LDH levels when compared to the saline-treated groups [NS: 570.00 (487.00–761.50) IU/L; PS 538.00 (498.00–549.00) IU/L; $p < 0.05$].

Cholesterol and high-density lipoprotein (HDL) levels were significantly decreased in the PAVP [cholesterol: 1.10 (1.10–1.30) mmol/L; HDL: 0.65 (0.64–0.68) mmol/L] and NAVP groups [cholesterol: 1.20 (1.00–1.20) mmol/L; HDL:0.77 (0.70–0.79) mmol/L] *vs* the PS group [cholesterol: 1.29 (1.25–1.32) mmol/L; HDL: 0.94

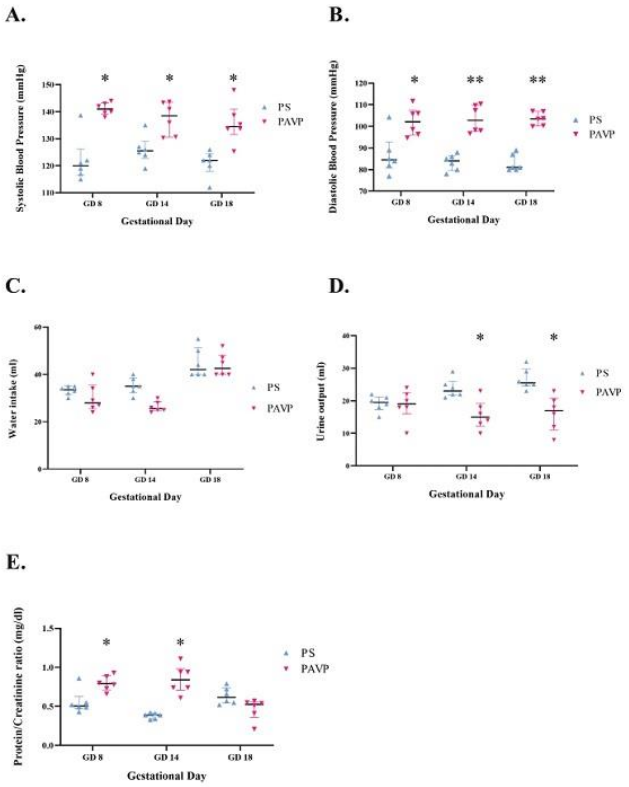

Figure 2. Clinical parameters (A-B) systolic and diastolic blood pressures (mmHg); (C) water intake (ml); (D) urinary output (ml) and (E) urine protein:creatinine ratio levels (mg/dL) across all study groups. Systolic (A) and diastolic (B) blood pressure was significantly increased in AVP-treated groups in comparison to saline groups. (C) Water intake is significantly upregulated in the pregnant groups in comparison to the non-pregnant rat groups. (D) Urine output is significantly reduced in the pregnant AVP-treated group as compared to the pregnant saline group (E) AVP infusion in pregnant rats induced significant elevations in urine protein: creatinine ratio levels in comparison to saline infusion on GD 8 and 14. Kruskal-Wallis and Dunn's post hoc multiple comparison tests were used to compare the medians between groups. Data is shown as median and interquartile range, *$p < 0.05$, **$p < 0.001$. # Data for non-pregnant (NS and NAVP) groups were within normal ranges and are not illustrated.

(0.91–0.96) mmol/L; $p < 0.05$; Figure 3D–E]. Triglyceride concentrations increased significantly in the PAVP [2.69 (2.16–3.05) mmol/L] compared to the NAVP [0.64 (0.58–0.69) mmol/L], NS [0.84 (0.47–1.09) mmol/L] and PS [1.27 (0.98–1.30) mmol/L] groups ($p < 0.05$; Figure 3F).

Blood glucose levels decreased significantly in the PAVP [4.35 (3.90–4.50) mmol/L] group in comparison with the NAVP [5.65 (5.40–6.40) mmol/L] and NS [6.30 (6.05–6.50) mmol/L] groups on GD18 ($p < 0.05$; Figure 3C). Non-significant reductions were noted for red blood cell (RBC) count, platelets, and hematocrit levels in the PAVP group compared to the NAVP group ($p > 0.05$; Table 1), the levels were above the normal reference range.

A significant reduction in the serum concentrations of sodium [139.00 (138.00–143.00) mmol/L *vs* 143.00 (141.00–143.00) mmol/L]; urea [4.50 (3.50–5.80) mmol/L *vs* 5.60 (5.50–6.00) mmol/L]; and chloride [98.00 (97.00–100.00) mmol/L *vs* 105.00 (104.00–106.00) mmol/L] was noted between the PAVP group compared to the NAVP group ($p < 0.05$; **Table 2**). Potassium levels decreased significantly in the PAVP

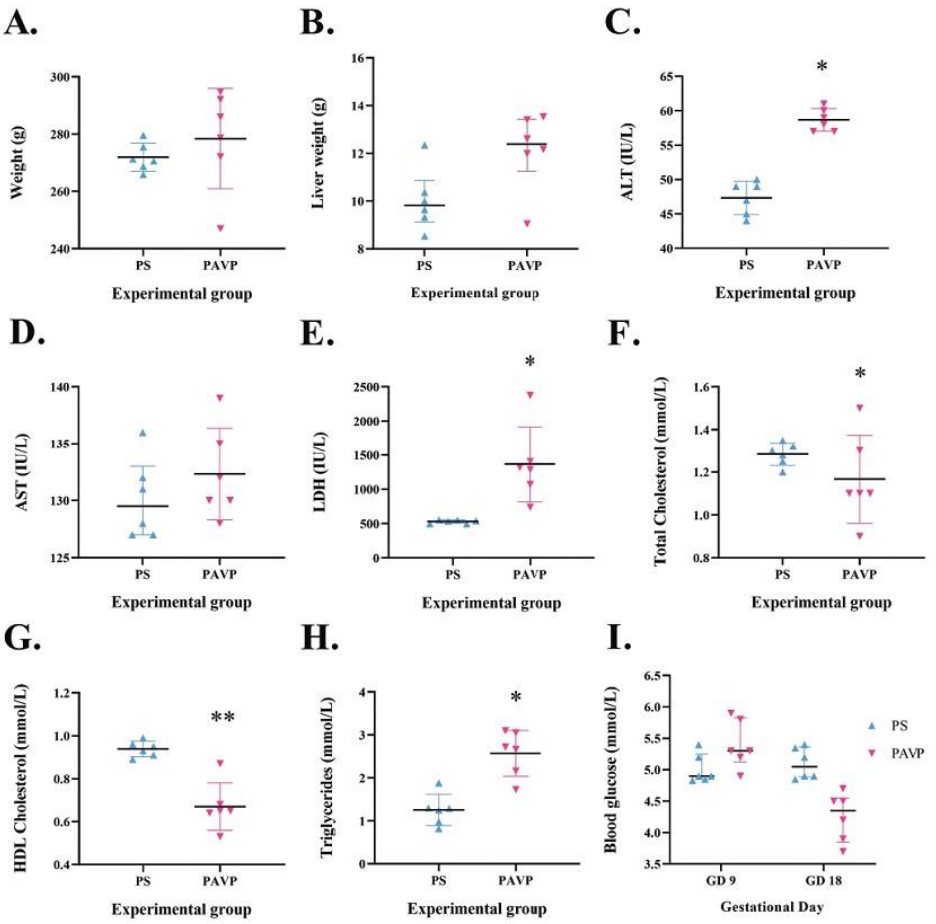

Figure 3. Metabolic parameters (A) weight (g) and (B) liver weight (g); and biochemical parameters (C) alanine aminotransferase (ALT, mmol/L); (D) aspartate aminotransferase (AST, mmol/L); (E) lactic acid dehydrogenase (LDH, mmol/L); (F) total cholesterol (mmol/L); (G) HDL cholesterol (mmol/L); (H) triglycerides (mmol/L) and (I) blood glucose (mmol/L), measured across all study groups on GD 18. (A) Pregnant rat groups demonstrate significant elevations in weight in comparison to the non-pregnant control groups. (B) Liver weight is increased in the PAVP group in comparison to the PS group. (C) ALT concentrations were significantly elevated in the PAVP *vs* the PS group. (D) AST concentrations are elevated in the PAVP group in comparison to the PS group. (E) LDH levels are significantly increased in the PAVP group in comparison to the PS group. (F) Total cholesterol and (G) HDL cholesterol is significantly reduced in the PAVP group in comparison to the PS group, while (H) Triglyceride levels in the PAVP group are significantly elevated as compared to the PS group. (I) Blood glucose levels are higher in the PAVP group when compared to the PS group on GD 9, conversely the PAVP group is reduced in comparison to the PS group on GD 18. Kruskal-Wallis and Dunn's post hoc multiple comparison tests were used to compare the medians between groups. Data is shown as median and interquartile range, $*p < 0.05$, $**p < 0.001$. # Data for non-pregnant (NS and NAVP) groups were within normal ranges and are not illustrated.

Table 1. Hematological parameters between the non-pregnant AVP and pregnant AVP groups on GD 18.

	Rat Group		
	Non-pregnant AVP (n = 6)	Pregnant AVP (n = 6)	*p* value
Complete Blood Count			
Red Cell Count (10^{12}/L)	8.00 (6.20–8.44)	7.10 (6.23–8.73)	0.909
MCV (fL)	63.10 (60.90–65.00)	64.20 (61.40–64.20)	0.909
RDW (%)	14.70 (13.10–15.90)	13.20 (12.50–16.30)	0.732
Hemoglobin (g/dL)	14.90 (12.00–15.90)	13.40 (12.60–14.20)	0.423
MCHC (g/dL)	29.60 (29.20–29.90)	29.30 (28.90–29.80)	0.424
MCH (pg)	19.00 (18.40–19.40)	18.80 (17.80–19.20)	0.566
White Cell Count (10^9/L)	4.72 (2.57–6.14)	5.07 (2.52–6.17)	0.909
Basophil count (10^9/L)	0.01 (0.00–0.01)	0.01 (0.00–0.01)	1.00
Eosinophil count (10^9/L)	0.11 (0.03–0.21)	0.10 (0.04–0.13)	0.732
Lymphocyte count (10^9/L)	3.33 (1.74–4.18)	4.03 (1.46–4.31)	0.569
Monocyte count (10^9/L)	0.26 (0.09–0.56)	0.28 (0.16–0.29)	0.909
Neutrophil count (10^9/L)	0.71 (0.71–1.38)	0.74 (0.49–1.71)	0.729
Hematocrit (%)	51.20 (46.20–53.00)	49.70 (48.10–52.30)	0.871
Platelets (10^9/L)	916.00 (856.00–973.00)	908.00 (895.00–929.00)	1.00

* $p < 0.05$ was considered statistically significant
Note: Data are presented as median (25^{th} percentile – 75^{th} percentile)
saline hematological control data were within normal range

Table 2. Serum electrolyte levels across experimental groups on GD 18.

	Rat Group				
	Non-pregnant Saline (n = 6)	Non-pregnant AVP (n = 6)	Pregnant Saline (n = 6)	Pregnant AVP (n = 6)	*p* value
Calcium (mmol/L)	2.42 (2.36–2.45)	2.36 (2.34–2.40) [d]	2.41 (2.40–2.42)	2.36 (2.35–2.36) [c g]	0.076
Chloride (mmol/L)	103.00 (100.00–104.00)	105.00 (104.00–106.00) [a d]	103.00 (99.00–104.00)	98.00 (97.00–100.00) [f]	0.012*
Creatinine (µmol/L)	35.50 (33.50–38.00)	29.00 (27.00–33.00) [a]	29.50 (27.00–34.00) [b]	28.50 (24.00–30.00) [c]	0.049*
Potassium (mmol/L)	5.00 (4.80–5.40)	4.40 (4.20–4.80) [a]	4.40 (4.20–4.70) [b]	4.55 (3.90–4.80) [c]	0.018*
Sodium (mmol/L)	140.00 (139.00–141.00)	143.00 (141.00–143.00) [a]	141.50 (139.00–145.00)	139.00 (138.00–143.00) [e]	0.113
Urea (mmol/L)	5.70 (5.45–5.95)	5.60 (5.50–6.00) [d]	4.50 (4.30–4.90) [b]	4.50 (3.50–5.80) [c e]	0.046*

* $p < 0.05$ was considered statistically significant
NS vs NAVP: [a] $p < 0.05$; NS vs PS: [b] $p < 0.05$; NS vs PAVP: [c] $p < 0.05$; NAVP vs PS: [d] $p < 0.05$; NAVP vs PAVP: [e] $p < 0.05$, [f] $p < 0.001$; PS vs PAVP: [g] $p < 0.05$
Note: Data are presented as median (25^{th} percentile – 75^{th} percentile)

Table 3. Fetal and placental parameters.

	Rat Group		
	Pregnant Saline (n = 6)	Pregnant AVP (n = 6)	*p* value
Total Placenta weight (g)	5.42 (4.68–5.94)	4.00 (3.38–5.16)*	0.04
Individual placenta weight (g)	0.59 (0.55–0.63)	0.39 (0.31–0.46)*	0.002
Individual Pup weight (g)	1.79 (1.70–1.85)	1.57 (1.45–1.67)*	0.02
Number of pups	9.00 (8.00–10.00)	12.00 (11.00–12.00)*	0.04

Note: Data are presented as median (25^{th} percentile – 75^{th} percentile)
* $p < 0.05$ was considered statistically significant

[4.55 (3.90–4.80) mmol/L] and NAVP [4.40 (4.20–4.80) mmol/L] *vs* the NS group [5.00 (4.80–5.40) mmol/L; $p < 0.05$; Table 2]. A reduction in creatinine levels was observed in both AVP groups [NAVP: 29.00 (27.00–33.00) µmol/L; PAVP: 28.50 (24.00–30.00) µmol/L; $p < 0.05$; Table 2].

Pup numbers increased significantly in the PAVP [12.00 (11.00–12.00)] *vs* PS groups [9.00 (8.00–10.00); $p < 0.05$; Table 3], however, the weight per pup was significantly lower in the PAVP [1.57 (1.45–1.67) g *vs* the PS groups [1.79 (1.70–1.85) g; $p < 0.05$]. We also demonstrate significantly lower individual placental weights in the PAVP [0.39 (0.31–0.46) g] *vs* the PS groups [0.59 (0.55–0.63) g; $p < 0.05$; Table 3].

Histological evaluation of liver, kidney and placenta

The histological examination of the liver in both the untreated and AVP treated groups appeared normal. In the kidney, mild mesangial cell increase together with an increase in mesangium (Figure 4A-B) was noted in the AVP-treated (NAVP and PAVP) in contrast to the untreated groups. Mild focal cellular glomerular

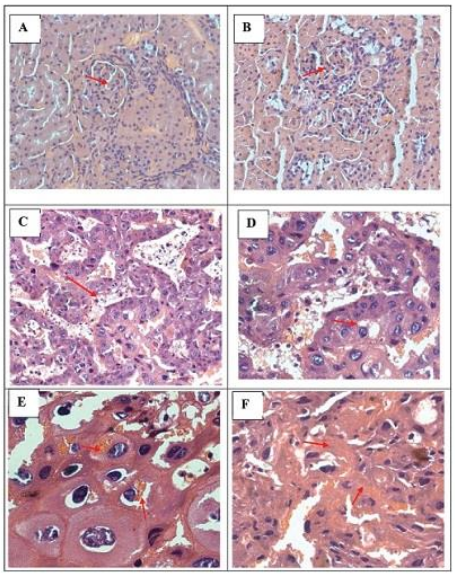

Figure 4. Histopathological examination of tissue sections from pregnant AVP treated rats (H&E stain) displaying (from top left plate clockwise; red arrows): (**A**-B: Kidney): mild mesangial cell increase, increase in mesangium and reduction in the Bowmans space, 40x magnification; (**C**-F: Placenta): occasional vacuolation along labyrinth trophoblast cells, 20x magnification; degenerative spongiotrophoblasts, 40x magnification; red blood cell phagocytosis, 40x; and fibrinoids, 40x respectively.

crescents with concurrent reduction in the Bowmans space was also evident (Figure 4A-B). In the placenta, the labyrinth trophoblast cells lining the vascular channels appeared normal with occasional vacuolation while the spongiotrophoblasts were frequently degenerative in the AVP-treated group *vs* the untreated group (Figure 4C-F). The spongiotrophoblasts were often necrotic, with phagocytic material visible (Figure 4E-F).

Discussion

This study demonstrates that AVP infusion (150 ng/h) resulted in significantly elevated blood pressure and urinary protein, features that typifies PE. Both systolic and diastolic blood pressure were significantly elevated throughout pregnancy in the PAVP *vs* the PS group. Similarly, chronic AVP infusion of 24 ng/h in a murine model demonstrated increased systolic blood pressure and negatively impacted fetal growth and development (Santillan et al. 2014). The short biological half-life of AVP prevents the direct measurement of secreted AVP (Santillan et al. 2014), hence, copeptin, a stable protein by-product of AVP synthesis released in a 1:1 ratio with AVP, is the substitute biomarker for measuring AVP secretion (Dobsa and Edozien 2013; Sandgren et al. 2015). In humans, higher plasma and serum copeptin levels in the preeclamptic compared to normotensive pregnancies is indicative of higher AVP levels as well [(serum: normotensive: 1.69 ± 1.02 ng/mL vs PE: 2.27 ± 2.00 ng/mL) (Yeung et al. 2014) and (plasma: normotensive: 0.31 ± 0.09 ng/mL vs mild PE: 0.62 ± 0.16 ng/mL) (Zulfikaroglu et al. 2011)].

Earlier studies also linked AVP to arterial blood pressure regulation (Jablonskis and Howe 1993; Song and Martin 2006; Li et al. 2012). The elevations in both systolic and diastolic blood pressure in our study throughout pregnancy in the PAVP group, suggests that arginine vasopressin stimulates the renin-angiotensin aldosterone system (RAAS). This results in vasoconstriction, which is mediated via the V1a receptor and consequently increases peripheral resistance and systemic blood pressure as observed in our study (Qian 2018). Myocardial atrial contraction results in an atrial-induced increase in end-diastolic pressure, which subsequently enhances ventricular contraction. Arginine vasopressin increases the impact of norepinephrine and Ang II on cardiac muscle and blood vessels thus altering hemodynamic function (Lee et al. 2003), and negatively affects myocardial contraction (Goldsmith 2005; Goldsmith and Gheorghiade 2005). Chronic hypertension results in diastolic dysfunction and consequent left ventricular hypertrophy thereby reducing cardiac compliance (Lorell and Carabello 2000). This results in a higher diastolic pressure–volume relationship where even minor elevations in left ventricular end-diastolic volume induces a significant rise in left ventricular end diastolic pressure (Gutierrez and Blanchard 2004). The pronounced effect of AVP on diastolic pressure may be due to the exaggerated interaction of AVP with the V1A and V2 receptors on peripheral blood vessels (Goldsmith 2005; Goldsmith and Gheorghiade 2005).

Despite the exclusion of proteinuria as a characterizing factor (ACOG 2013), it remains significant in PE diagnosis (Guida et al. 2018; Özkara et al. 2018; Tanacan et al. 2019). We report significantly increased urinary protein: creatinine ratios in the PAVP group compared to the pregnant saline group on GD 8 and 14. It is possible that AVP affects the control of glomerular filtration rate via the macula densa and the tubulo-glomerular feedback mechanism

as a result of an increase in intraglomerular pressure. The macula densa cells detects the increased tubular ion content subsequently activating the RAAS, which inhibits the juxtaglomerular release of nitric oxide. This results in arteriolar vasoconstriction and increases glomerular and systemic blood pressure, causing glomerular hyperfiltration and proteinuria. A similar effect was previously demonstrated in diabetic rats (Bankir et al. 2001; Bolignano and Zoccali 2010).

Likewise, chronic AVP administration in pregnant mice induces glomerular endotheliosis and consequent proteinuria (Sandgren et al. 2018). In a nitric oxide synthase inhibition rat PE model, elevations in systolic blood pressure were associated with increased glomerular pressure and consequent glomerular damage and proteinuria (Baijnath et al. 2014). Shedding of the podocyte barrier due to the intermittent separation of the foot processes from the basement membrane may result in proteinuria (Koop et al. 2003). An angiogenic balance between vascular endothelial growth factor (VEGF) and its receptor sFlt-1 maintains the integrity and function of glomerular filtration barrier (al Embarazo et al. 2007). In PE, increased levels of sFlt-1, an antagonist of the VEGF receptor, offsets this balance by inhibiting its receptor binding and reducing VEGF levels (Müller-Deile and Schiffer 2011; Govender et al. 2012), negatively impacting the glomerular structural integrity resulting in proteinuria (Baijnath et al. 2017). Moreover, elevations in urinary mRNA levels of podocin and nephrin in early onset PE suggest glomerular damage and podocyte loss characteristic of PE development (Baijnath et al. 2017).

Glomerular damage increases glomerular permeability, thereby permitting entry of larger-sized proteins into the filtrate. The increase in peripheral vascular resistance due to AVP treatment, elevates systemic BP with consequent glomerular injury (Santillan et al. 2014) as demonstrated by the higher urinary protein levels observed in the AVP-treated rats in our study. Kidney histology as shown in Figure 4 highlights the effects of mild increase in mesangium, mild glomerular crescents and reduction in the Bowman's space in AVP-treated rats (NAVP and PAVP). The Bowman's capsular space protects glomerular function by acting as a shield against leukocyte infiltration (Chen et al. 2018). Glomerular epithelial crescents produced by the aggregation of inflammatory cells and proliferating epithelial cells in the Bowman's space, are characteristic of glomerulonephritis (D'Souza et al. 2013). Thus, our data suggest an AVP-induced leukocyte infiltration and accompanying glomerular damage may contribute to increased urinary protein levels in the AVP- treated groups. In our study, water intake increased proportionally with gestational days amongst the pregnant (PAVP and PS) and NAVP groups, while urinary output reduced significantly in PAVP rats compared to the PS rats. Reductions in urinary output may be attributed to the antidiuretic effect of AVP, via V2 receptor activation and increased expression of aquaporin-2 channels resulting in water retention and reduced urinary output (Guelinckx et al. 2016).

In our study, we notice a significant reduction in the serum concentrations of sodium, urea, and chloride was noted between the PAVP group compared to the NAVP group. Potassium levels decreased significantly in the PAVP and NAVP *vs* the NS group. Sodium, chloride, and creatinine levels in the PAVP group showed a lower trend in comparison with the PS group, however, these differences were not significant. Reduced serum sodium and potassium levels suggest a dysregulation in their transport across the vascular smooth-muscle cell membrane (Indumati et al. 2011). Sodium reabsorption mediated by AVP within renal tubules, creates a salt-sensitive rise in blood pressure while urea retention may increase plasma urea levels, indirectly increasing glomerular filtration rate (Qian 2018). Our results corroborate others (Anastasio et al. 2001; Bankir et al. 2017), and supports a direct relationship between increased GFR and urine osmolality in both humans and rats. Elevated serum chloride levels in PE may possibly inhibit vessel dilation due to increased osmolality (Barrett et al. 2010).

The observed reduction in creatinine levels in both AVP groups contradicts other reports of elevated creatinine levels in PE (Manjareeka and Nanda 2013; Patil et al. 2016). However, creatinine levels may be influenced by many other factors including the glomerular filtration rate and differences in filtration fraction (Huang et al. 2011), since higher filtration fractions reduce efferent blood flow resulting in decreased tubular creatinine secretion. Moreover, vasoconstriction of the efferent arteriole may be due to increased RAAS activation induced by AVP, which increases angiotensin II that significantly influences serum creatinine levels.

In addition, we also investigated serum biochemical parameters in the AVP model of PE. We observed significantly higher serum ALT levels in the PAVP compared to the NAVP and saline-treated groups, with liver weight also significantly higher in the former group. Conversely, AST levels were significantly downregulated in the PAVP compared to both the NS and NAVP groups. A non-significant increase in AST levels was noted in the PAVP group in comparison with the PS group. Despite the noticeable dysregulation of transaminase levels in our study, the liver histology of the PS

and PAVP groups was normal, suggesting that while a 150 ng/h infusion of AVP could elevate the level of liver enzymes, it was not high enough to cause liver damage of hemolysis, elevated liver enzymes, low platelet (HELLP) associated with PE. Despite down-regulated LDH levels in the PAVP *vs* the NAVP group, both AVP-treated groups displayed significantly higher LDH levels when compared to the saline-treated groups.

Liver diseases are reported to affect approximately 3% of pregnancies, resulting in maternal and fetal mortality (Mikolasevic et al. 2018), hence it is important to not rule out these variations in the liver injury enzymes. It may be potential indicators of liver dysfunction such as intrahepatic cholestasis of pregnancy or acute fatty liver of pregnancy manifesting in late pregnancy (Mikolasevic et al. 2018). This is typical in patients who present with HELLP syndrome, which is seen in severe cases of PE, however, our data indicates mild AVP induced blood pressure elevations, suggestive of mild PE onset. HELLP syndrome is a pregnancy-associated liver disease and is a predisposing factor for the progression of PE to eclampsia (Barton and Sibai 2004; Jeyabalan 2013; Brown et al. 2018). Reproducing this HELLP phenotype in animal models of PE will support the investigation of mechanisms implicated in PE development and its progression from severe PE to eclampsia.

We recently reported higher serum levels of liver injury enzymes arginase, and 5′-nucleotidase in AVP-treated pregnant rats in comparison with saline-treated rats (Govender et al. 2021). Higher 5′-nucleotidase levels observed in the PAVP group is suggestive of possible hepatotoxicity and hepatobiliary disease as previously reported (Dixon and Purdom 1954; Carakostas et al. 1990). The observed increase in these enzyme levels are indicative of liver injury induced by AVP, however, the extent of the liver injury displayed in this model was insufficient to produce the hepatic dysfunction associated with the HELLP syndrome (Govender et al. 2021). Our results are in accordance with Sandgren's group, who reported similar findings (Sandgren et al. 2018). The abnormal liver markers and elevated transaminase levels observed in our study may be indicative of acute pregnancy-initiated liver dysfunction.

There is limited data associated with the AVP-induced rat PE model. Nonetheless, higher serum ALT, AST, LDH, and aminotransferase levels occur in PE compared to healthy pregnant women (Dacaj et al. 2016; Gupta et al. 2019). Chronic AVP possibly creates a hypoxic environment that alters liver function and increases serum AST and ALT levels. Additionally, endothelial dysfunction, a pathognomonic PE feature may alter the prostacyclin: thromboxane ratio with consequent elevations in thromboxane causing hepato-vascular vasoconstriction (Dacaj et al. 2016). Moreover, hypoxia in PE may increase glycolysis consequently increasing LDH activity (Lu et al. 2005), indicating cellular damage and their potential use to evaluate disease severity (Qublan et al. 2005).

Our data also highlight a reduction in cholesterol and HDL levels in the PAVP and NAVP groups in comparison with the PS group. Reduced HDL levels in our study may be linked to the endothelial dysfunction as previously reported (ACOG 2013; White et al. 2019). However, reduced cholesterol levels in AVP-treated rats in comparison with pregnant saline rats contradicts others (Gohil et al. 2011; White et al. 2019), which indicate elevated cholesterol levels in PE. Triglyceride concentrations increased significantly in the PAVP compared to the NAVP- and saline-treated groups. Maternal serum triglyceride levels increase during pregnancy to accommodate the fetal nutritional needs (Cortés-Vásquez et al. 2018), however, an excess in triglyceride levels are associated with PE development (Huang et al. 2013). Arginine vasopressin exerts an anti-lipolytic effect via V1a receptors through the down-regulation of tissue lipase, thereby inhibiting triglyceride breakdown (Hiroyama et al. 2007). Elevations in triglyceride levels may interfere with placental vascular development resulting in inadequate implantation and/or poor placental perfusion (Mayret-Mesquiti et al. 2007; Vrijkotte et al. 2012; El Khouly et al. 2016). Additionally, increased triglyceride levels may promote oxidative stress via peroxidation of placental lipids and trophoblasts resulting in endothelial cell damage (Niromanesh et al. 2012; El Khouly et al. 2016). Others also demonstrated higher triglyceride levels in PE compared to normotensive pregnancies (Lima et al. 2011; Huang et al. 2013; Saha et al. 2013), suggestive that triglyceride elevation may be linked to spiral artery atherosclerosis/thrombosis and consequent endothelial dysfunction (Gilbert et al. 2008; Saha et al. 2013).

Blood glucose levels decreased significantly in the PAVP group in comparison with the NAVP and NS groups on GD18. Acute AVP infusion in rats produces a temporary rise in blood glucose concentration (Hems et al. 1975; Taveau et al. 2016), however, the reductions noted in our AVP- treated rats suggest that AVP most probably inhibited gluconeogenesis and glycogenolysis, a process that warrants further investigation.

Despite the non-significant reductions noted for RBC count, platelets, and hematocrit levels in the PAVP group compared to the NAVP group, the levels were above normal. Notably, AVP induces the proliferation and

differentiation of red blood cell precursors (Mayer et al. 2017), accounting for the elevation noted for RBC counts and hematocrit. Increased hematocrit and RBC levels increase blood viscosity and peripheral resistance with consequent elevations in blood pressure (Emamian et al. 2017). Decreased platelet count is associated with the development of the HELLP syndrome in severe PE, which is characterized by hemolysis, elevated liver enzyme, and low platelet count (Weiner et al. 2016). High platelet levels observed in the AVP-treated groups are likely due to the mild AVP-induced elevations in blood pressure (120–140 mmHg), which was unable to excessively alter platelet levels. Interestingly, our findings are corroborated by others, who also reported no significant changes to hematological parameters between PE and normal pregnant women (Makuyana et al. 2002; Hershkovitz et al. 2005; Siddiqui et al. 2011).

Despite a significant increase in pup numbers in the PAVP *vs* PS group, the weight per pup was significantly lower in the PAVP in contrast to the PS group. Abnormal placentation characteristic of preeclamptic placentae leads to reduced placental perfusion and hypoxia (Cheng and Wang 2009) with consequent fetal growth restriction (Cotechini et al. 2014). We also demonstrate significantly lower individual placental weights in the PAVP *vs* the PS groups. The smaller placentae may be associated with reduced uteroplacental blood flow due to vasoconstriction of spiral arterioles, which directly influences fetal growth (Ferrazzani et al. 2011), mirroring human PE. Placental spongiotrophoblasts are responsible for increasing nutrient availability to the placenta in response to the maternal metabolic adjustments to pregnancy (Hu and Cross 2009; Eaton et al. 2020). Our histology data demonstrate that the labyrinth trophoblast cells lining the vascular channels appeared normal with occasional vacuolation while the spongiotrophoblasts were frequently degenerative in the AVP-treated group *vs* the untreated group. The spongiotrophoblasts were often necrotic, with phagocytic material visible, which correlates with the reduced relative size of the basal/junctional zone. It is possible that AVP-induced necrosis and cellular degeneration leading to decreased spongiotrophoblast proliferation and inadequate placental development on the fetal side, which corresponds to the lower individual pup weights observed in the treated groups (Eaton et al. 2020).

Despite higher pup numbers observed in the PAVP group, the weight per pup was smaller, indicative of restricted fetal development in comparison to the PS group. The placenta is integral in transporting glucose from the maternal to fetal circulation (Furukawa et al. 2011). The AVP-induced glucose reduction and placental impairment observed in our study may have consequently altered glucose transport to the fetus, which correlates with the lower pup weights shown in the AVP- treated groups. Similar findings are reported by various others (Crews et al. 2000; Li et al. 2012; Macdonald-Wallis et al. 2014; Santillan et al. 2014). Likewise, lower birth weight and intrauterine growth restriction were reported in C57BL/6 J pregnant mice chronically infused with AVP (Santillan et al. 2014). Similarly, in a RUPP rat PE model, uteroplacental perfusion was reduced by almost 40% *in vivo*, resulting in late gestational hypertension, proteinuria, smaller litter sizes, and fetal growth restriction (Crews et al. 2000; Li et al. 2012). Elevated maternal blood pressure occurring early in pregnancy is thus associated with reduced birth weight and small-for-gestational -age babies (Macdonald-Wallis et al. 2014). However, an earlier study reported that preeclampsia is associated with both pregnancies with normal placental function as well as those with abnormal placentation and intrauterine growth restriction (Rasmussen and Irgens 2003). Late-onset PE pregnancies (>34 weeks) are usually linked to normal birth weights (Odegård et al. 2000), whereas in early-onset PE pregnancies, the presence or absence of fetal growth restriction did not induce significant differences in the degree of hypertension or maternal complications (Haddad et al. 2007).

To our knowledge, this is the first study to successfully replicate an AVP-induced mouse PE model to a Sprague-Dawley rat model. We demonstrate significantly increased systolic and diastolic blood pressures and proteinuria in the AVP-treated rats in comparison with the untreated rats. The physiological and hematological data confirm that this AVP-induced rat model has the potential to adequately reproduce the clinical PE features of elevated blood pressure, proteinuria, fetal growth restriction together with elevations in several blood and lipid parameters. We demonstrate significantly higher serum levels of ALT and triglycerides along with reduced HDL levels in the AVP-treated pregnant group, confirming the potential role of AVP in the development of PE.

Materials and methods

Animal welfare

Sprague-Dawley rats aged 10–12 weeks (160–180 g) were housed in polycarbonate cages under standard laboratory conditions of temperature (22 to 24°C), humidity (60%) and illumination (12 h light/dark cycles) with *ad libitum* access to standard rat chow (Meadows Feeds, Pietermaritzburg, South Africa) and normal drinking water.

Preliminary dose-response study

A pilot dose-response study used three AVP doses (50, 100, and 150 ng/h) to determine the optimum and safe dose required to produce the clinical symptoms of PE. Six pregnant female Sprague-Dawley rats (n = 2 per dose) were surgically implanted with ALZET mini-osmotic pumps (model 2004; Durect Corporation, Cupertino, CA), on GD 1 to subcutaneously deliver the selected doses of AVP. The mini osmotic pumps remained implanted until sacrifice. Physiological parameters (weight, systolic and diastolic blood pressure) were measured at GD 3, 13, 14, 18, and 20 using the MRBP tail-cuff BP monitor (IITC Life Sciences Inc., USA). Urine samples (24 h) were collected on GD 10, 17, and 21 to measure urinary proteins, using the M-TP Microprotein Kit (Beckman Coulter, CA, USA) and read using the SYNCHRON LX® System (Beckman Coulter, CA, USA).

Experimental study

Twenty-four pregnant female Sprague-Dawley rats (200–220 g) were randomly grouped as follows:

- Group 1: (n = 6) Non-pregnant with saline delivery **(NS)**
- Group 2: (n = 6) Non-pregnant with AVP delivery **(NAVP)**
- Group 3: (n = 6) Pregnant with saline delivery **(PS)**
- Group 4: (n = 6) Pregnant with AVP delivery **(PAVP)**

Based on the pilot study outcomes, an AVP dose of 150 ng/h was chosen since it produced the most significant changes in blood pressure and proteinuria compared to the lower doses tested. The ALZET mini osmotic pumps were subcutaneously implanted on GD 1 and remained implanted until sacrifice (GD 18) in all study groups. Saline and AVP (150 ng/h) were subcutaneously delivered throughout gestation, to the control and experimental groups respectively and pumps were removed at sacrifice (GD 18). Systolic and diastolic blood pressure was measured on GD 8, 14, and 18 using the MRBP tail-cuff BP monitor (IITC Life Sciences Inc., USA), by placing animals in a suitably sized restrainer. Normal blood pressure was defined as systolic ≤120 mmHg and diastolic ≤80 mmHg. Hypertension in rats was defined as systolic ≥140 mmHg and diastolic ≥90 mmHg (Brown et al. 2018). Animals were housed in metabolic cages (Techniplast, Italy) on GD 8, 14, and 18, for the measurement of water intake and urinary output; and collection of 24 h urine samples. Urinary protein content was measured using the M-TP Microprotein Kit (Beckman Coulter, CA, USA) and read using the SYNCHRON LX® System (Beckman Coulter, CA, USA). Animals were then euthanized on GD 18, via isoflurane overdose (Safeline Pharmaceuticals, South Africa). Blood samples were collected via cardiac puncture and centrifuged for 15 min at 3500 rpm at 4°C. The number and weight of placentae and pups were recorded. Biochemical and hematological analysis was carried out by a pathology laboratory using rodent reference ranges.

Histological evaluation of the liver, kidney, and placenta

Liver, kidney, and placenta samples were fixed in 10% buffered formaldehyde and embedded into paraffin wax blocks as per standard laboratory procedure (Burton et al. 2014). Sections of liver, kidney, and placental tissue were cut (3 µm) using a rotary microtome and mounted onto frosted glass slides (ISOLAB GmbH Herstellung und Vertrieb von Laborgeräten, Eschau, Germany). Tissue sections were de-paraffinized and rehydrated for hematoxylin and eosin staining (Fischer et al. 2008). Stained sections were permanently mounted. Tissue sections were examined using the ZEISS Axio Imager 2 and images captured using Zen (Blue edition) software (Carl-Zeiss-Strasse, Oberkochen, Germany).

Statistical analysis

All statistical analyses were carried out using Stata (Version 12). All data were non-parametric and are summarized as median and interquartile range (IQR). The biochemical/hematological parameters in pregnant rats were compared with non-pregnant rats using the Mann–Whitney test. The Kruskal–Wallis and Dunn's post hoc test was used to compare the medians between groups. A probability value of $p < 0.05$ was considered statistically significant.

Ethics approval for animal study

This study was approved by the Animal Research Ethics Committee, University of KwaZulu-Natal (UKZN) (AREC/046/017). All procedures were conducted as per the approved institutional standard protocols for animal research.

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Disclosure statement

The author(s) report no conflict of interest.

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Authors' contributions

Conception and study design: NG, SB, BNM, SS. Conduction of experiments and data collection: SR, VP. Analysis and interpretation of the data: NG, SR, SB. Development and finalisation of the manuscript: NG, SR, TN, SB BNM, SS.

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CHAPTER 4

Research Article: Kidney injury molecule-1 and podocalyxin dysregulation in an arginine vasopressin induced rodent model of preeclampsia

The following manuscript investigated kidney injury in the AVP rat model by determining urinary expression of KIM-1, renal immunolocalization of KIM-1 and podocalyxin along with a histological and ultrastructural evaluation of the kidneys of pregnant AVP-treated rats.

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Full length article

Kidney injury molecule-1 and podocalyxin dysregulation in an arginine vasopressin induced rodent model of preeclampsia

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ABSTRACT

Objective: To assess renal injury in an arginine vasopressin (AVP) rodent model of preeclampsia.
Study design: Urinary expression of kidney injury molecule-1 (KIM-1), urinary protein and creatinine was determined in rodents (n = 24; pregnant AVP, pregnant saline, non-pregnant AVP and non-pregnant saline), which received a continuous dose of either AVP or saline via subcutaneous mini osmotic pumps for 18 days, using a Multiplex kidney toxicity immunoassay. Renal morphology was assessed using haematoxylin and eosin staining and transmission electron microscopy. The immunolocalization of KIM-1 and podocalyxin was qualitatively evaluated using immunohistochemistry.
Results: Urinary KIM-1 and urinary protein levels were significantly increased in treated *vs.* untreated rats on gestational days 8 ($p < 0.05$), 14 ($p < 0.001$) and 18 ($p < 0.001$). The pregnant rats displayed a lower trend of creatinine compared to the non-pregnant groups, albeit non-significantly. KIM-1 was immunolocalized in the proximal convoluted tubules in AVP treated *vs.* untreated groups. In contrast, podocalyxin was weakly immunostained within glomeruli of pregnant AVP treated *vs.* pregnant untreated rats. Histological evaluation revealed reduced Bowman's space, with some tubular and blood vessel necrosis in the pregnant treated group. Ultrastructural observations included effacement and fusion of podocyte foot processes, glomerular basement membrane abnormalities, podocyte nuclear crenations, mitochondrial oedema and cristae degeneration with cytoplasmic lysis within treated tissue.
Conclusion: Our findings demonstrate region-specific kidney injury particularly glomerular impairment and endothelial injury in AVP-treated rats. The findings highlight the utility of this model in studying the mechanisms driving renal damage in a rodent model of preeclampsia.

Introduction

The structural integrity of the glomerular filtration apparatus (GFA) facilitates glomerular blood filtration and arterial blood pressure regulation [1]. Alteration of endothelial fenestration and glomerular charge affects function [2]. Preeclampsia, a multi-system hypertensive disorder, is associated with glomerular dysfunction [3,4]. While there are uncertainties regarding the aetiology of preeclampsia, inadequate placental vascular development associated with placental oxidative stress, the release of inflammatory mediators, and extensive systemic endothelial dysfunction have been implicated as major contributing factors [5].

Renal pathogenesis in preeclampsia is reportedly associated with structural modifications in the kidney, atypical renal blood flow and the dysfunctional regulation of molecules and ions in the kidney [2]. Kidney dysfunction is associated with glomerular endotheliosis [6], a pathognomonic characteristic of preeclampsia pathology. These features predispose glomerular volume increase, distension of endothelial cells, abnormal glomerular charge and obstruction of capillary lumens [7]. Hence, a disturbed GFA impairs glomerular filtration with consequent loss of structural proteins from both viable and apoptotic podocytes into the urine [6]. More importantly, glomerular injury in preeclampsia is associated with proteinuria as a consequence of GFA dysfunction [8]. This impairment may result from alterations in podocyte structure, such as the enlargement of the foot processes and their separation from the glomerular basement membrane [6,9]. Additionally, preeclampsia is

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reported to increase the susceptibility of developing chronic kidney disease and/or end-stage renal disease later in life [10]. Understanding the processes involved in this vulnerability will assist in the establishment and implementation of pre-emptive and treatment strategies renal complications in preeclampsia.

In a healthy kidney, low levels of kidney injury molecule-1 (KIM-1) is observed, however these levels significantly increase following ischemic–reperfusion injury [11]. Kidney injury molecule-1 is a 38.7 kDa type l transmembrane glycoprotein that contains an extracellular immunoglobulin-like domain topping a long mucin-like domain [12]. This protein possesses a transmembrane domain as well as a short intracellular domain containing a signalling protein for tyrosine phosphorylation [13]. In kidneys exhibiting injury, KIM-1 is mainly localized in the proximal tubule cells in rodents [14] as well as in humans [12]. KIM-1 is also identified as a marker of proliferation and regeneration in proximal tubules [15], as well as a phosphatidylserine receptor during phagocytosis of apoptotic cells in the post-ischemic kidney [16]. Higher urinary levels of KIM-1 in preeclampsia compared to normotensive pregnant women supports its role as potential biomarker for preeclampsia detection [17].

In contrast, the podocyte-specific protein podocalyxin is abundantly expressed on the apical surface of glomerular podocytes [18]. Podocalyxin is a 140–165 kDa protein that is a constituent of the CD34 transmembrane sialomucin family [19,20]. Podocalyxin levels are significantly increased in urine [9] and plasma [21] in preeclampsia compared to normotensive pregnancies. A recent study confirmed an increase in podocyturia and podocalyxin in maternal urine in preeclampsia compared to normal pregnancies [22].

Our group recently demonstrated that arginine vasopressin (AVP) is able to successfully reproduce a human preeclampsia-like syndrome in the Sprague-Dawley pregnant rat [23]. Furthermore, our data highlights significant elevations in circulating levels of transforming growth factor beta-2 and the liver injury enzymes arginase and 5′-nucleotidase in the AVP treated pregnant rats compared to untreated rats [24]. This study aimed to investigate the applicability of an AVP induced rodent model in the study of preeclampsia, by determining the urinary levels of KIM-1 as well as by the immunolocalization of podocalyxin and KIM-1, as indicators of kidney injury and to validate the model against clinical presentation of the disorder. The morphological and ultrastructural changes in the kidney were also evaluated in order to determine the usefulness of the model in studying the kidney injury in preeclampsia development.

Materials and methods

This study was approved by the Animal Research Ethics Committee, University of KwaZulu-Natal (AREC/046/017) and carried out based on a previously published protocol [23]. The study groups were inclusive of non-pregnant rats with saline delivery **(NS, n = 6)**; non-pregnant rats with AVP delivery **(NAVP, n = 6)**; pregnant rats with saline delivery **(PS, n = 6)** and pregnant rats with AVP delivery **(PAVP, n = 6)**. Urinary KIM-1 and creatinine concentrations as well as urinary protein levels were determined using 24-hour urine samples collected on GD8, 14 and 18 respectively. Histological assessment was conducted using kidney tissue stored in buffered formalin. Ultrastructural evaluation was done using kidney tissue that was stored in 0.1% glutaraldehyde/4% paraformaldehyde fixative in 0.2 M phosphate buffer overnight at 4 °C (pH 7.2).

Determination of urinary protein and creatinine levels

Urinary protein and creatinine levels were determined using the M−TP Microprotein Kit (Beckman Coulter, CA, USA) and Creatinine reagent (Beckman Coulter, CA, USA) respectively; and read using the SYNCHRON LXR System (Beckman Coulter, CA, USA).

Multiplex ELISA assay

Urinary concentration of KIM-1 was determined using the kidney toxicity panel 1 (Cat. #RKTX1MAG-37 K, Merck Millipore, Darmstadt, Germany) multiplex immunoassay according to the manufacturer's instructions, with undiluted samples. The assay plate was read using the Bio-Plex MAGPIX® Multiplex reader (Bio-Rad Laboratories Inc., USA) with xPONENT® v.3.2 software and analysed with MILLIPLEX® Analyst 5.1 software (Merck Millipore, Darmstadt, Germany).

Histological evaluation of the kidney

Kidney tissue was dehydrated, cleared with xylene and infiltrated with paraffin wax using an automated tissue processor (Sakura 5, Torrance, California, USA). The tissue was then embedded into the paraffin wax using an embedding station (Leica EG 1160 embedding station; Germany). A rotary microtome (Leica RM2135, UK) was used to cut 3 μm sections of kidney tissue, which were floated in a 50 °C water bath (Leica HI1210, Leica Biosystems, UK). Sections were thereafter mounted onto frosted glass slides (ISOLAB GmbH Herstellung und Vertrieb von Laborgeräten, Eschau, Germany) and HistoBond® adhesive charged slides (Marienfeld, Germany) for haematoxylin and eosin and immunohistochemical staining respectively.

Haematoxylin and eosin staining

Tissue sections underwent de-paraffinization and rehydration steps for haematoxylin and eosin (H&E) staining [25]. Stained sections were permanently mounted, examined and images were captured using the ZEISS Axio Imager 2 (Carl-Zeiss- Strasse, Oberkochen, Germany).

Immunohistochemistry

The immunolocalization of KIM-1 and podocalyxin in both the untreated and AVP treated rats was done using the VECTASTAIN elite ABC-HRP Kit, Peroxidase (Rabbit IgG) (PK-6101, Vector Laboratories, CA, USA). Slides were baked overnight at 60 °C, thereafter, dewaxed in xylene and rehydrated using a descending series of ethanol prior to antigen retrieval. Thereafter, slides were incubated in a pre-heated tris-based (pH 9.0) antigen unmasking solution (H-3301, Vector Laboratories, CA, USA) and subsequently cooled to room temperature (15 min). Following rinsing and incubation (5 min) in a wash buffer solution of phosphate buffered saline (PBS), pH 7.5 (BR0014G, Oxoid, England), sections were blocked with an endogenous hydrogen peroxide blocking reagent (ab64218, Abcam, UK) for 10 min in a humidity chamber. Slides were then washed with PBS and further blocked with normal goat serum (20 min) (PK-6101, Vector Laboratories, CA, USA) to prevent non-specific binding. Sections were thereafter incubated with primary antibodies: anti-KIM-1 (ab47635, Abcam, UK; 1:40 dilution) and anti-PODXL (ab205350, Abcam, UK; 1:25 dilution) overnight at 4 °C.

This was then followed by PBS washing and incubation with biotinylated secondary antibody (PK-6101, Vector Laboratories, CA, USA) for 30 min in a humidity chamber. Slides were washed with PBS and thereafter incubated with VECTASTAIN elite ABC reagent (PK-6101, Vector Laboratories, CA, USA) for 30 min. Following washing, sections were incubated with a diaminobenzidine (DAB) chromogen (SK-4105, Vector Laboratories, CA, USA) to visualize antibody expression. The slides were rinsed in deionized water (dH_2O), counterstained with Mayer's haematoxylin (30sec) and mounted in dibutylphthalate xylene (DPX). Replacement of the primary antibody with normal goat serum as the primary served as the method control. The immunolocalization of KIM-1 and podocalyxin were qualitatively determined in the proximal tubules and cortical glomeruli respectively, using the ZEISS Axio Imager 2 (Carl-Zeiss- Strasse, Oberkochen, Germany). An intensity scoring system was used to semi-qualitatively assess the immunolocalization of KIM-1 and podocalyxin in proximal tubules and cortical glomeruli respectively. The staining intensity was determined as follows: 0 (no staining), 1 (weak staining), 2 (moderate staining) and 3 (strong

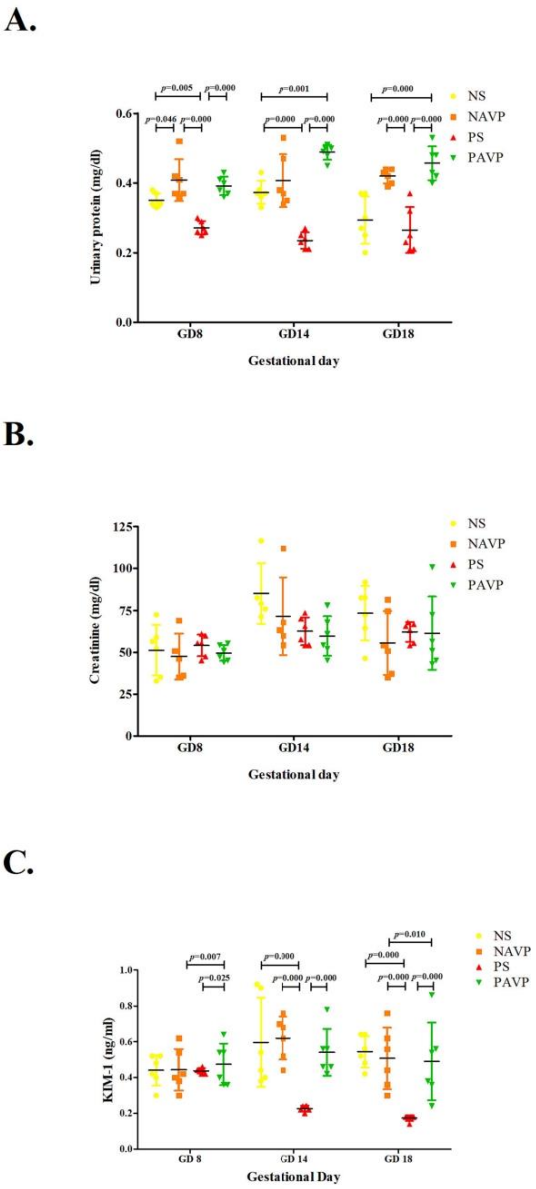

Fig. 1. Urinary expression of (A) protein, (B) creatinine and (C) Kidney injury molecule-1 (KIM-1) across all study groups. Data is expressed as mean ± standard deviation (n = 6 per group). NS: non-pregnant saline; NAVP: non-pregnant AVP; PS: pregnant saline; PAVP: Pregnant AVP.

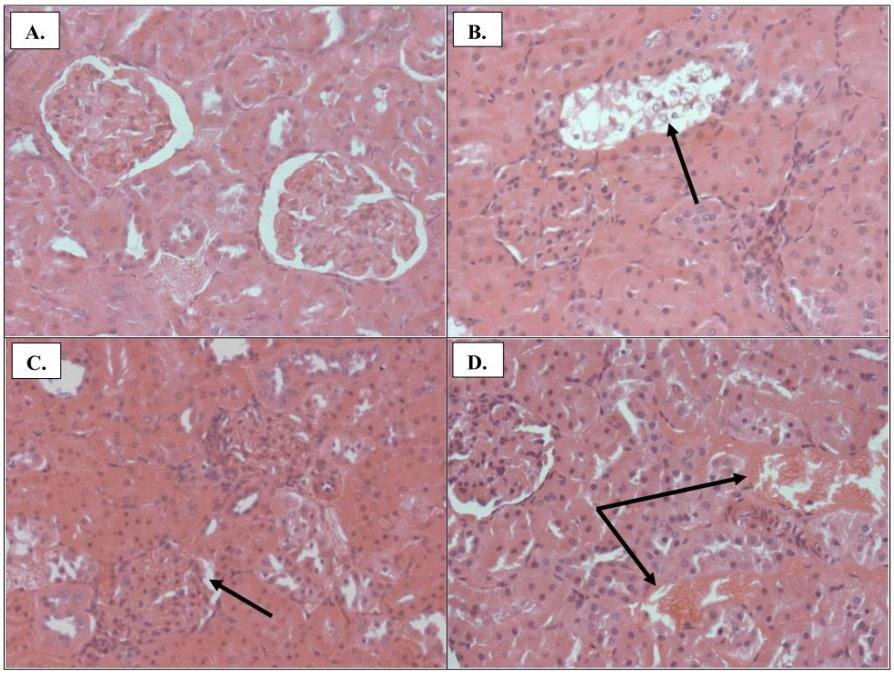

Fig. 2. H & E histological changes in pregnant saline and AVP treated rat kidneys. (A) No visible changes observed in the saline treated kidneys, ×40. AVP treated pregnant kidneys demonstrate (B) tubular necrosis (black arrow), ×40 (C) reduced Bowman's space (black arrow), ×40 and (D) vascular necrosis (black arrow), ×40.

staining). Proximal tubules and glomeruli which were assigned scores between 0 and 1 were considered negative whereas scores of 2 and 3 were considered positive.

Transmission electron microscopy

Fixed kidney tissue were cut into 1 mm^3 cubes and re-immersed into fresh 0.1% glutaraldehyde/4% paraformaldehyde fixative. The specimens were then washed in 0.2 M phosphate buffer (pH 7.2; 4 °C) and dehydrated through an ascending series of ethanol, propylene oxide and thereafter infiltrated with Spurr's epoxy resin (Sigma, USA) (Spurr, 1969). This was followed by embedding specimens in capsules (size 00, BEEM, USA) and polymerisation for 48hrs at 60 °C. Sections (1 μm) were cut using an ultramicrotome (Reichert S/Ultracut R, Leica, Germany), collected onto glass slides, heat-fixed, and stained with a 1% toluidine blue solution. Fields of interest containing glomeruli were selected and thereafter ultrathin sections (50–60 nm) were cut and collected onto 200 mesh copper grids (Sigma, UK). The ultrastructural features of the cortical glomeruli and tubules was qualitatively evaluated using the Jeol 1011 transmission electron microscope (Jeol 1011, Japan).

Data analysis

All data was analysed using STATA (version 12, STATACORP). Urinary KIM-1 data was summarized as means and standard deviation. A one-way ANOVA was used to determine if a significant difference existed between the four treatment groups. This was followed by the Tukey's post-hoc test to determine the level of significance between pairs of group means. A *p*-value of < 0.05 was considered as statistically significant. The Pearson's correlation was used to determine if an association existed between KIM-1, urinary protein and creatinine.

Results and discussion

Our findings confirm significant kidney injury in AVP treated pregnant rats compared to untreated rats. We previously demonstrated mild elevation of blood pressure in the pregnant AVP treated groups *vs.* untreated groups [23]. These findings may be a result of AVP induced activation of the renin-angiotensin aldosterone system and consequent increase in glomerular pressure, glomerular filtration rate, glomerular damage and proteinuria. We now demonstrate significantly higher urinary levels of KIM-1 in the PAVP *vs.* the PS group (Fig. 1C). Our findings suggest that AVP up-regulates KIM-1 levels as early as GD8 ($p < 0.05$), remained elevated on GD14 ($p < 0.001$), and decreased minimally on GD18 ($p < 0.001$) compared to the PS group. Moreover, KIM-1 levels were lower in the PS compared to the NS groups on GD14 ($p < 0.001$) and GD18 ($p < 0.001$). On GD8 and 18, KIM-1 levels increased significantly in the PAVP versus the NAVP groups ($p < 0.05$), and was unchanged on GD14. Elevations in urinary KIM-1 levels are associated with acute kidney injury [26] and ischemic renal injury [27]. Our data are corroborated by others [6,28,29], who report higher urinary KIM-1 levels in preeclampsia compared to normotensive pregnancies.

Similarly, urinary protein concentrations were significantly elevated on GD8, 14 and 18 in the PAVP *vs.* PS groups ($p < 0.001$; Fig. 1A). On GD14 and GD18, significantly higher urinary protein levels were observed in the PAVP compared to the NS and NAVP groups ($p < 0.001$). Despite the lack of statistical significance observed between the PAVP

Table 1
Qualitative assessment of KIM-1 and podocalyxin immunolocalization in the kidney.

	KIM-1				Podocalyxin			
	NS	NAVP	PS	PAVP	NS	NAVP	PS	PAVP
Glomerulus	0	0	0	0	3	2	2	1
Proximal convoluted tubule	0	1	1	3	0	0	0	0

0: no staining; 1: weak staining; 2: moderate staining; 3: strong staining.
NS: non-pregnant saline; NAVP: non-pregnant AVP; PS: pregnant saline; PAVP: pregnant AVP.

and PS groups (Fig. 1B), the pregnant groups do demonstrate lower creatinine levels in comparison to non-pregnant control groups on GD8, 14 and 18. Gaurang (2015) also reported reduced urinary creatinine levels in preeclampsia in comparison to normotensive pregnancies [30]. However, our data is contradictory to others, [31–33], which reports elevated urinary creatinine levels in preeclamptic women. Our findings may be clinically valuable if the use of urinary KIM-1 is considered in conjunction with urinary protein expression, in predicting kidney injury associated with preeclampsia.

The correlation analysis demonstrated weak positive associations between KIM-1 and urinary protein on GD8 ($r = 0.33$, $p = 0.52$), GD14 ($r = 0.30$, $p = 0.56$) and GD18 ($r = 0.13$, $p = 0.81$). No significant associations were observed between KIM-1 and creatinine on GD8 ($r = 0.48$, $p = 0.33$) and GD14 ($r = -0.06$, $p = 0.90$), however a stronger association was noted on GD18 ($r = 0.80$, $p = 0.06$). Notably, no correlation was also noted between serum KIM-1 and creatinine levels amongst patients that developed contrast-induced acute kidney injury and those that didn't [34].

We also demonstrate morphological changes in the kidney (Fig. 2A-D), *viz.*, tubular necrosis (Fig. 2B) and vasculitis (Fig. 2D) in PAVP treated compared to non-pregnant saline groups. In human preeclampsia, significant reduction in both glomerular filtration rate and renal plasma flow with concomitant sustained renal hypoperfusion results in tubular necrosis in severe preeclampsia [35]. We previously demonstrated a mild mesangial cell increase along with mild focal cellular glomerular crescents associated with this model [23].

The immunohistochemical localization of KIM-1 and podocalyxin was qualitatively assessed in the tubules and cortical glomeruli (Table 1, Fig. 3). KIM-1 was prominently immunolocalized in the brush border of the proximal convoluted tubules of AVP treated rats suggestive of an AVP-induced tubular dysfunction (Fig. 3A-B). Similarly, a positive correlation between urinary KIM-1 levels and tissue expression was observed in patients with acute tubular injury, indicative of the severity of renal damage [36]. The clinical biomarker value of KIM-1 in acute kidney injury and acute tubular necrosis has been reported [37]. Furthermore, Kramer et al. demonstrated that elevations in urinary and kidney KIM-1 levels correlates with the degree of renal dysfunction in the adriamycin-induced nephropathy rat model. Likewise in women diagnosed with severe preeclampsia, renal matrix metallopeptidase production is up-regulated by KIM-1 expression, which exacerbates glomerular barrier dysfunction and podocyte loss in preeclampsia [6].

Moreover, KIM-1 is implicated in the reparative processes in the kidney, as well as in the proliferation and regeneration processes in the proximal tubules [15]. A marked increase in KIM-1 expression is associated with the regeneration of the proximal tubular epithelium and its transition into the differentiated cells that border areas of injury [11]. Moreover, KIM-1 facilitates the phagocytosis of apoptotic bodies and cell debris in proximal tubule epithelial cells by functioning as an epithelial phosphatidylserine receptor [38,39]. This phagocytic action

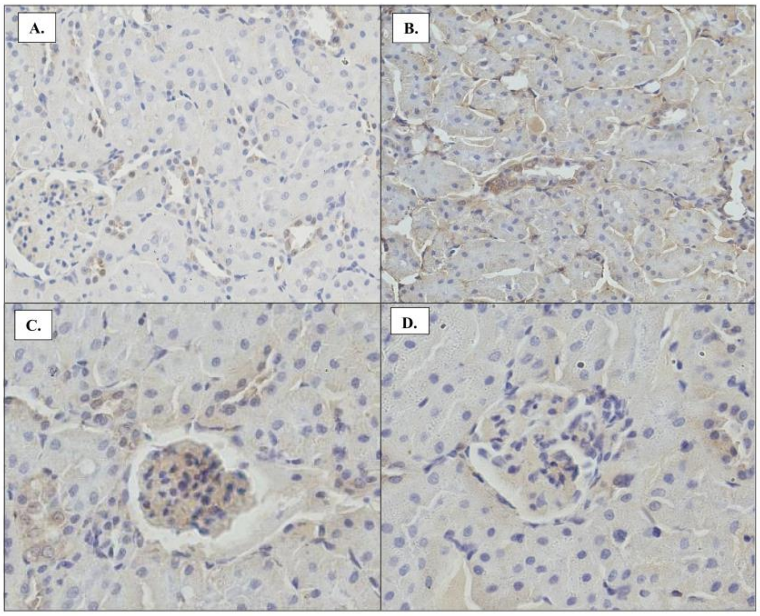

Fig. 3. Immunolocalization of KIM-1 in proximal convoluted tubules of (A) pregnant saline treated rat kidneys ×40, (B) pregnant rat kidneys treated with AVP, ×40; and podocalyxin in the glomerulus of (C) pregnant saline treated rat kidneys ×40 and (D) pregnant AVP-treated rat kidneys ×40.

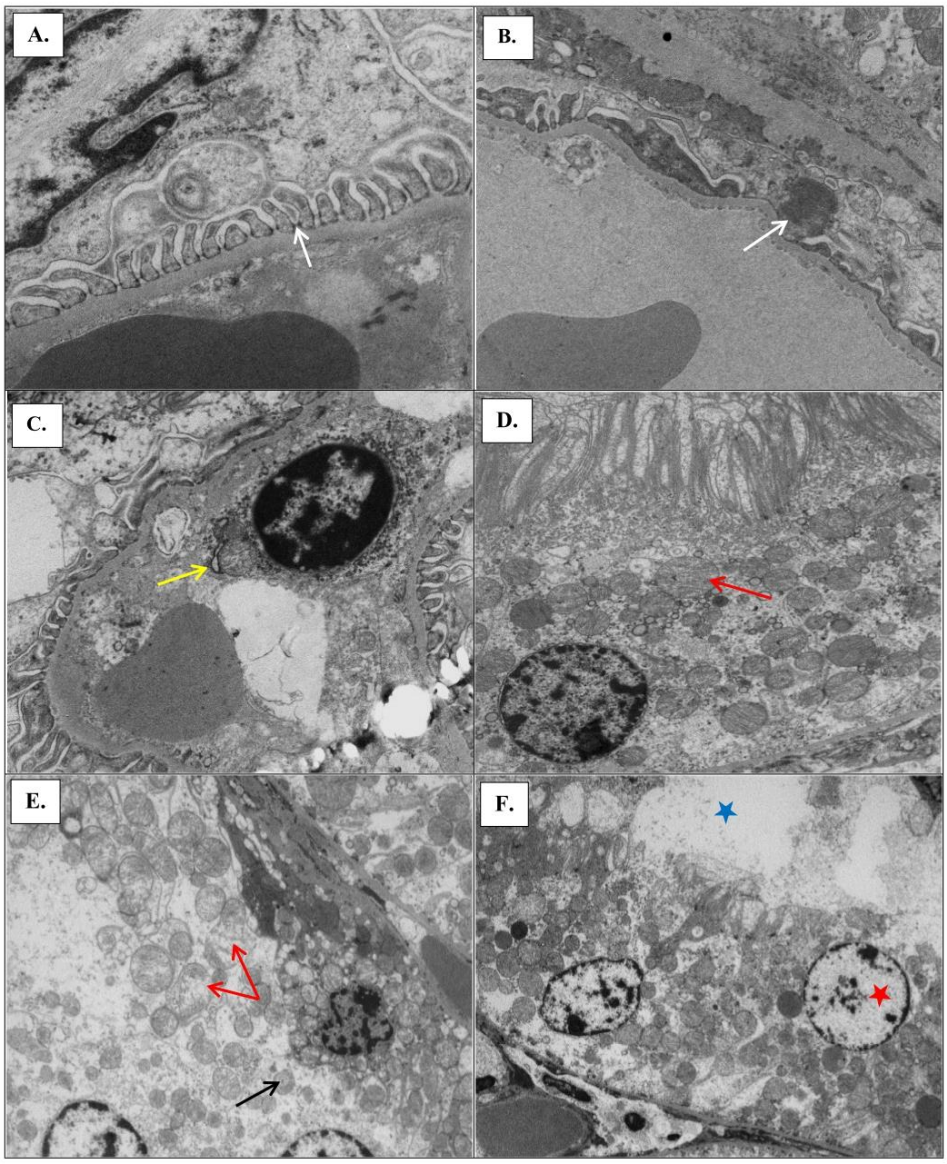

Fig. 4. Subcellular changes in the nephron from untreated and PAVP treated kidney tissue (A) Normal appearance of glomerular filtration apparatus in untreated tissue with visible slit diaphragms (white arrow) between adjacent foot processes ×20000. Glomeruli from PAVP kidney tissue showing (B) Focal adhesion of foot processes with granular deposits (white arrow) ×10000, (C) irregular shape of basement membrane, fusion of foot processes and dilation of endoplasmic reticulum (yellow arrow) in endothelial cell ×10000, (D) nuclear changes in proximal tubule and mitochondrial swelling (red arrow) ×6000, (E) cristae degradation (red arrows), formation of "donut" mitochondria (black arrow), cytoplasmic lysis and electron lucent cytoplasm ×6000, and (F) nuclear (less granular chromatin, red star) and cytoplasmic changes (cytoplasmic lysis, blue star) in proximal tubule, ×4000. PAVP: pregnant AVP treated rats. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

potentially reduces inflammation and supports renal repair [40]. The downregulation of KIM-1 observed in our pregnant saline group may be due to early renal injury occurring during the onset of pregnancy, followed by renal repair facilitated by KIM-1, compared to the sustained renal damage observed in pregnant AVP treated rats.

Since urinary podocyte excretion appears to occur prior to microalbuminuria and proteinuria, podocyturia is considered as an early biomarker for preeclampsia diagnosis [41], exhibiting a sensitivity and specificity of 100% in early diagnosis of preeclampsia [42]. Elevation in urinary podocalyxin expression in preeclamptic women are associated with podocyte injury [9,43]. Our qualitative immunohistochemical findings of podocalyxin confirmed a positive membranous localization in the podocytes in the PS group (Table 1; Fig. 3C). In contrast, podocalyxin immunolocalization was less pronounced in the cortical glomeruli of pregnant AVP treated groups (Fig. 3D). Since podocalyxin is the major glycocalyx protein of podocytes, its negative charge is implicated as a charge barrier in glomerular filtration and it maintains the open intercellular spaces via its anti-adhesive properties [44]. Hence, a reduced podocalyxin expression is suggestive of a loss of podocyte integrity plus a disruption of glomerular organization and consequent kidney injury. Our findings suggest that AVP has the potential to induce kidney injury and dysregulate podocalyxin expression. This imbalance disrupts the anti-adhesive properties that ensure spatial separation of the foot processes resulting in collapse of the filtration slits, effacement and reduced filtration capacity.

Our ultrastructural examination of untreated kidney tissue confirms a normal appearance of the glomerular basement membrane, the mature podocyte foot processes and slit diaphragms (Fig. 4A). Albeit in the PAVP group, we noted an effacement of foot processes indicative of AVP induced kidney injury (Fig. 4B). Despite the paucity of data regarding immunoexpression of podocalyxin in preeclampsia, elevations in urine and serum levels in preeclamptic pregnancies are noteworthy. Urinary podocalyxin levels are significantly higher in preeclamptic compared to normotensive pregnancies [9,45]. Furthermore, serum podocalyxin levels are higher at 11–13 weeks of gestation in women who subsequently developed preeclampsia [46]; and increased as gestation progressed in preeclamptic compared to normotensive pregnancies, [21].

Our ultrastructural data viz., the irregular "frilling" of the glomerular basement membrane (Fig. 4B-C), granular deposits and podocyte changes of dilation of the endoplasmic reticulum (ER; Fig. 4C), mitochondrial swelling, lysis and cristae degradation along with nuclear crenations and low euchromatin distribution (Fig. 4D–F), cytoplasmic lysis (Fig. 4E–F), further confirm renal abnormalities in the PAVP group. Mitochondrial swelling observed in the treated tissue may be attributed to the opening of the mitochondrial permeability transition pore (mPTP) during hypoxia that precedes preeclampsia development [47]. The appearance of "donut" shaped mitochondria and ER dilation may be a consequence of hypoxia, oxidative stress and osmotic pressure changes, triggered by the opening of mPTP or potassium (K^+) channels, including mitochondrial adenosine triphosphate (ATP)-activated K^+ channels and mitochondrial calcium (Ca^{2+})-activated K^+ channels [48].

Earlier data suggests that AVP may elevate mitochondrial Ca^{2+} levels [49]. It is plausible that the administration of AVP in this model promotes the mitochondrial swelling and appearance of "donut" shaped features observed in our study, through the opening of the mPTP channels and elevated Ca^{2+} levels. These mitochondrial abnormalities may also inhibit podocyte functioning as large quantities of ATP are required to sustain the high surface area of tertiary foot processes, and the loss of ATP may lead to the breakdown of the glomerular filtration barrier [50].

Conclusion

Our findings demonstrate a pronounced KIM-1 tubular immunolocalization and a significant upregulation in urinary KIM-1 levels in the pregnant AVP compared to the pregnant saline groups, in contrast to the reduced prominence of podocalyxin in the AVP treated group. The histological changes observed in pregnant AVP treated rats are consistent with preeclampsia development. Our results suggest that AVP administration perchance induces glomerular impairment and endothelial dysfunction, associated with preeclampsia development, because of its association with elevations in peripheral resistance and systemic blood pressure. These findings demonstrate the potential value of KIM-1 and podocalyxin to detect renal injury associated with preeclampsia. Further study of the kidney injury induced by AVP is required to fully understand its role in the development and progression of preeclampsia. Future work on the application of morphometric image analysis of glomeruli and tubules is currently in progress.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CHAPTER 5

Research Article: Renal biomarker expression in an arginine vasopressin induced rat model of preeclampsia

This article evaluates the urinary expression of glomerular and tubular markers of injury associated with the AVP rat model

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Renal biomarker expression in an arginine vasopressin induced rat model of preeclampsia

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Abstract:	Objective Kidney dysfunction plays an important role in the pathophysiology of preeclampsia (PE). Thus, the identification of an early biomarker of renal dysfunction is vital for the diagnosis and prevention of progression from PE to eclampsia. Kidney injury has not been fully explored the arginine vasopressin (AVP) model, therefore, this study aimed to determine kidney toxicity in the AVP-induced rat kidney. Study design Female Sprague Dawley rats (n=24; pregnant AVP, pregnant saline, non-pregnant AVP and non-pregnant saline) were infused with AVP or saline for 18 days. Urine samples were collected on GD8, 14 and 18 and used to determine the levels of albumin, VEGF-A, clusterin, NGAL/Lipocalin-2, KIM-1, cystatin C, TIMP-1, β2M and OPN via Multiplex ELISAs. Results Albumin, and NGAL/lipocalin-2 were significantly elevated in the PAVP vs. PS group on GD14 ($p<0.001$) and GD18 ($p<0.001$). VEGF-A significantly decreased in the pregnant vs. non-pregnant groups on GD14 ($p<0.001$) and 18 ($p<0.001$). Clusterin, cystatin C, β2M and KIM-1 are significantly higher in the PAVP vs. PS groups on GD8 ($p<0.05$), 14 ($p<0.001$) and 18 ($p<0.05$). Significantly higher concentrations of TIMP-1 and OPN were observed in the PAVP vs. PS groups on GD14 and 18 respectively. Conclusion Arginine vasopressin successfully elevated the urinary levels of the kidney injury biomarkers and replicated kidney injury associated with PE development. Glomerular impairment and endothelial dysfunction is associated with the imbalance in kidney injury markers. Hence, our findings confirm the utility of the AVP rat model in studying the mechanisms driving renal damage in PE development.
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Original Article

Renal biomarker expression in an arginine vasopressin induced rat model of preeclampsia

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Abstract

Objective: Kidney dysfunction plays an important role in the pathophysiology of preeclampsia (PE). Thus, the identification of an early biomarker of renal dysfunction is vital for the diagnosis and prevention of progression from PE to eclampsia. Kidney injury has not been fully explored the arginine vasopressin (AVP) model, therefore, this study aimed to determine kidney toxicity in the AVP-induced rat kidney.

Study design: Female Sprague Dawley rats (n=24; pregnant AVP, pregnant saline, non-pregnant AVP and non-pregnant saline) were infused with AVP or saline for 18 days. Urine samples were collected on GD8, 14 and 18 and used to determine the levels of albumin, VEGF-A, clusterin, NGAL/Lipocalin-2, KIM-1, cystatin C, TIMP-1, β2M and OPN via Multiplex ELISAs.

Results: Albumin, and NGAL/lipocalin-2 were significantly elevated in the PAVP *vs.* PS group on GD14 ($p<0.001$) and GD18 ($p<0.001$). VEGF-A significantly decreased in the pregnant *vs.* non-pregnant groups on GD14 ($p<0.001$) and 18 ($p<0.001$). Clusterin, cystatin C, β2M and KIM-1 are significantly higher in the PAVP *vs*. PS groups on GD8 ($p<0.05$), 14 ($p<0.001$) and 18 ($p<0.05$). Significantly higher concentrations of TIMP-1 and OPN were observed in the PAVP *vs*. PS groups on GD14 and 18 respectively.

Conclusion: Arginine vasopressin successfully elevated the urinary levels of the kidney injury biomarkers and replicated kidney injury associated with PE development. Glomerular impairment and endothelial dysfunction is associated with the imbalance in kidney injury markers. Hence, our findings confirm the utility of the AVP rat model in studying the mechanisms driving renal damage in PE development.

Key words: glomerular injury, hypertension, pregnancy, tubular dysfunction

Introduction

The kidney is one of the most affected organ during pregnancy as it induces a wide range of functional changes which modify renal hemodynamics [1]. These changes include a significant elevation in glomerular filtration rate (GFR) [2] and renal plasma flow [3] together with kidney hypertrophy due to fluid retention [4]. This is accompanied by an imbalance in fluid and electrolyte levels and consequent mild increase in proteinuria, glycosuria with concomitant decline in serum osmolality and sodium levels [4].

As such the kidney is highly susceptible to hypertensive disorders during pregnancy such as preeclampsia (PE), which manifests after 20 week of gestation as new onset hypertension, with or without proteinuria and widespread organ damage including the kidney, liver, brain, heart and lungs [5]. Kidney injury associated with PE is commonly considered reversible as it spontaneously resolves post-delivery [6]. Nonetheless, women with persistent kidney impairment are reported to be vulnerable to end-stage kidney disease [7, 8].

Serum creatinine evaluation is currently the gold standard used to diagnose acute kidney injury and chronic kidney disease [9]. However, a limitation of serum creatinine concentration as a diagnostic indicator for identifying early changes in kidney function is its inability to discriminate structural kidney damage from functional hemodynamic changes [9-11]. The need for specific and sensitive urinary rather than circulating biomarkers of kidney injury is thus urgently warranted [12]. Moreover, reliable and sensitive markers to predict PE identification and development will enhance treatment and prevent maternal renal failure as well as protect the fetus from future renal complications [13].

Kidney structural proteins such as neutrophil gelatinase-associated lipocalin (NGAL/lipocalin-2), kidney injury molecule 1 (KIM-1), beta-2-microglobulin (β2M), cystatin C, albumin, clusterin, tissue inhibitor of metalloproteinases-1 (TIMP-1), osteopontin (OPN) and vascular endothelial growth factor-A (VEGF-A) have shown potential in detecting kidney injury [14-16] and utility as markers of PE detection [9, 17-22]. In PE, urinary levels of NGAL/lipocalin-2 [17], KIM-1 [9], cystatin C [18], albumin

[19] and clusterin [20] are higher in comparison to normotensive pregnant women. Moreover, women with PE have elevated circulating levels of TIMP-1 [21] and VEGF-A [22] in comparison to normotensive pregnancies. Notably, the diagnostic utility of β2M [23] and OPN [24] as markers of renal dysfunction is reported, however limited clinical data precludes their predictor value for PE identification and development.

Our lab has recently demonstrated that arginine vasopressin (AVP) induced in pregnant Sprague-Dawley rats replicates the phenotypes of hypertension, proteinuria and fetal growth restriction associated with human PE development [25]. In spite of proteinuria no longer being mandatory for the diagnosis of PE [5], it still remains a significant indicator of kidney injury. This study thus aimed to determine the renal injury associated with the AVP rat model by evaluating the urinary levels of kidney toxicity indicators viz., albumin, clusterin, KIM-1, OPN, TIMP-1, VEGF-A, β2M, cystatin C, and NGAL/Lipocalin-2.

Materials and methods

Ethical considerations

This study protocol was approved by the Animal Research Ethics Committee, University of KwaZulu-Natal (AREC/046/017) and was conducted in accordance with a previously published protocol [25]. All animals were subdivided into non-pregnant rats with saline delivery **(NS**, n = 6**)**; non-pregnant rats with AVP delivery **(NAVP, n=6)**; pregnant rats with saline delivery **(PS, n=6)** and pregnant rats with AVP delivery **(PAVP, n=6)**. Twenty-four hour urine samples were collected on gestational days (GD) 8, 14 and 18 until further analyses.

Multiplex ELISA assay

Kidney damage induced by AVP was determined using a multiplex enzyme-linked immunosorbent assay. Kidney toxicity panels 1 (Cat.#RKTX1MAG-37K) and 2 (Cat.#RKTX2MAG-37K) were purchased from Merck Millipore (Darmstadt, Germany). Both panels were subsequently used to measure the urinary concentration of clusterin, KIM-1, OPN, TIMP-1, VEGF-A and albumin, β2M, cystatin C, and NGAL/lipocalin-2.

All multiplex assays were done in triplicate and performed according to the manufacturer's instructions.

Procedure Kidney Panel 1 and Panel 2

The assay plate was inclusive of sample, background, standards and control wells. An assay buffer (200 µL) was added to all wells and mixed on a plate shaker for 10 minutes at room temperature. Thereafter, 25 µL of each standard or control was added into the appropriate wells. The assay buffer (25 µL) was added to background and sample wells, while 25 µL of serum was added to background, standards and control wells. This was followed by the addition of 25 µL diluted samples for panel (1:2 dilution) and panel 2 (1:500) respectively to sample wells. Biotinylated albumin (25 µL) was added in each well for Panel 2. This was followed by the addition of mixed beads (25 µL) and plates were incubated overnight at 4°C with agitation. The plate was washed twice with wash buffer, followed by the addition of detection antibodies (50 µL) into each well and incubated for 1 hour at room temperature. Streptavidin-Phycoerythrin (50 µL) was added to each well and incubated for 30 minutes at room temperature. The plate was then washed twice and resuspended in sheath fluid (125 µL). Both assay plates were then analyzed with the Bio-Plex MAGPIX® Multiplex reader (Bio-Rad Laboratories Inc., USA) with xPONENT® v.3.2 software and further analyzed with MILLIPLEX® Analyst 5.1 software (Merck Millipore, Darmstadt, Germany).

Data analysis

All data was analyzed using STATA (version 12, STATACORP). Normality of data was tested using the skewness and kurtosis normality test, and all parametric data are summarized as means and standard deviation. A one-way ANOVA was used to determine if a significant difference existed between the groups, followed by a pair-wise comparison of means between the different groups to determine significance. The Pearson's correlation was used to determine the existence of relationships amongst kidney injury markers as well as to correlate the expression of kidney injury markers with systolic and diastolic blood pressure, urinary output, pup number, calcium, chloride, sodium, potassium and urea. A p-value < 0.05 indicated statistical significance.

Results

Urinary glomerular biomarkers of kidney injury

The mean urinary levels for albumin, VEGF-A, cystatin C and β2M are shown in Figure 1. There was no significant differences were observed for albumin between the PAVP, PS, NS and NAVP groups on GD 8. In contrast, the levels were significantly elevated in the PAVP group compared to the PS, NS and NAVP groups on GD 14 (p<0.001) and GD18 (p<0.001) respectively. For VEGF-A, no significant differences were displayed by the experimental groups on GD 8. However a significant reduction in VEGF-A levels were observed in the PAVP and PS groups in comparison to the non-pregnant groups on GD 14 (p<0.001) and 18 (p<0.001). No significant changes were seen between the PS and PAVP groups on GD 8, 14 and 18.

Urinary tubular biomarkers of kidney injury

The concentrations (means±SD) for urinary clusterin, NGAL/lipocalin-2, KIM-1, OPN and TIMP-1 are shown in Figure 2. The levels of clusterin were significantly higher in the treated (PAVP and NAVP) versus the PS groups on GD 8 (p<0.001), 14 (p<0.001) and 18 (p<0.001). Similarly elevations in cystatin-C levels were observed in the PAVP group compared to the PS, NS and NAVP groups on GD 8 (p<0.05), 14 (p<0.001) and 18 (p<0.05). Likewise, β2M levels was upregulated in the PAVP group versus the PS, NS and NAVP groups on GD 8 (p<0.001), 14 (p<0.001) and 18 (p<0.001).

A significantly lower concentration was observed for NGAL/lipocalin-2 in the PAVP compared to the PS group on GD 8 (p<0.05), as opposed to the up-regulation noted in the PAVP *vs* the PS group on GD 14 (p<0.001) and 18 (p<0.001). In contrast, urinary levels of KIM-1 was significantly elevated in the PAVP *vs* the PS group throughout gestation [*i.e.* GD 8 (p<0.05), 14 (p<0.001) and 18 (p<0.001)]. A similar trend was noted for TIMP-1, which highlights elevations in the PAVP and PS groups on GD 14 (p<0.001) as well as GD 18, albeit non-significant. In contrast, OPN levels was significantly reduced in the PAVP group compared to the PS and NAVP groups (p<0.001) on GD 8. However, OPN levels were significantly elevated in the PAVP *vs* the PS group on GD 18 (p<0.001).

Pearson's Correlation between analytes and clinical factors

The correlation analyses revealed a significant and positive correlation only between β2M and cystatin C (r = 0.9260, p = 0.0080), and between clusterin and diastolic blood pressure (r = 0.9720, p = 0.0050) on GD8. On GD 14, both systolic (r = 0.949, p = 0.051) and diastolic (r = -0.922, p = 0.026) blood pressures was positively correlated with cystatin C. However for KIM-1, a positive correlation was noted only for systolic blood pressure (r = 0.893, p = 0.042). In the PAVP group, albumin demonstrated a positive correlation with cystatin C on GD 14 (r = 0.9199, p = 0.0094) and GD 18 (r = 0.9457, p = 0.0043). Moreover, a positive correlation was observed between albumin and NGAL/Lipocalin-2 (r = 0.8927, p = 0.0167), OPN (r = 0.8442, p = 0.0345) and KIM-1 (r = 0.8284, p = 0.0416) in AVP treated pregnant rats on GD 18. A strong association was also noted between cystatin C and NGAL/Lipocalin-2 (r = 0.9522, p = 0.0034) on GD 18 in the PAVP group. In pregnant treated rats (GD 14), clusterin was positively associated with VEGF-A (r = 0.9326, p = 0.0067); however, on GD18, it was significantly associated with OPN (r = 0.8969, p = 0.0154).

Off note, calcium was positively associated with OPN on GD 18 (r = 0.8683, p = 0.0249) in treated pregnant rats. Moreover, in the AVP treated pregnant rats, a strong positive association was noted between chloride and albumin (r = 0.9120, p = 0.0113) as well as OPN (r = 0.9403, p = 0.0052). Similarly, sodium was strongly associated with albumin (r = 0.9611, p = 0.0022) and OPN (r = 0.8668, p = 0.0254), while potassium is positively associated with albumin (r = 0.8258, p = 0.0429) on GD 18.

On GD 18, urea demonstrated a strong, positive correlation with KIM-1 (r = 0.8532, p = 0.0308), OPN (r = 0.8465, p = 0.0335), albumin (r = 0.9917, p = 0.0001), cystatin C (r = 0.9330, p = 0.0066) and NGAL/Lipocalin-2 (r = 0.8951, p = 0.0159) in the PAVP group. In the treated pregnant groups, urinary output was inversely associated with cystatin C (r = -0.8702, p = 0.0242) and NGAL/Lipocalin-2 (r = -0.9138, p = 0.0108), while pup number was inversely correlated with TIMP-1 levels (r = -0.8237, p = 0.0439) on GD 18. A strong negative association was noted on GD 18, between KIM-1 and systolic (r = -0.895, p = 0.040) and diastolic blood pressure (r = -0.968, p = 0.007), while

β2M demonstrated a positive association only with systolic blood pressure ($r = 0.813$, $p = 0.049$).

Discussion

Our findings demonstrates significantly higher levels of urinary injury markers in the AVP treated pregnant groups compared with untreated groups. Our data illustrating glomerular injury confirm significant elevations in albumin, with a concomitant reduction in VEGF-A throughout gestation. Albumin levels are significantly higher in pregnant AVP treated rats as early as GD 14 in comparison to those infused with saline. Moreover, earlier studies support the use of albumin excretion as a reflection of glomerular damage and systemic endothelial cell dysfunction than total protein excretion [15]. Our findings of elevated albumin levels in the pregnant AVP-treated rats confirm glomerular injury and may be a result of impaired glomerular filtration and consequent reduction in tubular reabsorption. It is possible that the albuminuria coincides with glomerular damage and subsequent podocyturia in response to AVP-induction. Similar findings were demonstrated in women with severe PE diagnosed with albuminuria [19]. The frequency of sustained microalbuminuria also appears to be almost 4-times higher in women with a history of PE compared to normotensive pregnant women [26]. Additionally the proximal tubule cells facilitate the renal reabsorption of all filtered proteins, including albumin which also competes for reabsorption with other low-molecular-weight proteins such as β2M, cystatin C and NGAL/Lipocalin-2 in order to be returned to the circulation to maintain plasma albumin levels [19]. The significant positive correlations between albumin and NGAL/Lipocalin-2 and cystatin C in our study suggest that those with albuminuria also exhibit high levels of NGAL/Lipocalin-2 and cystatin C and consequently competency for reabsorption.

In the kidney, VEGF-A/VEGF is produced by podocytes and is transferred across the glomerular basement membrane where it aids in the maintenance of the glomerular filtration barrier by regulating the survival and structural integrity of endothelial cells [27]. Hence, kidney injury may be attributed to an imbalance in glomerular VEGF-A expression [28]. Moreover, circulating levels of VEGF-A are significantly higher in

preeclamptic women compared to normotensive pregnancies [22], as well as in preeclamptic women giving birth to small gestational aged infants [29]. In our study, the reduced VEGF-A in pregnant AVP-treated rats is indicative of an angiogenic imbalance, associated with the VEGF inhibitor, soluble fms-like tyrosine kinase (sflt-1) [30, 31]. The administration of sFlt-1 to diabetic mice is reported to reverse kidney damage and albuminuria and subsequently reduces mesangial proliferation [32]. Patients with diabetic nephropathy exhibit lower levels of renal VEGF-A which correlate with disease progression [33].

Since proximal tubular cells of the nephron is where injury is noted, urinary examination is critical in detecting and confirming kidney dysfunction as early as the first day of renal diagnosis, and may be a more accurate indicator than blood biomarkers [12]. Our tubular data displays significant elevations observed for clusterin, cystatin C, β2M, NGAL/lipocalin-2, KIM-1, OPN and TIMP-1 concentration in AVP treated pregnant rats is suggestive of tubular injury. Kidney disease has shown an association with increased tubular expression of clusterin which is subsequently released into the lumen [18, 34]. Elevated circulating levels of clusterin often result from tissue specific injury and tissue remodeling [35]. Our findings, demonstrating an elevation in urinary clusterin levels observed in the PAVP compared to the PS groups, may be a compensatory response for the defective spiral artery remodelling and inadequate placental development emanating from the shallow trophoblastic invasion in PE [36]. Our data also indicates a positive association between clusterin and diastolic blood pressure in the PAVP group, suggestive that diastolic pressure elevations induced by the AVP may be responsible for increasing the levels of clusterin. Our findings are corroborated by significant differences in clusterin levels between PE and normotensive groups in both urine [20] and serum [37].

The proximal tubular injury markers NGAL/Lipocalin-2, KIM-1, cystatin C, β2M and OPN was also significantly elevated in the PAVP compared to the PS group. It is possible that AVP stimulated NGAL/lipocalin-2 release from tubular epithelial cells to protect the kidney against ischemic and nephrotoxic injury [38]. Urinary NGAL/lipocalin-2 is upregulated in the first trimester in pregnancies complicated with type 1 diabetes mellitus prior to PE development compared to normotensive pregnancies [39]. Additionally, we

report a strong correlation between cystatin C and NGAL/Lipocalin-2, which may be indicative of a relationship between reduced GFR and tubular damage induced by AVP. Increased urinary KIM-1 levels is reported to be associated with acute kidney injury [16, 40] and ischemic renal injury [41]. Our results are similar to the high urinary NGAL/Lipocalin-2 and KIM-1 reported in women with mild and severe PE compared to normotensive pregnancies complicated by chronic hypertension [42]. These findings suggest that AVP may have induced tubular dysfunction and/or glomerular endotheliosis and/or podocyturia. Our significant positive correlations between KIM-1 and blood pressure on GD 14 and GD 18 once again suggests that AVP induces elevations in BP which consequently stimulates KIM-1 release from the tubular cells, and consequent tubular injury.

Cystatin C and β2M are freely filtered by the glomerulus and are reabsorbed and metabolized by the proximal tubules, [23, 43]. They are degraded and not reabsorbed into circulation [44], thus an increase in the urinary excretion of both cystatin C and β2M observed in our study is reflective of proximal tubular injury and hence supports their use in predicting kidney injury prior to histopathological changes [14]. In our study, urinary cystatin C is directly associated with systolic and diastolic blood pressure on GD 14, whilst β2M was positively associated with systolic blood pressure on GD 18. The elevations in both cystatin C and β2M in the AVP treated groups in our study is indicative of AVP induced proximal tubular injury which is believed to inhibit reabsorption via megalin receptors [45] and their subsequent catabolism and loss in the urine [46]. Urinary levels of cystatin C are significantly higher in PE compared to normotensive pregnancies [18], and is closely correlated with impaired glomerular filtration and acute kidney injury [19]. Off note, in contrast to their urine expression, the serum levels of cystatin C and β2M have been proposed as serum markers of reduced GFR due to their minimal presence in the urine under normal conditions [47, 48].

Osteopontin (OPN) is widely expressed in bone, immune, smooth muscle, epithelial and endothelial cells [49], as well as human and murine placental tissue [50]. Hence, their expression in placental tissue implicates them in trophoblast proliferation and invasion during early placentation. Preeclamptic patients with extensive endothelial injury are

reported to have higher plasma OPN levels [24], whereas urinary OPN levels are significantly increased in diabetic patients with renal impairment [51]. It was recently established that elevated urinary OPN is associated with proteinuria, reduced creatinine clearance, fibrosis and macrophage and T-cell infiltration [52]. Osteopontin and β2M are also implicated in modulating the immune response, thereby enhancing the T helper cell 1 (T_H1)-mediated response [52]. The elevations in OPN and β2M levels observed in our study may be associated with the immunoregulatory role of AVP in T_H1 cell predominance, dendritic cell activation, and T helper 17 cell activation, characteristic of early PE development [53]. Scroggins et al., (2018) also demonstrated an increased pro-inflammatory T_H1- associated interferon gamma (IFN-ɤ) response in a PE mouse model subcutaneously infused with AVP throughout gestation [54].

Our correlation analyses confirm a significant direct relationship between OPN and calcium, chloride, sodium and urea respectively. The kidney tubules regulate serum electrolyte levels viz., sodium, potassium, and calcium, which subsequently maintains homeostasis [55]. The positive associations observed between OPN and calcium, chloride, sodium as well as urea is indicative of a possible electrolyte imbalance resulting from tubular injury. Our findings are also suggestive of a potential inhibitory role of OPN on the formation, growth and aggregation of calcium oxalate crystals which is a primary component of kidney stones [56]. Furthermore, the kidney also maintains chloride homeostasis, through reabsorption thereby regulating extracellular fluid volume [57]. The positive association demonstrated between OPN and chloride levels is thus reflective of tubular injury and chloride imbalance as a consequence of AVP induction in pregnant-treated rats.

Our data also demonstrates increased TIMP-1 levels in the PAVP group as a consequence of AVP infusion. The elevated TIMP-1 levels may prevent extracellular matrix remodelling in the kidney by inhibiting the action of matrix metalloproteinases (MMP) [58]. Elevations in TIMP-1 is also associated with chronic renal disease [58]. In PE, significant elevations in TIMP-1 expression was observed in the placenta [59] as well as in serum [21, 60, 61]. This may have a contributory role in impairing trophoblast invasion

and consequent poorly perfused fetoplacental unit during the early stages of PE development, which is also influenced by a decrease in MMP-2 and MMP-9, [58].

Conclusion

This study confirms a significant upregulation in the urinary levels of albumin, clusterin, NGAL/Lipocalin-2, KIM-1, OPN, β2M, cystatin C and TIMP-1 with a reduction in VEGF-A in the AVP treated pregnant group compared to untreated pregnant group. This suggests a role of AVP in inducing substantial renal injury, characteristic of PE development as well as demonstrates their value as early predictors for PE development. Moreover, these kidney injury biomarkers may be useful in identifying the specific area of injury in the nephron, offering the advantage of differentiating between kidney injury and altered kidney functioning. Further investigations are however required to fully elucidate the role of AVP in the mechanisms that initiate kidney injury in PE development.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Author contributions

NG, TN, SB: protocol/project development. SR: data collection. NG, TN, SB, SR: data analysis. NG, TN, SB, SR: manuscript writing/editing

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List of figure captions

1. **Figure 1: Markers of glomerular dysfunction.** *p < 0.001: NS *vs* PS; **p < 0.001: NAVP *vs* PS; ***p < 0.001: PAVP *vs* PS; # p < 0.001: PAVP *vs* NS; ## p < 0.001: PAVP *vs* NAVP.

2. **Figure 2: Markers of tubular injury.** *p < 0.05: PAVP *vs* PS;**p < 0.001: PAVP *vs* PS; # p < 0.05: PAVP *vs* NS; ## p < 0.05: PAVP *vs* NAVP.

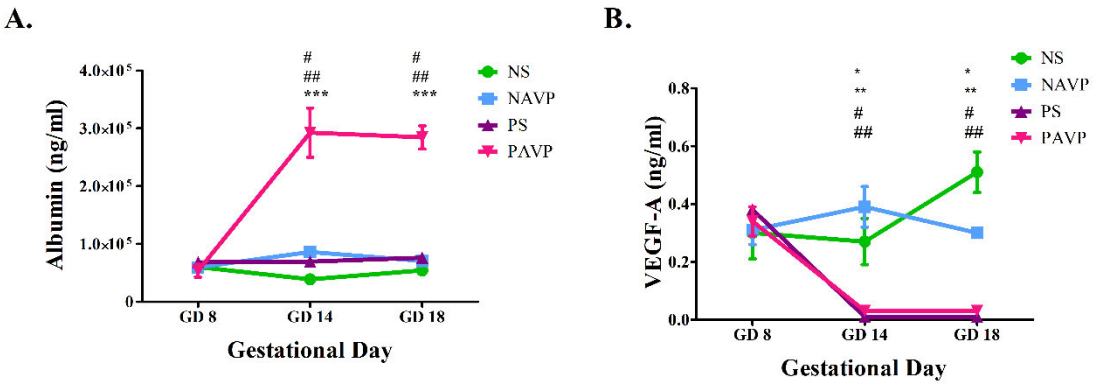

Figure 1: Markers of glomerular dysfunction.

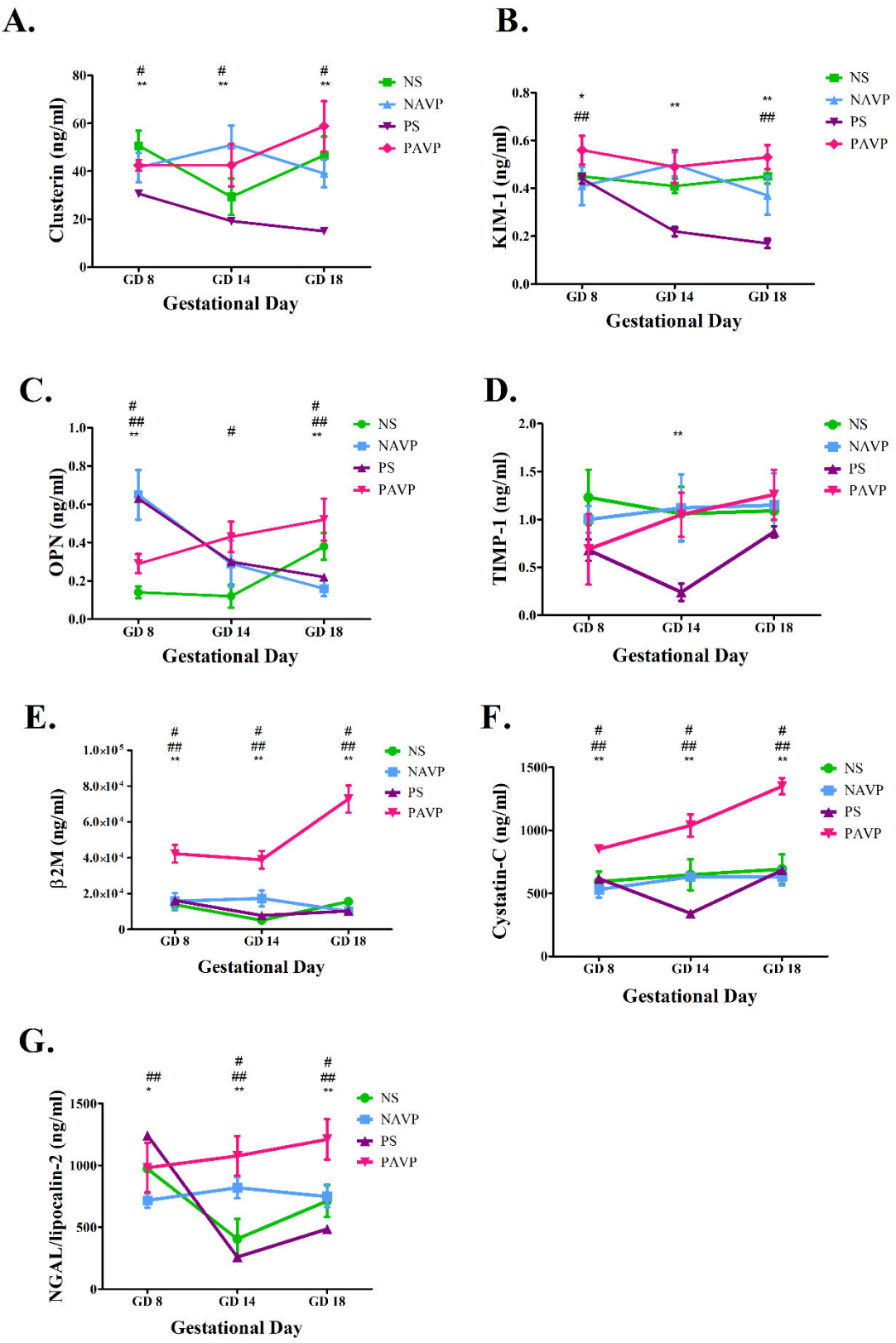

Figure 2: Markers of tubular injury.

CHAPTER 6

Conference Abstracts

Abstracts generated from this study were presented at the following conferences:

Ramdin, S., Baijnath, S., Naicker, T., Singh, S.D. and Govender, N., 2021. Does arginine vasopressin induce kidney injury in pregnant Sprague Dawley rats? *Keystone Symposia eSymposia meeting Maternal-Fetal Newborn Immunity 22EK1*. Virtual attendance, 28-29th October 2021.

Ramdin, S., Baijnath, S., Naicker, T. and Govender, N., 2022. Renal injury associated with arginine vasopressin induction in a rat model of preeclampsia. *The 29th Scientific Meeting of the International Society of Hypertension.* Kyoto, Japan, 12-16th October 2022.

Does arginine vasopressin induce kidney injury in pregnant Sprague Dawley rats?

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Introduction: Preeclampsia (PE) is a leading cause of maternal and fetal morbidity and mortality. Arginine vasopressin (AVP) has been implicated in PE pathogenesis, therefore this study investigates the physiological effects of AVP on the kidneys of pregnant rats in an attempt to elucidate its role in PE development.

Methods: Twenty-four female Sprague Dawley rats were surgically implanted with osmotic pumps infusing with either saline or AVP for 18 days. The rats were divided into four groups: non-pregnant saline, pregnant saline, non-pregnant AVP and pregnant AVP. Blood pressure measurements and urine samples were taken on gestational days (GD) 8, 14 and 18. Placental and fetal weights were recorded on GD 18 following sacrifice. Morphological changes in the kidney were visualized using hematoxylin and eosin staining. Urinary levels of cystatin C and kidney injury molecule-1 (KIM-1) were determined using Rat kidney toxicity immunoassay panels 1 and 2.

Results: Blood pressure and urinary protein levels were significantly elevated in the pregnant AVP *vs* pregnant saline group ($p<0.05$). The pregnant AVP group also displayed significantly reduced placental and individual pup weight compared to the pregnant saline group ($p<0.05$). Qualitative analysis of kidney morphology revealed a slight increase in glomerular mesangium and mesangial cells, as well as a decrease in the Bowman's space in the pregnant AVP group *vs* pregnant saline group. Urinary levels of cystatin C and KIM-1 were also significantly higher in the pregnant AVP group compared to the pregnant saline group.

Conclusion: AVP infusion increases blood pressure, urine protein levels urinary expression of cystatin C and KIM-1, with concomitant glomerular pathology. Our findings suggest significant kidney damage associated with AVP exposure, indicative of its utility of this model in studying the mechanisms underlying renal damage in PE.

Methods: Male Sprague-Dawley rats were divided into Sham, 5/6 nephrectomy + sedentary, and 5/6 nephrectomy + exercise training groups. 5/6 nephrectomy + exercise training group underwent treadmill running for 12weeks. Systolic blood pressure and urinary protein were measured every 2 weeks. Renal function, renal histology and oxidative stress-related factors were examined after completion of the exercise protocol.

Results: 5/6 nephrectomized rats exhibited hypertension, proteinuria, renal dysfunction glomerulosclerosis, renal interstitial fibrosis, and exercise training ameliorated them. 5/6 nephrectomy increased urinary malondialdehyde, renal cortical NADPH oxidase and xanthine oxidase activities and exercise training inhibited the 5/6 nephrectomy-increased urinary malondialdehyde, renal cortical NADPH oxidase and xanthine oxidase activities. Plasma malondialdehyde was not affected by 5/6 nephrectomy or exercise.

Conclusions: In rats with chronic kidney disease, long-term exercise prevented hypertension, renal dysfunction, glomerulosclerosis, and renal interstitial fibrosis. Mechanism for the antihypertensive and renal protective effects of long-term exercise in chronic kidney disease may involve the reduction of renal oxidative stress.

PS-BPB06-5 URINARY CELL TRANSCRIPTOMICS: A NON-INVASIVE EXPRESSION READOUT OF KIDNEY GENES OF RELEVANCE TO HYPERTENSION

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Objective: Hypertension affects 35% of the global adult population and is the leading preventable risk factor for premature death globally. The kidney is the key organ of blood pressure regulation in the body. Here, we compare the gene expression profile from renal cells shed naturally into the urine and kidney tissue to determine if urine can provide a non-invasive readout of kidney gene expression and whether hypertension-associated genes can be quantified in urine.

Design and Method: Urinary cell and kidney tissue samples were collected from 33 human participants and were both profiled by poly-A RNA-sequencing, generating an average of 30 million paired reads per sample. These were quantified using the standard Genotype Tissue Expression (GTEx) project pipeline and were compared against 43 different human tissues and other bodily fluids using transcriptomic correlation. RNA-sequencing quality metrics were calculated by RNA-SeQC software. Gene set overrepresentation analysis for Gene Ontology (GO) terms and Kyoto Encyclopaedia of Genes and Genomes (KEGG) employed Fishers exact test. Enriched and enhanced kidney genes were collected from the Human Protein Atlas (HPA).

Results: Our RNA-sequencing metric analysis revealed that urinary cells can generate robust data of comparable or superior quality to that of saliva at similar read coverage. The top one hundred most highly expressed urinary cell genes show an enrichment for biological themes shared with the renal transcriptome, including immunity ($P = 1.2x10^{-7}$), glucose metabolism ($P = 1.3x10^{-5}$) and renal mineral reabsorption ($P = 9.8x10^{-4}$). In an analysis all protein-coding genes across 43 human tissues/cell-types, urinary cells showed the highest level of transcriptomic correlation with kidney cortex ($r^2 = 0.65$) and kidney medulla ($r^2 = 0.64$). This correlation between urinary cells and kidney tissue was particularly strong ($r^2 = 0.72$) in an analysis restricted to highly specific kidney genes (including uromodulin and the Na-K-Cl cotransporter NKCC2 [loop diuretic target]). 98% (176 out of 179) of kidney genes with a known causal association to blood pressure were expressed in urinary cells. Their urinary expression demonstrated strong correlation with their abundance in kidney cortex ($r^2 = 0.68$) and medulla ($r^2 = 0.64$).

Conclusions: Standard poly-A RNA-sequencing of cells harvested from urinary sediments produces robust gene expression profiles. These profiles provide a non-invasive insight into transcriptome of the kidney and permit measuring expression of kidney genes of relevance to BP regulation and hypertension.

PS-BPB06-6 RENAL INJURY ASSOCIATED WITH ARGININE VASOPRESSIN INDUCTION IN A RAT MODEL OF PREECLAMPSIA

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Objective: Worldwide, preeclampsia (PE) accounts for 2–8% of pregnancies and is a main cause of maternal and fetal morbidity and mortality. *In vivo* murine studies confirm the use of arginine vasopressin (AVP) in replicating the clinical symptoms of PE development, suggestive of a role for PE prediction. The renal effects that AVP may have in PE remains elusive, hence this study aimed to explore the physiological effects of AVP on the kidneys of pregnant rats in an effort to determine its role in PE development.

Design and method: Twenty-four female Sprague Dawley rats (non-pregnant saline, pregnant saline, non-pregnant AVP and pregnant AVP) were surgically implanted with ALZET mini osmotic pumps infusing with either saline or AVP for 18 days. Blood pressure measurements and urine samples were taken on gestational days (GD) 8, 14 and 18. Placental and fetal weights were recorded following sacrifice on GD18. Urinary levels of kidney injury molecule-1 (KIM-1) were determined using the rat kidney toxicity immunoassay panel 1 (#RKTX-1MAG-37K). Immunohistochemistry was used to determine the expression of KIM-1 in rat kidney tissue.

Results: Systolic [143.00 (139.67–144.00) mmHg *vs* 125.00 (124.00–127.00) mmHg; $p < 0.05$] and diastolic blood pressures [103.50 (100.33–106.88) mmHg *vs* 81.00 (80.00–87.00) mmHg; $p < 0.001$] were significantly higher in the pregnant AVP *vs* pregnant saline group. Urinary protein levels in pregnant AVP rats [0.45 (0.42–0.48) g/L] were significantly elevated in comparison to pregnant saline rats [0.24 (0.21–0.32) g/L; $p < 0.05$]. Pregnant AVP rats also demonstrated a significant decrease in individual placental [0.39 (0.31–0.46) g *vs* 0.59 (0.55–0.63) g; $p < 0.05$] and pup weights [1.57 (1.45–1.67) g *vs* 1.79 (1.70–1.85) g; $p < 0.05$] in comparison to pregnant saline rats. Urinary KIM-1 levels are significantly elevated in the pregnant AVP (0.53 ± 0.05 ng/mL) *vs* pregnant saline group (0.17 ± 0.02 ng/mL; $p < 0.001$). Qualitative analysis revealed that KIM-1 expression is markedly upregulated on the apical membrane surface of epithelial cells of the proximal convoluted tubules in AVP treated rats.

Conclusions: AVP infusion increases blood pressure, urine protein levels and urinary and tissue expression of KIM-1. Our findings indicate that there is significant kidney injury associated with AVP treatment, indicative of the utility of this model in studying the mechanisms driving renal damage in PE.

PS-BPB06-7 NOVEL MOLECULAR MECHANISMS OF CARDIO-RENAL INTERACTION MEDIATED BY INCREASED SERUM MODIFIED NUCLEOSIDES ASSOCIATED WITH RENAL FAILURE

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Objective: The number of patients with chronic kidney disease (CKD) continues to rise by population aging. Moreover, CKD patients progress to end-stage renal failure and require renal replacement therapy. Many cohort studies have shown that impaired renal function itself is a risk factor for the development of cardiovascular disease, rather than comorbidities such as diabetes or hypertension. However, the molecular mechanisms are not fully understood. We focused on chemical modifications in RNA and their pathophysiological significance. Previously, we have shown that modified nucleosides, the metabolites of modified RNA, have unique physiological effects. For example, we reported that N6-methyladenosine is a true ligand for the A3 adenosine receptor, and that 1-methylguanosine promotes Zika virus replication in the host. In addition, it was found that a number of modified nucleosides are present in serum and excreted into extracellular spaces like urine. Therefore, we hypothesized that the decreased urinary excretion of modified nucleosides upon renal failure may cause abnormally high levels of modified nucleosides in the blood, which may exert detrimental effects on the heart and vasculature.

Methods and Results: First, serum samples were collected from 210 CKD patients, and the correlation between serum modified nucleoside levels and eGFR

CHAPTER 7

7.1. SYNTHESIS, CONCLUSIONS AND FUTURE RESEARCH

7.1.1 Problem statement

Preeclampsia (PE), a hypertensive disorder of pregnancy, is the main direct cause of maternal and fetal morbidity and mortality in SA (South African National Department of Health, 2017), accounting for an estimated prevalence rate of 18% (Moodley *et al.*, 2016; Moodley, 2018). The multifactorial nature of this disorder renders it difficult to ascertain its exact etiology. However, it is believed that its pathogenesis arises from reduced trophoblast invasion, non-physiological transformation of spiral arteries with subsequent reduced placental perfusion and resultant placental and fetal hypoxia (Pankiewicz *et al.*, 2019). At present, there is no definitive cure or efficient predictive biomarkers for this disorder. To this extent, the use of animal models has been limited yet valuable in PE study. Animal models have led to novel discoveries regarding the pathways involved in the pathogenesis of this disorder, as well as new methods for screening, diagnosis, and treatment.

Kidney injury associated with PE is characterized by pathognomic glomerular endotheliosis (Wang *et al.*, 2015). Additionally, preeclamptic patients display glomerular sclerosis, capillary occlusion, macrophage infiltration, and reduced density of endothelial fenestrae (Karumanchi *et al.*, 2005; Strevens *et al.*, 2003; Stillman and Karumanchi, 2007). Of note, PE is proposed to increase the susceptibility to future risk of kidney disease and has also been linked to a higher risk of albuminuria (McDonald *et al.*, 2010; Kattah *et al.*, 2013), chronic kidney disease (Wang *et al.*, 2013), and end-stage renal disease (Vikse *et al.*, 2008; Wang *et al.*, 2013; Kattah *et al.*, 2017; Covella *et al.*, 2019). Therefore, a greater understanding of the association between PE and kidney injury will assist in the discovery of novel biomarkers for PE prediction and innovative treatment strategies.

Research conducted in pregnant women is challenging due to the potential danger posed to maternal and fetal health. Additionally, the ethical limitations faced by human studies renders animal models a more feasible and valuable option to study the mechanisms associated with PE and to develop and test innovative treatment strategies. Of the

numerous animal models of PE available, the AVP mouse model displays features that closely model human PE. Infusion of AVP into pregnant mice cause pregnancy-specific hypertension, elevated urinary protein expression, glomerular endotheliosis and fetal growth restriction (Santillan *et al.*, 2014). Further study of this model also revealed that higher AVP levels lead to changes in placental morphology, reactive oxygen species and angiogenic imbalance (Sandgren *et al.*, 2015). These findings propose the potential value that AVP dysregulation may have in future PE research.

Hence, this study sought to reproduce these findings in a rodent model as their placental vasculature shares close similarities with human placentation. Additionally, the AVP mouse model did not fully explore the renal effects of AVP in pregnant mice. Therefore, this study provides a comprehensive morphological investigation into the composite AVP effects induced on the kidneys of pregnant rats.

The aim of the study was accomplished through the completion of the following objectives:

1. Determining the biochemical, haematological and fetal parameters in the AVP treated pregnant rats to provide a complete physiological characterization of the model
2. Determining the morphological changes in the kidneys of AVP-treated rats using Haematoxylin and Eosin **(**H/E) staining.
3. Determining the ultrastructural features of the glomerular filtration apparatus in rats infused with AVP, using transmission electron microscopy.
4. Immunolocalizing the structural proteins of the glomerular filtration apparatus in the kidney and kidney injury markers viz., podocalyxin and KIM-1 in the AVP-treated rat kidney using immunohistochemistry.
5. Investigating the urinary expression of albumin, VEGF-A, clusterin, cystatin C, β2M, KIM-1, NGAL/lipocalin-2, OPN and TIMP-1 in the AVP-induced rat kidney using the Multiplex ELISA immunoassay procedure.

The details presented in this chapter are a summary of each objective and the manuscripts associated with it. Four manuscripts emanated from this study, of which 1 is published and 3 are under review (1 is currently under 2nd review in Current Hypertension Reports, following minor revisions).

7.1.2 Manuscript 1 – Chapter 2 (aligned with the Literature review)

The clinical value of rodent models in understanding preeclampsia development and progression [Published in Current Hypertension Reports (Impact factor: 4.59), DOI: https://doi.org/10.1007/s11906-023-01233-9]

This chapter provides a review of the current rodent models of PE and correlates their value to the human condition. At present there are limited treatment options available for the management of PE. These include extending the duration of pregnancy, anti-convulsive treatment to avert the occurrence of seizures and lastly the premature delivery of the fetus and placenta, which is the most effective form of treatment for PE (Dekker, 2014). As global prevalence of PE continues to increase, there is a greater need to expand PE research to unravel the pathogenic processes involved in this disorder and to discover and develop novel forms of treatment that can be used to ensure the wellbeing of both the mother and fetus (Taylor and George, 2022).

In this regard, a variety of animal models have been developed to study PE pathogenesis and ascertain potential therapeutic targets. To-date, numerous methodologies have been utilized in the quest to create a suitable animal model that fully replicates all aspects of human physiology in PE. Unfortunately, the complex multi-system nature of this disorder makes developing a gold standard model to study PE extremely challenging.

These models have been created via surgical (Alexander *et al.*, 2001; Khalil and Granger, 2002; LaMarca *et al.*, 2016), pharmacological intervention (Ramesar *et al.*, 2011; Santillan *et al.*, 2014), genetic and immunological modification (Doridot *et al.*, 2012; Maynard *et al.*, 2003; Nishizawa *et al.*, 2008; Santillan *et al.*, 2014), and through the utilization of animals with pre-existing hypertension that develop superimposed PE (Davisson *et al.*, 2002; Gillis *et al.*, 2015). These models are able to replicate clinical

characteristics consistent with human PE such as pregnancy-specific hypertension, proteinuria, renal dysfunction, placental ischemia and fetal growth restriction (Gatford *et al.*, 2020).

While these models are associated with various limitations, they have contributed significantly to the development of therapeutic and diagnostic strategies. The more widely studied rodent and murine models of PE include the RUPP, sFlt rat model, BPH5 mouse model and L-NAME models and have been used to explore therapeutic interventions. Pravastatin administration in the RUPP model lowers blood pressure, reactive oxygen species and aids in restoring angiogenic balance (Bauer *et al.*, 2013). Unfortunately, these findings were not reflected in human trials in early-onset preeclamptic women, who demonstrated that while pravastatin treatment lowered adverse fetal outcomes it does not affect sFlt-1 concentration in plasma or alter the sFlt-1:PlGF ratio in comparison to the placebo group (Ahmed *et al.*, 2020; Costantine *et al.*, 2021). In contrast, RUPP rats treated with PlGF have been shown to exhibit down-regulated levels of sFlt-1, blood pressure, and urinary protein (Spradley *et al.*, 2016).

Additionally, the administration of the endothelin type A receptor antagonist ameliorates mean arterial pressure reduced in the RUPP (Alexander *et al.*, 2001), sFlt-1 infusion (Murphy *et al.*, 2010), TNF infusion (LaMarca *et al.*, 2005), and AT1-AA infusion models (LaMarca *et al.*, 2009). Further testing of the RUPP model demonstrates that vitamin D treatment reduces blood pressure, endothelin-1, sFlt-1, and AT1-AA levels but does not influence fetal outcomes (Darby *et al.*, 2013; Faulkner *et al.*, 2016; Tian *et al.*, 2016). The sFlt-1 rat model has contributed to the development of angiogenic screening platforms and immunoassays to assist PE diagnosis (Maynard *et al.*, 2003). The Triage PlGF and the Elecsys immunoassay sFlt-1/PlGF ratio tests have clinical potential in excluding PE diagnosis in women presenting with suspected symptoms between 20 and 34 weeks (NICE, 2021).

We report that no single model of PE mimics the phenotypes of human PE such as abnormal placentation, fetal growth restriction, pregnancy-specific hypertension, proteinuria, endothelial dysfunction, and an imbalance of angiogenic factors. Our

findings recommend the use of a combination of different rodent models to study and uncover new aspects of PE pathogenesis and development.

7.1.3 Manuscript 2 – Chapter 3 (Associated with Objective 1)

Physiological characterization of an arginine vasopressin rat model of preeclampsia [Published in Systems Biology in Reproductive Medicine (Impact factor: 2.96), DOI: 10.1080/19396368.2021.1981486]

Chapter 3 provides a complete physiological characterization of the AVP Sprague Dawley rat model following its successful transfer from the AVP mouse model (Ramdin *et al.*, 2022). Arginine vasopressin is a vasoconstrictor and anti-diuretic hormone used to induce a PE-like syndrome in pregnant Sprague Dawley rats. Chronic administration of AVP (150ng/hr) throughout gestation (GD1-GD18) replicates the phenotype associated with human PE, viz., elevated blood pressure (≥140/90 mmHg) (Magee *et al.*, 2022), increased urinary protein levels and fetal growth restriction. Our findings corroborate those reported by Santillan and co-workers (Santillan *et al.*, 2014).

Arginine vasopressin maintains blood pressure homeostasis via the modulation of body fluid osmolarity and volume (Jadli *et al.*, 2015). The hypertension displayed by treated pregnant rats in our study may be attributed to the AVP-mediated reabsorption of water in the kidney tubules and arteriole constriction, resulting in an elevation in peripheral vascular resistance (Erfanian *et al.*, 2019). An increase in systolic blood pressure is associated with increased glomerular pressure, increased glomerular filtration rate and glomerular damage with resultant proteinuria (Baijnath et al., 2014). We demonstrate significantly higher urinary protein:creatinine ratios in the PAVP group *vs.* PS group on GD 8 and 14. It is plausible that AVP influences the macula densa and tubulo-glomerular feedback mechanism in response to an elevation in intraglomerular pressure which leads to a dysregulation in GFR. This in turn stimulates the activation of the RAAS which down-regulates nitric oxide release, resulting in arteriolar vasoconstriction and elevated glomerular and systemic blood pressure, leading to glomerular hyperfiltration and proteinuria.

Our data is corroborated by Sandgren (2018) who demonstrated that pregnant mice chronically infused with AVP display glomerular endotheliosis and proteinuria (Sandgren *et al.*, 2018a). Elevated circulating levels of AVP result in a rise in peripheral vascular resistance, which increases systemic blood pressure along with glomerular injury (Santillan *et al.*, 2014). This is evidenced by the higher urinary protein concentration exhibited by the AVP-treated rats in our study. Additionally, a recent study evaluated the significance of the urine protein:creatinine ratio in the diagnosis of proteinuria and determined its association with poor pregnancy outcomes in PE typified by kidney injury (Xiao *et al.*, 2022). In preeclamptic women with kidney injury, 24hr proteinuria showed a significant positive correlation with the protein:creatinine ratio, while the protein:creatinine ratio was determined to be an in independent predictive factor for poor maternal and fetal outcomes. They propose the use of protein:creatinine ratio-based diagnosis of proteinuria in preeclamptic patients as a more effective and simpler method in comparison to 24hr proteinuria measurement (Xiao *et al.*, 2022).

We also report significantly higher serum ALT and triglyceride levels along with reduced HDL levels in pregnant AVP-treated rats. Higher levels of ALT are observed in women with PE in comparison to normotensive preeclamptic women (Dacaj *et al.*, 2016). We propose that AVP potentially dysregulates liver function by promoting a hypoxic environment which results in higher ALT levels. The excess triglyceride levels reported in our study may result from the anti-lipolytic function of AVP, which induces the inhibition of tissue lipase which is involved in the degradation of triglycerides (Hiroyama *et al.*, 2007). We also demonstrate lower HDL levels in the treated groups in comparison to non-treated groups, which may result from endothelial dysfunction (White *et al.*, 2019).

Infusion of AVP significantly reduced individual placental and pup weights as well as the number of pups produced in pregnant AVP-treated rats compared to pregnant saline-treated rats. These findings may be a result of inadequate perfusion emanating from the vasoconstrictive action of AVP; emulating foetal growth restriction that occurs in PE. Our data corroborates those reported by other studies (Crews *et al.*, 2000; Alladin and Harrison, 2012; Macdonald-Wallis *et al.*, 2014; Santillan *et al.*, 2014). Histopathological

analyses of the placenta demonstrated a high frequency of degenerative spongiotrophoblasts in rats of the PAVP group compared to the untreated groups. Furthermore, a qualitative examination of kidney morphology revealed a mild increase in mesangium, mild glomerular crescents and reduced Bowman's space in AVP treated rats. These findings endorse the ability of the AVP rat model to sufficiently replicate the clinical features of human PE and verifies the possible role of AVP dysregulation in the development of PE. Our model also demonstrates that blood pressure modulators are active in early pregnancy and could therefore provide new insights to the etiology of PE development.

7.1.4 Manuscript 3 – Chapter 4 (aligned with objective 2, 3 & 4)

Kidney injury molecule-1 and podocalyxin dysregulation in an arginine vasopressin induced rodent model of preeclampsia [Published in European Journal of Obstetrics & Gynecology and Reproductive Biology (Impact factor: 2.83), DOI: https://doi.org/10.1016/j.ejogrb.2023.03.012]

The kidney is a major target organ in PE due to the adaptive physiological modification it undergoes during pregnancy. As a regulator of water balance and urine osmolarity, AVP regulation is essential to ensure the optimum functioning of the kidneys. In order to explore the involvement of AVP in kidney injury associated with PE development, we evaluated urinary KIM-1 levels, immunolocalization of tissue markers of kidney injury together with a qualitative histological and ultrastructural assessment. We demonstrate a prominent KIM-1 tubular immunolocalization, coupled with a significant rise in urinary KIM-1 levels in pregnant AVP-treated rats compared to the pregnant saline group. Conversely, the expression of podocalyxin is less pronounced in the AVP treated group. The histological changes observed in pregnant AVP treated rats are consistent with PE development.

The pregnant AVP group exhibits significantly higher levels of KIM-1 in early pregnancy, implying that AVP elevates urinary KIM-1 expression as early as GD 8. This increase in urinary KIM-1 levels corresponds with prominent KIM-1 immunolocalization in the kidney and is indicative of renal ischemic injury (Sabbisetti *et*

al., 2014; Lopez-Giacoman *et al.*, 2015). The proximal tubular epithelial cells express significantly raised levels of KIM-1 in response to acute and chronic kidney injury (Luft, 2020; Ichimura *et al.*, 2012). Urinary KIM-1 levels are significantly higher in women with severe PE in comparison to normotensive pregnant women (Wang *et al.*, 2015). Similarly, the studies by Kamel (2020) and Berenji (2022) assessed urinary KIM-1 levels in preeclamptic women. They reported significant elevations in KIM-1 concentrations in women with PE in comparison to normal pregnant women (Kamel *et al.*, 2020; Berenji *et al.*, 2022). Renal KIM-1 expression is also shown to aid in the restorative procedures in the kidney and is implicated in the regeneration of the proximal tubule epithelial cells (Ichimura *et al.*, 1998; Ichimura *et al.*, 2012).

Additionally, we evaluated urinary protein and creatinine levels in the pregnant and non-pregnant rat groups. Similar to urinary KIM-1 levels, urinary protein levels were significantly higher in pregnant AVP-treated rats in comparison to pregnant saline rats. In contrast, although non-significant both pregnant AVP and saline treated rats display lower levels of creatinine in comparison to non-pregnant rats. Of note, correlation analyses did not reveal significant associations between KIM-1, urinary protein and creatinine. While our creatinine findings are in conflict with those reported in preeclamptic women (Kuromoto *et al.*, 2010; Shilpa *et al.*, 2014; Vasava *et al.*, 2018), our data may still have predictive potential if urinary KIM-1 and urinary protein levels are used in combination to predict renal dysfunction linked to PE.

Podocalyxin is the principal podocyte surface antigen and is found on the apical surface of podocyte foot processes (Asao *et al.*, 2012). It is essential in maintaining the structural integrity of the podocyte and reduced expression of podocalyxin negatively impacts glomerular filtration function (Habara *et al.*, 2008; Doyonnas *et al.*, 2001). Our findings of reduced podocalyxin immunostaining in kidney tissue of the PAVP compared to the PS group is indicative of podocyte dysfunction. Podocyturia is present in PE and is evidenced by the irregular shedding of podocytes in the urine (Garovic *et al.*, 2007b; Aita *et al.*, 2009; Karumanchi and Lindheimer, 2007). The loss of podocalyxin compromises podocyte integrity affecting glomerular organization and function.

Our data proposes a possibility that AVP impairs podocalyxin expression by dysregulating the anti-adhesive properties which ensure separation of the podocyte foot processes (Takeda *et al.*, 2000). The loss of this separation reduces the structural integrity of the filtration slits and diminishes filtration capacity. These findings are further corroborated by the morphological alterations observed in the kidney ultrastructure of pregnant AVP-treated rats which demonstrate effacement and fusion of the podocyte foot processes and the presence of irregularly shaped glomerular basement membranes. Notably Takeda *et al.* (2000) demonstrate that chronic infusion of AVP is able to reproduce many aspects of kidney dysfunction that occur in human PE. While data concerning immunoexpression of podocalyxin in PE is scarce, podocalyxin expression in urine (Wang *et al.*, 2012; de Franco *et al.*, 2014) and serum (Chen *et al.*, 2017) are significantly increased in preeclamptic compared to normotensive pregnant women.

We further demonstrate AVP-induced renal irregularities in pregnant treated rats via our ultrastructural findings of the abnormal "frilling" of the glomerular basement membrane, granular deposits, distention of the endoplasmic reticulum, mitochondrial swelling, lysis and cristae degradation along with nuclear modifications, cytoplasmic lysis and less granular chromatin. In addition to histological changes observed in pregnant treated rats in Chapter 3, we further report the presence of tubular necrosis and vasculitis. These findings are supported by observations of women with severe PE who display tubular necrosis as a result of diminished GFR and renal plasma flow (Karumanchi *et al.*, 2005).

7.1.5 Manuscript 4 – Chapter 5 (aligned with objective 5)

Renal biomarker expression in an arginine vasopressin induced rat model of preeclampsia [Currently under review in Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health (Impact factor: 2.49; Manuscript ID: PREGHY-S-22-00376)]

Chapter 5 further explores kidney injury in the AVP model by evaluating the expression of several urinary biomarkers of kidney injury. We demonstrate that infusion of AVP in pregnant Sprague Dawley rats altered the levels of several urinary markers associated with kidney injury. Urinary levels of clusterin, KIM-1, OPN, TIMP-1, albumin, β2M,

cystatin C and NGAL/Lipocalin-2 were significantly increased in the PAVP compared to the PS group. In contrast VEGF-A levels were significantly reduced in both the PAVP and PS groups in comparison to the NS and NAVP groups.

The most commonly used method to diagnose kidney dysfunction is through the measurement of serum creatinine levels (Moresco *et al.*, 2018). Unfortunately, there are limitations associated with this method as changes in serum creatinine levels may not accurately reflect changes in GFR (Adiyanti and Loho, 2012), which may lead to a delay in clinical diagnosis. Additionally, serum creatinine lacks the ability to discriminate structural kidney damage from functional hemodynamic changes and tubular secretion (Wagener *et al.*, 2011; Medić *et al.*, 2016; Thongprayoon *et al.*, 2016; Moresco *et al.*, 2018) and also vary by muscle mass and diet (Baxmann *et al.*, 2008). Therefore, there is a need for non-invasive urinary markers with high specificity and sensitivity (Treacy *et al.*, 2019). Furthermore, reliable and sensitive markers to predict PE development will greatly aid in the treatment and assist in the prevention of maternal renal failure as well as protect the fetus from future renal complications (Ruggajo *et al.*, 2016).

Due to serum creatinine concentration being affected by creatinine secretion or extra-renal secretion, the GFR must deteriorate by approximately 50% in order to detect increases in serum creatinine levels (Levey *et al.*, 2003). In contrast to this, our data shows that clusterin, KIM-1, cystatin C, β2M, NGAL/lipocalin-1 and OPN are elevated in early pregnancy, as early as GD8, while albumin levels are significantly higher on GD 14 in the pregnant AVP in comparison to the pregnant saline group.

These proteins are potential predictive biomarkers of PE development as elevations in urinary levels of NGAL/lipocalin-2 (Kelly *et al.*, 2018), KIM-1 (Wang *et al.*, 2015), cystatin C (Lopez-Hernandez *et al.*, 2016), albumin (Burwick *et al.*, 2014) and clusterin (Odun-Ayo *et al.*, 2018) have been reported in preeclamptic compared to normotensive pregnant women. Women with PE also have elevated serum (Palei *et al.*, 2012) and placental (Zhu *et al.*, 2014) TIMP-1 expression. Moreover, VEGF-A expression is down-regulated in the kidneys in comparison to normotensive pregnancies (Baelde *et al.*, 2007). Plasma levels of β2M are significantly higher in PE compared to normotensive

pregnant women (Elsayed *et al.*, 2020). Higher plasma OPN levels are observed in PE patients with extensive endothelial injury (Stenczer *et al.*, 2010), while urinary OPN levels are significantly increased in diabetic patients with renal impairment (Qin *et al.*, 2004). Nonetheless, despite β2M and OPN been promising biomarkers of renal dysfunction, there is a dire paucity of data investigating their urinary clinical value in PE.

Proximal tubular cells contain a widespread endocytotic apparatus which is essential for the reabsorption and degradation of filtered proteins through the endosomal/lysosomal pathway (Marshansky *et al.*, 2002; Christensen and Birn, 2002). In the proximal tubule, low-molecular weight plasma proteins such as albumin, NGAL/lipocalin-2, cystatin C and β2M are reabsorbed via endocytosis and subsequently undergo lysosomal degradation (Cui *et al.*, 1996; Nielsen *et al.*, 2016). This pathway of reabsorption and degradation is facilitated by megalin and cubilin which are multiligand-binding receptors, located on the apical surface of the proximal tubule epithelial cells (Christensen and Birn, 2002). Additionally, studies have shown that down-regulated megalin expression results in impaired tubular function coupled with proteinuria and the reduced apical endocytic apparatus of the tubule epithelial cells (Christensen and Willnow, 1999; Leheste *et al.*, 1999).Therefore it is possible that proximal tubule injury caused by AVP leads to impaired endocytic function arising from megalin and cubilin receptor dysfunction, as evidenced by increased excretion of albumin, NGAL/lipocalin-2, cystatin C and β2M into the urine.

Our ultrastructural data highlighted in Chapter 4 demonstrates mitochondrial swelling, lysis and cristae degradation in the kidneys of pregnant AVP-infused rats. Mitochondrial function is critical for the optimal functioning of the proximal tubule as the reabsorption of ions requires active transport (Soltoff, 1986). Mitochondria have also been implicated in the pathological processes of kidney diseases such as acute kidney injury, diabetic nephropathy, and chronic kidney disease (Bhargava and Schnellmann, 2017; Jiang *et al.*, 2020; Zhang *et al.*, 2021). These findings may also negatively impact reabsorption of proteins from the filtrate due to tubular injury in the AVP model.

7.2 CONCLUSION

The main finding of our review of various rodent and murine models that replicate PE development highlights the absence of a rodent model that fully mimics the clinical hallmarks associated with human PE. We therefore recommend that a combination of different models be used concomitantly to mirror PE milieu.

Our study is novel because it successfully transfers an arginine vasopressin-induced mouse PE model to a Sprague Dawley rat model. It demonstrates for the first time that the arginine vasopressin rat model successfully replicates kidney injury such as glomerular injury and endothelial dysfunction, features that characterize PE development. Moreover, AVP infusion elevates peripheral resistance, resulting in higher systemic blood pressure and glomerular dysfunction as evidenced by proteinuria and increased urinary biomarker levels with concomitant reduction of VEGF-A concentration. The markers are advantageous as they can be used isolate specific regions of damage within the nephron and have the ability to distinguish between renal damage and altered renal function.

Furthermore, we report that the AVP model displays KIM-1 tubular immunolocalization in pregnant AVP-treated *vs.* pregnant saline-treated rats. In contrast, podocalyxin immunolocalization is prominent in pregnant saline-treated rats in comparison to the pregnant AVP-treated group, indicating a loss of structural integrity of podocytes that impact glomerular filtration. Additionally, renal morphological changes of pregnant treated rats indicated a mild increase in mesangium, glomerular crescents, reduced Bowman's space, tubular necrosis and vasculitis. The ultrastructural changes observed in pregnant AVP treated rats including the effacement of the podocyte foot processes, glomerular basement membrane abnormalities and mitochondrial dysfunction are consistent with PE development. This data also indicates the clinical potential of renal KIM-1 and podocalyxin immunolocalization as biomarkers for the early detection of PE as well as indicators of significant kidney injury related to PE.

Finally, we recommend the use of the AVP rat model for further study into the mechanisms contributing to PE development and progression. However, since our data

reports a mild case of PE development and does not induce the more severe features associated with PE development, a stronger AVP dosage is required to further evaluate whether a more severe form is produced. Our findings also implicate kidney pathology in the development of PE and demonstrates their value as early detection biomarkers for PE development, as well as in predicting the severity of renal injury in PE.

7.3 LIMITATIONS

Our study does model a mild case of PE development and does not induce the more severe features associated with PE development, albeit the immunolocalization of KIM-1 and podocalyxin was not quantitatively assessed using morphometric image analysis due to infrastructural constraints.

7.4 FUTURE RESEARCH

Further investigations are required to determine the exact mechanisms by which the AVP pathway contributes to the development of PE. As the dose of AVP used to create the AVP rat model produced a mild form of PE, it would be beneficial for future studies using this model to use a wider range of doses to enable the study of the more severe features of PE development. Kidney injury associated with this model may be expanded by investigating the expression of other kidney structural proteins associated with the glomerular filtration apparatus such as nephrin and podocin. Future work on the application of morphometric image analysis of glomeruli and tubules should be undertaken to further study the renal injury associated with this model and its application in PE development. Additionally, future investigations should confirm the angiogenic levels of PlGF, sEng and sFlt-1 which are commonly associated with PE development.

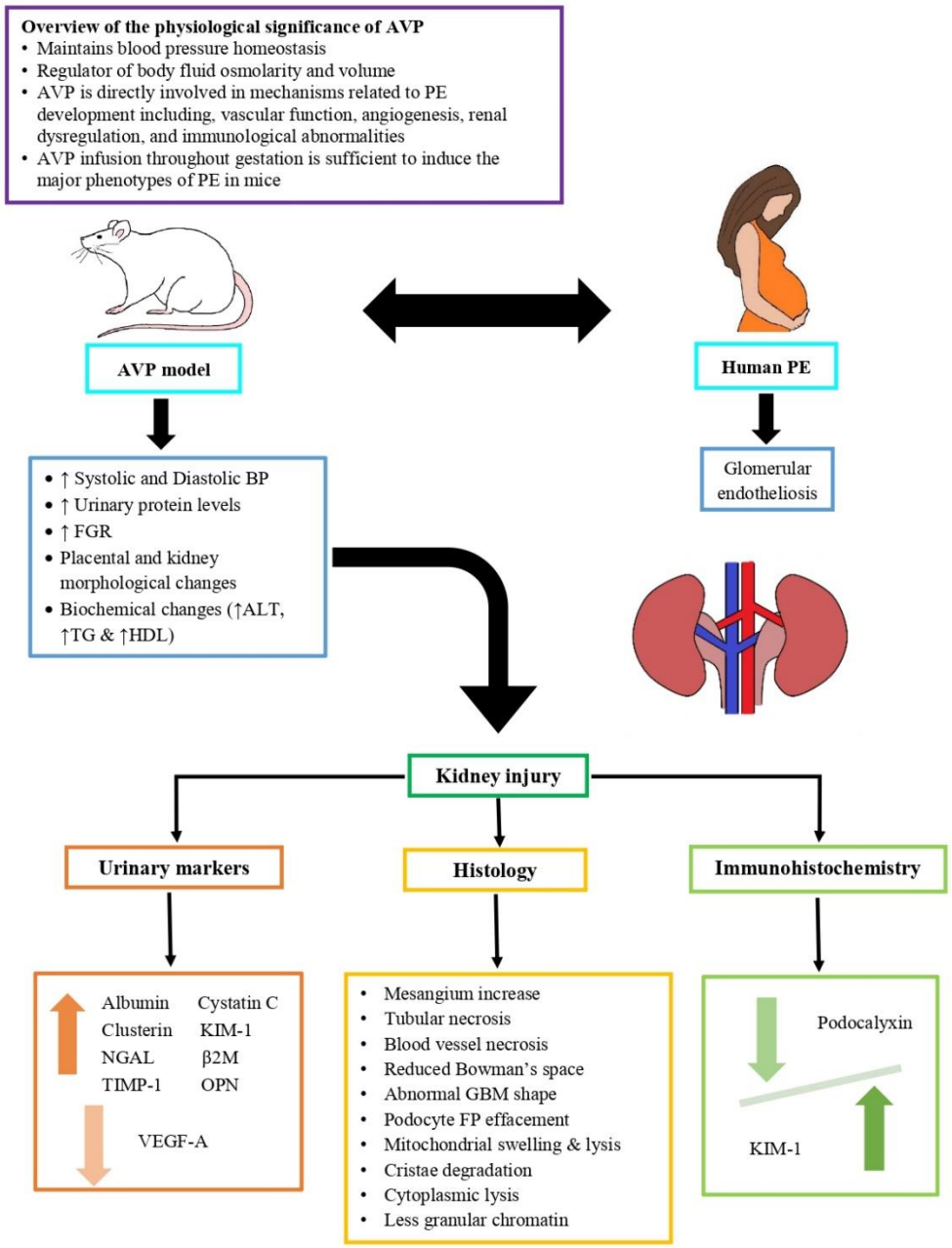

Figure 7.1. Illustration representing the overall findings of the study. Infusion of AVP into pregnant Sprague Dawley rats effectively reproduces pathological phenotypes of human PE. Additionally, significant kidney is observed in this model as evidenced by changes in the levels of structural and injury markers in urine and kidney tissue as well as abnormalities in kidney morphology. ALT: alanine transaminase; BP: blood pressure; FGR: fetal growth restriction; FP: foot process; GBM: glomerular basement membrane; HDL: high-density lipoprotein; TG: triglyceride.

CHAPTER 8

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CHAPTER 9

Appendix 1 – Faculty Research Committee Approval Letter

19 November, 2020

Ms S Ramdin
Student No: 22063558

P.O. Box Umzinto
4200

Dear Ms Ramdin

PHD IN HEALTH SCIENCES

I am pleased to advise that:

1. The Faculty Research Committee approved the following:

    (i) Your research proposal and dissertation title, being:

Nephrotoxicity associated with preeclampsia in an AVP induced rat pregnancy model

Please note: ANY PROPOSED CHANGES in the DISSERTATION TITLE require the approval of your supervisor and the Faculty Research Committee.

    (ii) Supervisor – Prof T Naicker
    (iii) Co-supervisor – Dr N Govender

2. Your request for funding totalling **R 15 000.00** subject to any literature referred to in Section A of the PG 4a form being accessioned by this University, and any equipment purchased shall become the property of the department.

NOTE: - This funding is not paid directly to you but is controlled by the Faculty Office. Any proposed changes to this funding allocation needs the approval of your supervisor, and Faculty Research Committee

The University Research Committee has stipulated that:

(a) Ownership of any patent registered in respect of the results of your studies is retained by you as the initiator of the project;

(b) Should you make any drift from the results of your studies, you will be required to repay

pro rata, the **R 15 000.00** investment which the University Research Committee has made in approving your request for funding;

(c) If the Durban University of Technology provided the equipment/materials for the creation of artefacts, this cost would be refunded to the University if such artefacts were sold and

(d) Durban University of Technology is given first refusal in respect of any possible future sale by you of any patent that may be registered in respect of your said project.

(e) All journal articles, referenced in your dissertation, are to accompany your ring-bound copies when submitting for examination purposes.

Should you experience any problems relating to your research studies, your supervisor must be informed as soon as possible. If the difficulty persists, you must then approach your Head of Department and thereafter the Executive Dean of the Faculty.

Yours sincerely

Ms S Perumal
FACULTY RESEARCH OFFICER

19 Nov 2020

Student's signature in acceptance of the conditions contained herein.

Date:

Appendix 2 - Animal ethics was approved for use of archived specimens

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

07 July 2020

Dr Nalini Govender (50002247)
Department of Basic Medical Sciences
Durban University of Technology
Ritson Campus

Dear Dr Govender,

Protocol reference number: AREC/046/017
Project title: Role of Biomarkers in early detection of preeclampsia in under resourced countries

Full Approval – Renewal Application

With reference to your renewal application received on 23 June 2020. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

Please note the following:

1. **There must be adherence to national and institutional COVID-19 regulations and guidelines at all times.** Researchers will be personally responsible and liable for non-adherence to national regulations. If in doubt, please contact the Research Ethics Chair and/or the University Dean of Research for advice.
2. **Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.**
3. **For the next renewal in 2021, you will have submit a new application on the RIG online application system.**

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 07 July 2021.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

Digitally signed by Dr Dalene Vosloo
Date: 2020.07.09 14:05:35 +02'00'

Dr Dalene Vosloo
Deputy Chair: Animal Research Ethics Committee

/kr

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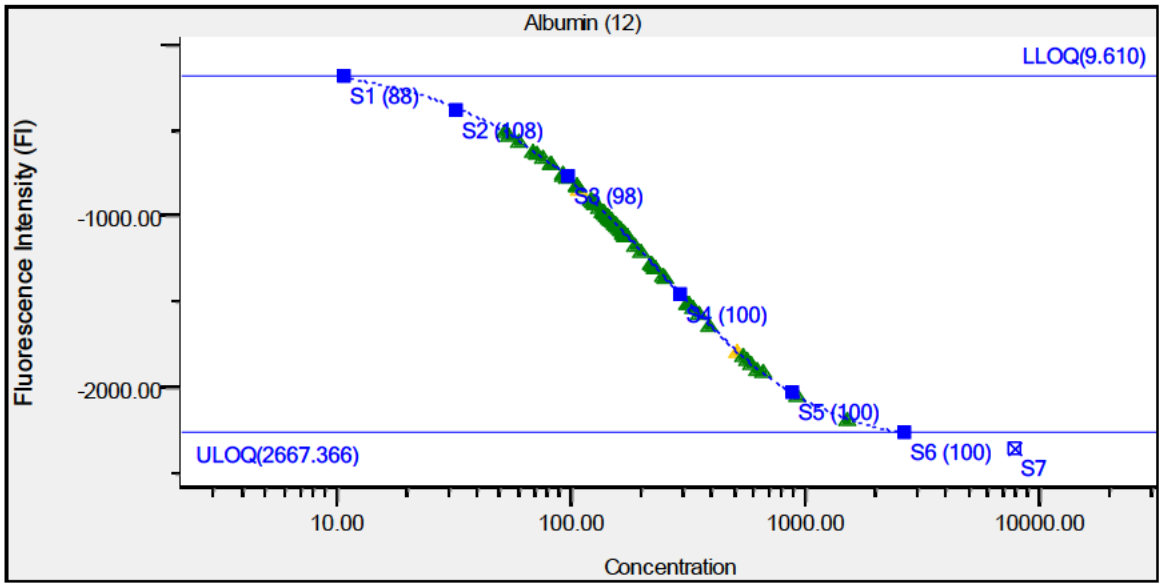
Medical School
Pietermaritzburg
Westville

Appendix 3 – Standard curve data for Multiplex immunoassays

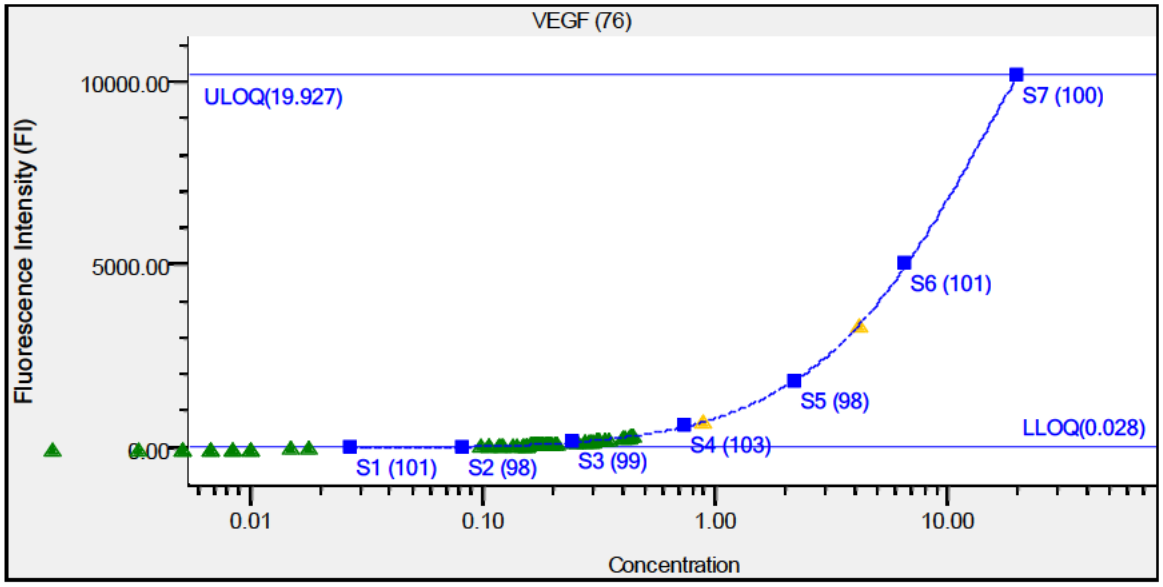


Standard **Partial Outlier** **Outlier**
Unknown **Control**

Regression Type: Logistic - 5PL

Std. Curve: FI = -2325.77 + (-97.0717 + 2325.77) / ((1 + (Conc / 359.369)^1.04258))^1.5603

FitProb. = 0.7539, ResVar. = 0.0983



Standard **Partial Outlier** **Outlier**
Unknown **Control**

Regression Type: Logistic - 5PL

Std. Curve: FI = 4.35316 + (18286.6 - 4.35316) / ((1 + (Conc / 16.2232)^-1.10313))^0.992371

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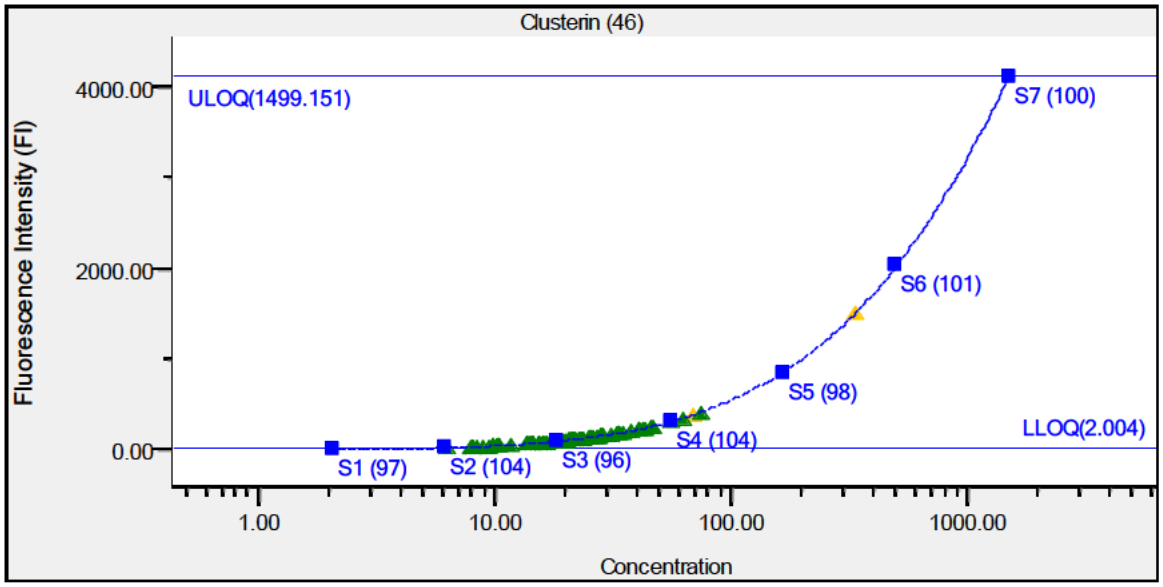
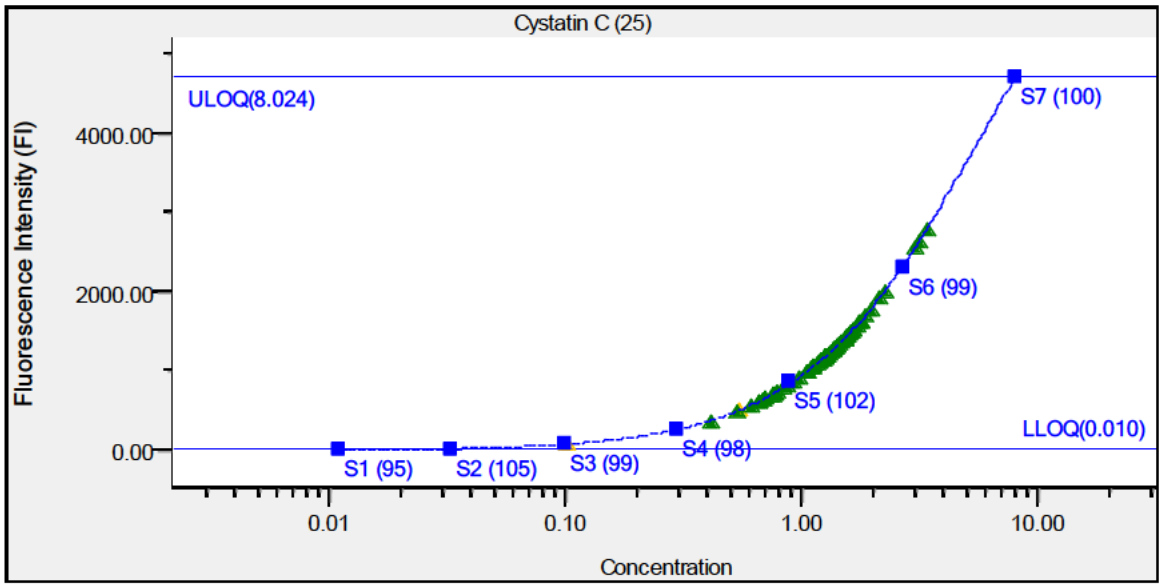
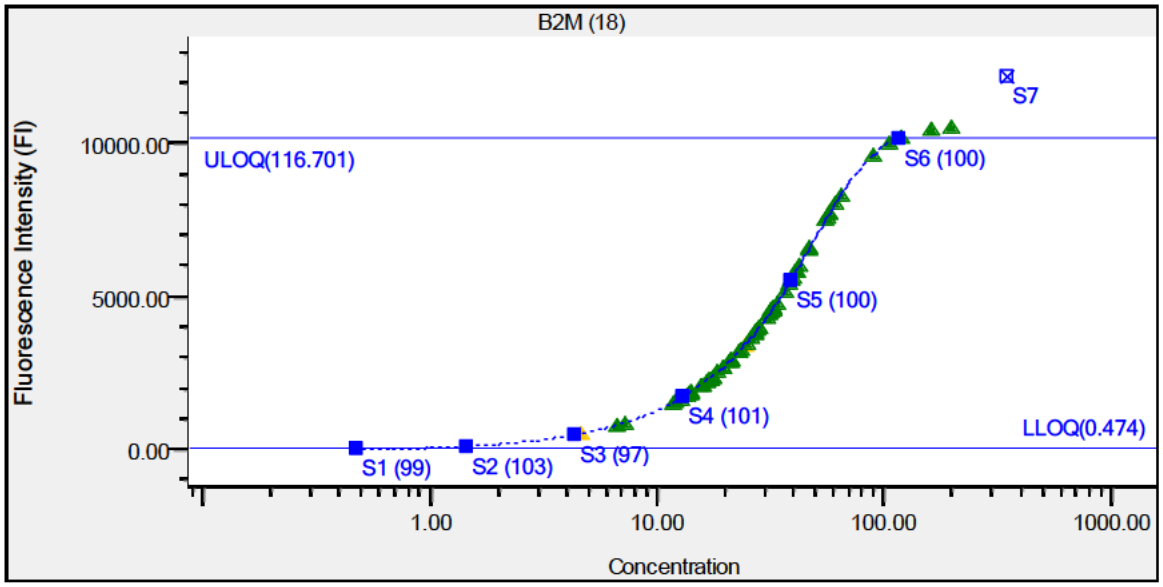


■ Standard ☐ Partial Outlier ☒ Outlier
▲ Unknown ▲ Control
Regression Type: Logistic - 5PL
Std. Curve: FI = 6.22348 + (37048.6 - 6.22348) / ((1 + (Conc / 414.022)^-0.336338))^4.38402
FitProb. = 0.5678, ResVar. = 0.5659



■ Standard ☐ Partial Outlier ☒ Outlier
▲ Unknown ▲ Control
Regression Type: Logistic - 5PL
Std. Curve: FI = 1.67582 + (7295.6 - 1.67582) / ((1 + (Conc / 6.16118)^-1.33221))^0.812397
FitProb. = 0.7012, ResVar. = 0.3550

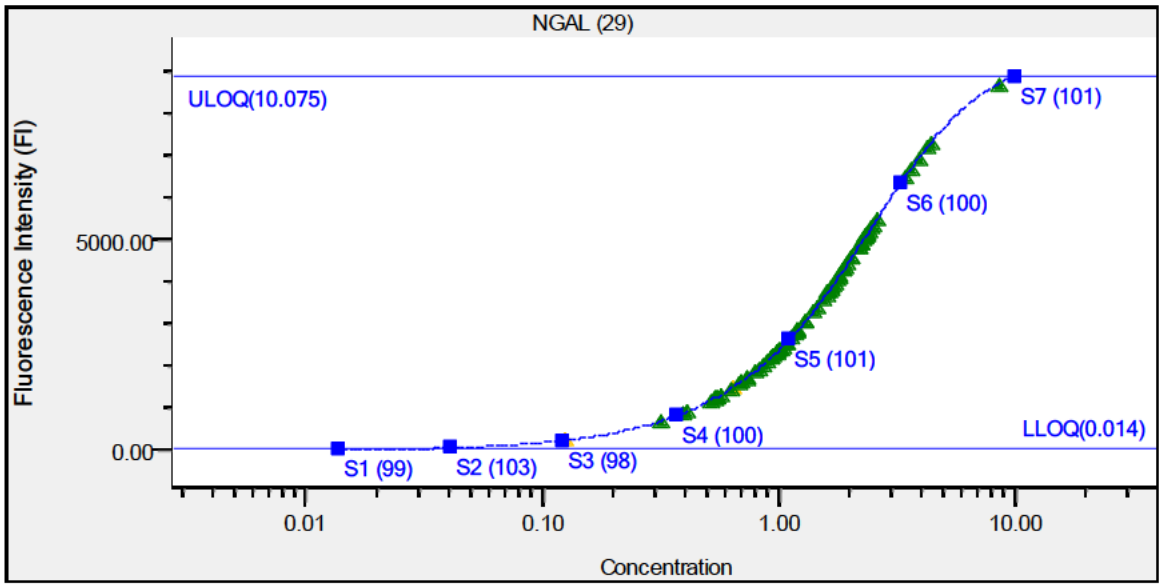


Standard **Partial Outlier** **Outlier**
Unknown **Control**

Regression Type: Logistic - 5PL

Std. Curve: FI = 6.22348 + (37048.6 - 6.22348) / ((1 + (Conc / 414.022)^-0.336338))^4.38402

FitProb. = 0.5678, ResVar. = 0.5659

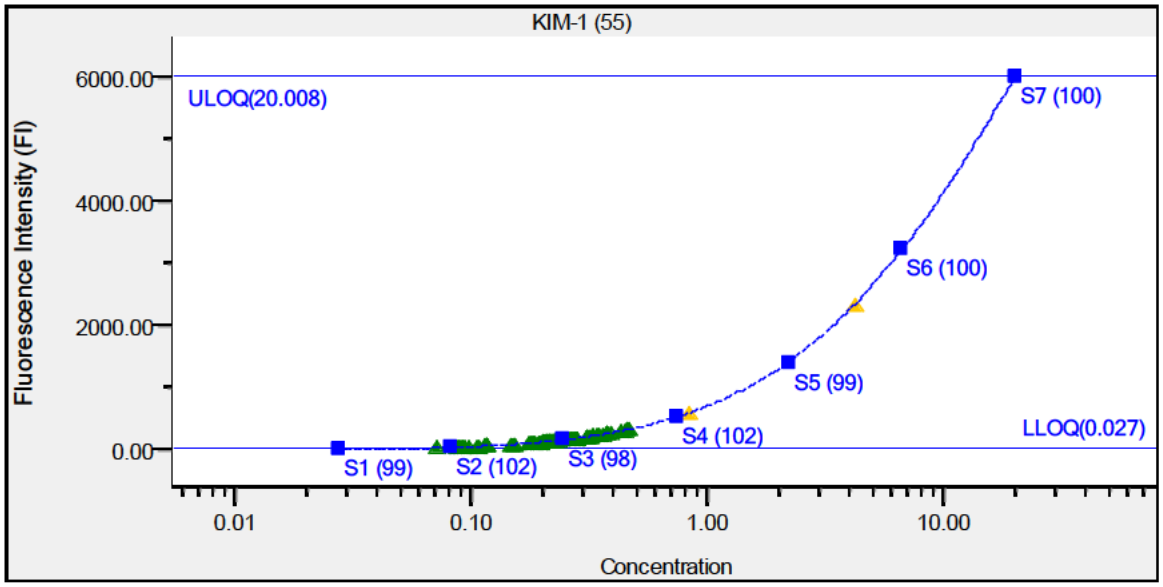


Standard **Partial Outlier** **Outlier**
Unknown **Control**

Regression Type: Logistic - 5PL

Std. Curve: FI = 5.07037 + (9489.08 - 5.07037) / ((1 + (Conc / 3.33619)^-1.96449))^0.562109

FitProb. = 0.8822, ResVar. = 0.1253

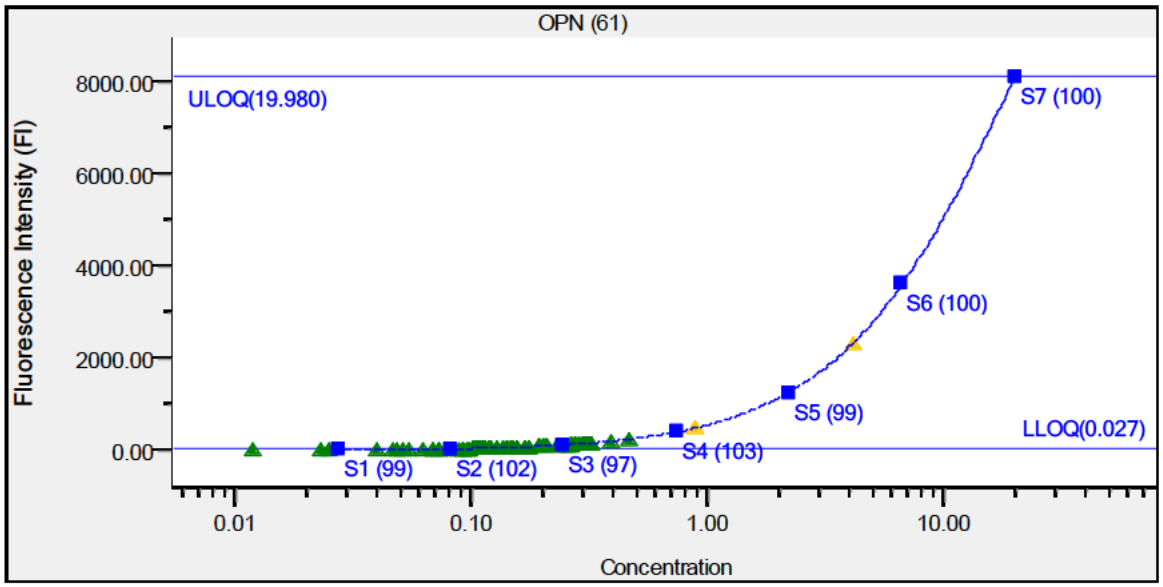


Standard **Partial Outlier** **Outlier**
Unknown **Control**

Regression Type: Logistic - 5PL

Std. Curve: FI = 8.13948 + (19178.1 - 8.13948) / ((1 + (Conc / 8.29899)^-0.524224))^2.36078

FitProb. = 0.8460, ResVar. = 0.1672

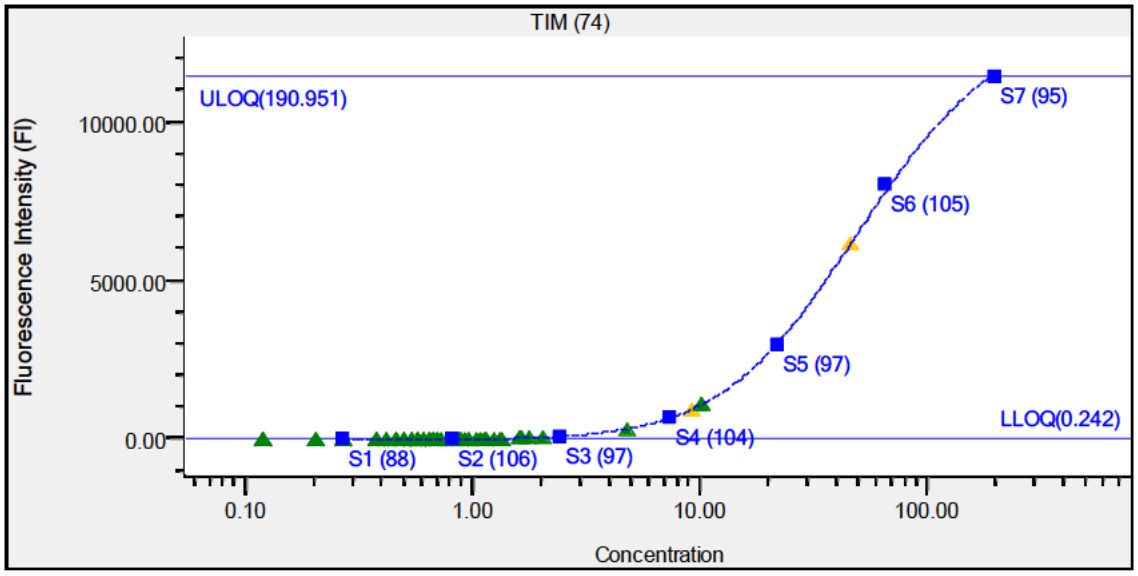


Standard **Partial Outlier** **Outlier**
Unknown **Control**

Regression Type: Logistic - 5PL

Std. Curve: FI = 3.58807 + (15083.8 - 3.58807) / ((1 + (Conc / 21.7718)^-1.29639))^0.824696

FitProb. = 0.7718, ResVar. = 0.2590



Standard **Partial Outlier** **Outlier**
Unknown **Control**

Regression Type: Logistic - 5PL

Std. Curve: FI = 4.85198 + (13414.9 - 4.85198) / ((1 + (Conc / 37.9071)^-1.28474))^1.34517

FitProb. = 0.2576, ResVar. = 1.3562