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Original article

# Is renal dysfunction amplified in an arginine vasopressin induced rat model of preeclampsia?

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ABSTRACT

Renal dysfunction is important in preeclampsia (PE) pathophysiology and has not been fully explored in the arginine vasopressin (AVP) rat model of PE. This study aimed to determine kidney toxicity associated with this model. Female Sprague Dawley rats (n = 24) were subcutaneously infused with AVP or saline for 18 days. Urine samples (GD8, 14 and 18) were 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. Albumin, and NGAL/lipocalin-2 were significantly elevated in the PAVP *vs* PS group on GD14 and GD18 ($p < 0.001$) respectively. VEGF-A significantly decreased in the pregnant *vs* non-pregnant groups on GD14 and 18 ($p < 0.001$). Clusterin ($p < 0.001$) and OPN ($p < 0.05$) were significantly higher in the PAVP *vs* PS group on GD18. Cystatin C and KIM-1 are significantly upregulated in the PAVP *vs* PS groups throughout gestation ($p < 0.05$). β2M is significantly elevated in the PAVP *vs* PS group on GD14 and 18 ($p < 0.05$). AVP elevated the urinary levels of the kidney injury biomarkers and replicated the renal dysfunction associated with PE development. Our findings confirm the potential applications of this model in studying the mechanisms underlying renal damage in PE.

## 1. Introduction

The kidney is one of the most affected organs 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 weeks 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].

Serum creatinine evaluation is currently the gold standard used to diagnose acute kidney injury and chronic kidney disease [8]. 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 [8,9]. The need for specific and sensitive urinary, rather than circulating biomarkers, of kidney injury is thus urgently warranted [10]. 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 [11].

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

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endothelial growth factor-A (VEGF-A) have shown potential in detecting kidney injury [12–14] and utility as markers of PE detection [8,15–20]. In PE, urinary levels of NGAL/lipocalin-2 [15], KIM-1 [8], cystatin C [16], albumin [17] and clusterin [18] are higher in comparison to normotensive pregnant women. Moreover, women with PE have elevated circulating levels of TIMP-1 [19] and VEGF-A [20] in comparison to normotensive pregnancies. Notably, the diagnostic utility of β2M [21] and OPN [22] as markers of renal dysfunction is reported, however limited clinical data precludes their predictor value for PE identification and development.

Arginine vasopressin (AVP) has garnered interest as a potential biomarker in the development of PE. The role of AVP in pregnant PE mice was initially reported by Santillan and co-workers [23–25]. AVP is significantly elevated in pregnant PE mice, and visible in PE pregnancies as early as the sixth week of gestation [23–27]. This is indicative that high AVP concentrations released early in gestation, appears to remain throughout pregnancy. The successful demonstration of the AVP mouse model replicating the characteristic features of human PE *viz.*, pregnancy induced hypertension, proteinuria and intrauterine growth restriction [24] and an upregulation in pro-inflammatory TH1- associated interferon gamma (IFN-ϒ) response [25] are notable.

Since AVP is unstable and cannot be reliably measured, copeptin, the surrogate marker for AVP, is widely reported as being useful in the early prediction of PE development [23,25,28–31]. Studies confirm higher circulating levels of copeptin during late second trimester and early third trimester PE compared to normotensive pregnancies [29] and a positive association between copeptin and gestational age in both PE and normotensive pregnant groups [31]. Of note, women undergoing vaginal delivery demonstrated higher serum levels of copeptin compared to those who underwent a cesarean section (CS) [30]. Moreover, a significant upregulation in cord serum copeptin levels was observed for fetal distress in CS deliveries (elective and intrapartum) compared to vaginal deliveries (normotensive and mild PE) [30].

In our laboratory, we also confirmed that the subcutaneous delivery of AVP in pregnant Sprague-Dawley rats successfully replicates the phenotypes of hypertension, proteinuria and fetal growth restriction associated with human PE development [32], by demonstrating histological evidence of renal injury [33]. Even though proteinuria is no longer mandatory for PE diagnosis [5], it remains a significant indicator of kidney injury. In pregnant AVP treated rats, the kidney injury molecule (KIM-1) was prominently immunolocalized in the proximal tubules, whilst podocalyxin showed a weak immunolocaliztion in the glomerulus [33]. Furthermore, reductions in Bowman's space, tubular and blood vessel necrosis were observed in the pregnant treated group. Ultrastructural findings confirms an effacement and fusion of podocyte foot processes, glomerular basement membrane abnormalities, podocyte nuclear crenations, mitochondrial edema and cristae degeneration with cytoplasmic lysis within treated tissue, which are consistent with human PE development.

In view of the histological evidence of kidney injury associated with this model, this study aimed to determine the urinary levels of kidney toxicity indicators *viz.*, albumin, clusterin, KIM-1, OPN, TIMP-1, VEGF-A, β2M, cystatin C, and NGAL/Lipocalin-2.

## 2. Materials and methods

This study protocol was approved by the Animal Research Ethics Committee, University of KwaZulu-Natal (AREC/046/017). Twenty-four adult female Sprague Dawley rats aged 10–12 weeks (weighing 160–180 g) were obtained from the Biomedical Research Unit (BRU), University of Kwa-Zulu Natal, South Africa. All animals were housed in polycarbonate cages at the BRU under standard laboratory conditions of temperature (22–24 °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. Adult pregnant and non-pregnant female Sprague-Dawley rats (n = 24) were surgically implanted with ALZET mini-osmotic pumps for the duration of the pregnancy (model 2004; Durect Corporation, Cupertino, CA) on gestational day (GD) 1, to subcutaneously deliver either AVP at 150 ng/hr or saline.

Blood pressure (BP) and twenty-four hour urine samples were collected on GD 8, 14 and 18 as reported in our previous study [32]. Blood pressure measurements were carried out using the tail-cuff method (MRBP tail-cuff BP monitor, IITC Life Sciences Inc., USA). Prior to the pump insertion, a two week period of acclimatization, inclusive of environmental (cages) and restrainer (BP measurement holders) exposure was undertaken to familiarize the rats and reduce distress. The restrainers were placed in each cage and thus also served as a source of environmental enrichment for the rats. Blood pressure readings were recorded and following this, rats were placed into metabolic cages (Techniplast, Italy) to collect 24 h urine samples for each rat. Urinary specimens were collected from non-pregnant and pregnant Sprague-Dawley rats, *viz.*, non-pregnant with saline delivery **(NS, n = 6)**; non-pregnant with AVP delivery **(NAVP, n = 6)**; pregnant with saline delivery **(PS, n = 6)** and pregnant with AVP delivery **(PAVP, n = 6)**. All animals were euthanized on GD 18, *via* inhalation anesthesia using isoflurane (Safeline Pharmaceuticals, South Africa). Blood samples were collected *via* cardiac puncture into heparinized tubes and centrifuged for 15 min at 3500 rpm at 4 °C. The serum was collected and stored at − 80 °C for electrolyte analyses. Weight (fetal and placental) and fetal numbers were recorded at sacrifice.

### 2.1. Multiplex ELISA assay

Kidney damage induced by AVP was determined using a multiplex enzyme-linked immunosorbent assay. Kidney toxicity panels 1 (Cat. #RKTX1MAG-37 K) and 2 (Cat.#RKTX2MAG-37 K) 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.

#### 2.1.1. Procedure kidney Panel 1 and Panel 2

Urine samples stored at − 80 °C were thawed and centrifuged to remove any sediment or debris. Samples were then vortexed and added to plate wells. The assay plate was inclusive of sample, background, standards and control wells. The assay buffer (200 μL) was then added to all wells and mixed on a plate shaker for 10 min 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 matrix was added to background, standards and control wells. This was followed by the addition of 25 μL samples for panel 1 (undiluted) and diluted samples for 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 h at room temperature. Streptavidin-Phycoerythrin (50 μL) was added to each well and incubated for 30 min 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).

### 2.2. Serum electrolyte levels

Measurement of serum electrolyte levels were conducted by a commercial pathology laboratory, in triplicate and all values were averaged. Serum was analyzed using the Beckman Coulter kits #467935 (calcium,

chloride, potassium and sodium) and #442750 (urea) and read using the SYNCHRON LXR System (Beckman Coulter, CA, USA).

### 2.3. Data analysis

All data was analyzed using STATA (version 12, STATACORP) and GraphPad Prism 5. Normality of data was tested using the skewness and kurtosis normality test. All parametric data are summarized as means and standard deviation; and non-parametric data as median and interquartile range (IQR). The Kruskal–Wallis and Dunn's *post hoc* test was used to compare the medians between groups for blood pressure, urinary output and serum electrolyte levels. The fetal parameters between pregnant groups were compared using the Mann-Whitney test. A parametric repeated measures ANOVA was used to confirm the presence of a significant difference in kidney injury biomarker levels between the experimental groups across different time periods. This was then followed by conducting the Boniferroni's post-hoc test between pairs of group means to determine significance. The Pearson's correlation was

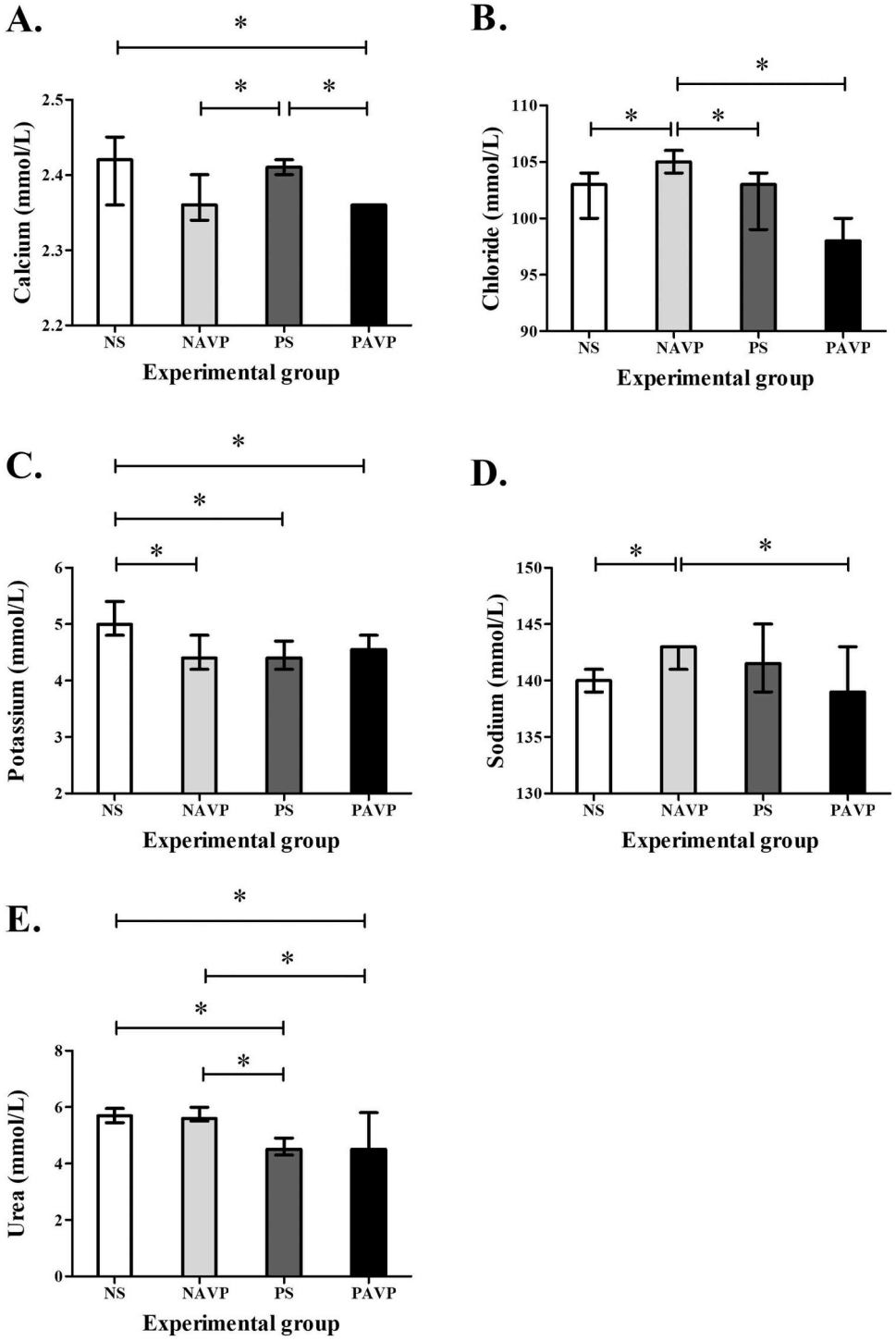


**Fig. 1.** Serum electrolyte concentrations of (A) Calcium, (B) Chloride, (C) Potassium, (D) Sodium and (E) urea, across all groups on gestational day 18. Kruskal-Wallis and Dunn's *post hoc* multiple comparison tests were used to compare the medians between groups. Data is shown as median (25th percentile – 75th percentile). NS: non-pregnant Saline; NAVP: non-pregnant AVP; PS: pregnant saline; PAVP: pregnant AVP, $*p < 0.05$; $**p < 0.001$; (n = 6 per group).

used to determine the existence of relationships amongst kidney injury markers. Additionally, the expression of these markers were correlated with systolic and diastolic blood pressures, urinary output, pup number, calcium, chloride, sodium, potassium and urea. A $p$-value $< 0.05$ indicated statistical significance.

## 3. Results

### 3.1. Clinical profile of study groups

Blood pressures (systolic and diastolic) were significantly increased on GD 8 and 14 in the PAVP *vs* the PS group and remained elevated at GD18 [135.33 (133.67–138.67) mmHg *vs* 122.00 (120.00–124.00) mmHg respectively; $p < 0.05$]. We previously reported higher blood pressures in the AVP treated rats compared to untreated rats, regardless of pregnancy status [32]. Systolic blood pressure remained unchanged between the NS, PS and NAVP groups on GD 8, 14 and 18. Diastolic pressure was increased in the NAVP group compared to the NS (GD 8, 14 and 18) and PS (GD 14 and 18) groups.

Lower urinary output volumes was observed in the PAVP *vs* PS groups on GD18 [17.00 (12.00–20.00) ml *vs* 25.50 (25.00–29.00) ml; $p < 0.05$]. Notably, a significantly lower urinary output was observed in the NAVP group compared to the NS [9.50 (8.00–15.00) ml *vs* 20.50 (20.00–23.50) ml; $p < 0.05$] and PS [9.50 (8.00–15.00) ml *vs* 25.50 (25.00–29.00) ml; $p < 0.001$] groups. Despite the lack of statistical significance, a marked decrease was observed in the NAVP *vs* the PAVP group.

At sacrifice (GD18), fetal numbers were significantly higher in the PAVP compared to the PS groups [12.00 (11.00–12.00) *vs* 9.00 (8.00–10.00); $p < 0.05$]. However, individual fetal weights were significantly reduced in the PAVP group in comparison to the PS group [1.57 (1.45–1.67) g *vs* 1.79 (1.70–1.85) g; $p < 0.05$]. Similarly, the individual placental weights were significantly lower in the PAVP group compared to the PS group [0.59 (0.55–0.63) *vs* 0.39 (0.31–0.46); $p < 0.05$].

Serum electrolyte concentrations measured on GD18 are shown in Fig. 1. A significant reduction was noted for calcium in the PAVP *vs* PS group ($p < 0.05$). Pregnant AVP-treated rats displayed a significant downregulation in the concentrations for sodium, urea and chloride in comparison to the NAVP group ($p < 0.05$). Lower sodium and chloride levels were observed in PAVP compared to the PS group, albeit non-significant. Noteworthy, urea levels remain unchanged between these groups, whereas potassium levels decreased significantly in the PAVP and NAVP *vs* NS groups ($p < 0.05$). A slight increase in potassium levels was observed in the PAVP compared to the PS groups, albeit non-significant.

### 3.2. Urinary glomerular biomarkers of kidney injury

The mean urinary levels for albumin and VEGF-A are shown in Fig. 2. No significant differences were observed for albumin across all study groups on GD8. However, a significant elevation in albumin was observed in the PAVP group compared to the PS and NS groups on GD14 and GD18 respectively ($p < 0.05$). Higher albumin levels were also observed in the PAVP group compared to the NAVP group, albeit non-significant. Similarly, no significant difference was noted in VEGF levels across all the groups on GD8. However a significant reduction was observed in the pregnant (PAVP and PS) *vs* the non-pregnant (NS and NAVP) groups on GD14 and GD18 respectively ($p < 0.001$). No significant changes were observed for VEGF between the PS and PAVP groups throughout gestation.

### 3.3. Urinary tubular biomarkers of kidney injury

The concentrations (means±SD) for urinary clusterin, cystatin C, β2M, NGAL/lipocalin-2, KIM-1, OPN and TIMP-1 are shown in Fig. 3. No significant differences were noted for clusterin between the experimental groups on GD8. However, there was a significant reduction in clusterin expression on GD14 in the PS *vs* NAVP group ($p < 0.05$) and significantly higher levels in the treated (PAVP and NAVP) *vs* the PS group on GD18 ($p < 0.001$).

Cystatin-C levels were significantly elevated in the PAVP compared to the PS and NS groups on GD8 ($p < 0.05$), 14 ($p < 0.001$) and 18 ($p < 0.05$). Additionally, cystatin-C levels were significantly upregulated in the PAVP *vs* NAVP group on GD8 and GD18 respectively ($p < 0.05$). However, β2M levels were only upregulated in the PAVP *vs* the PS and NS groups on GD14 and GD18 respectively ($p < 0.05$). A significantly higher β2M concentration was also observed in PAVP compared to the NAVP groups on GD18 ($p < 0.05$).

A lower concentration, albeit non-significant, was observed for NGAL/lipocalin-2 in the PAVP compared to the PS group on GD8, as opposed to the up-regulation noted in the PAVP *vs* PS group on GD14 ($p < 0.001$) and 18 ($p < 0.001$). In contrast, urinary levels of KIM-1 were significantly elevated in the PAVP *vs* PS group throughout gestation [*i.e.* GD8 ($p < 0.05$), 14 ($p < 0.001$) and 18 ($p < 0.001$)]. A similar trend was noted for TIMP-1, which highlights elevations in the PAVP *vs* PS group on GD8, 14 and 18, albeit non-significant. In contrast, despite non-significance, OPN levels were reduced in the PAVP group compared to the PS and NAVP groups on GD8. However, OPN levels were significantly elevated in the PAVP *vs* PS group on GD18 ($p < 0.05$).

### 3.4. Pearson's correlation between analytes and clinical factors

Several significant positive and negative associations were observed between the kidney injury molecules and selected clinical factors (Table 2). On GD18, significant, positive correlations were noted

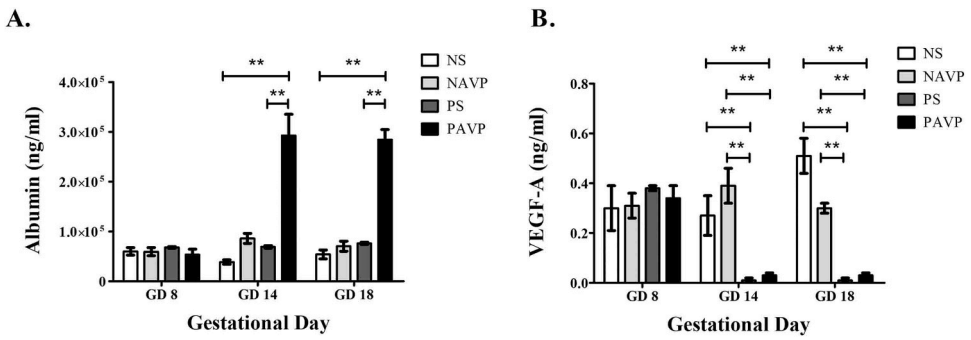


**Fig. 2.** Glomerular injury illustrated by biomarker concentrations (mean ± SD) on GD8, 14 and 18, of (A) Albumin and (B) VEGF-A. Repeated measures ANOVA and Boniferroni's post-hoc test was conducted between pairs of group means to determine significance. Data is shown as mean and standard deviation. NS: non-pregnant Saline; NAVP: non-pregnant AVP; PS: pregnant saline; PAVP: pregnant AVP, *$p < 0.05$; **$p < 0.001$; (n = 6 per group).

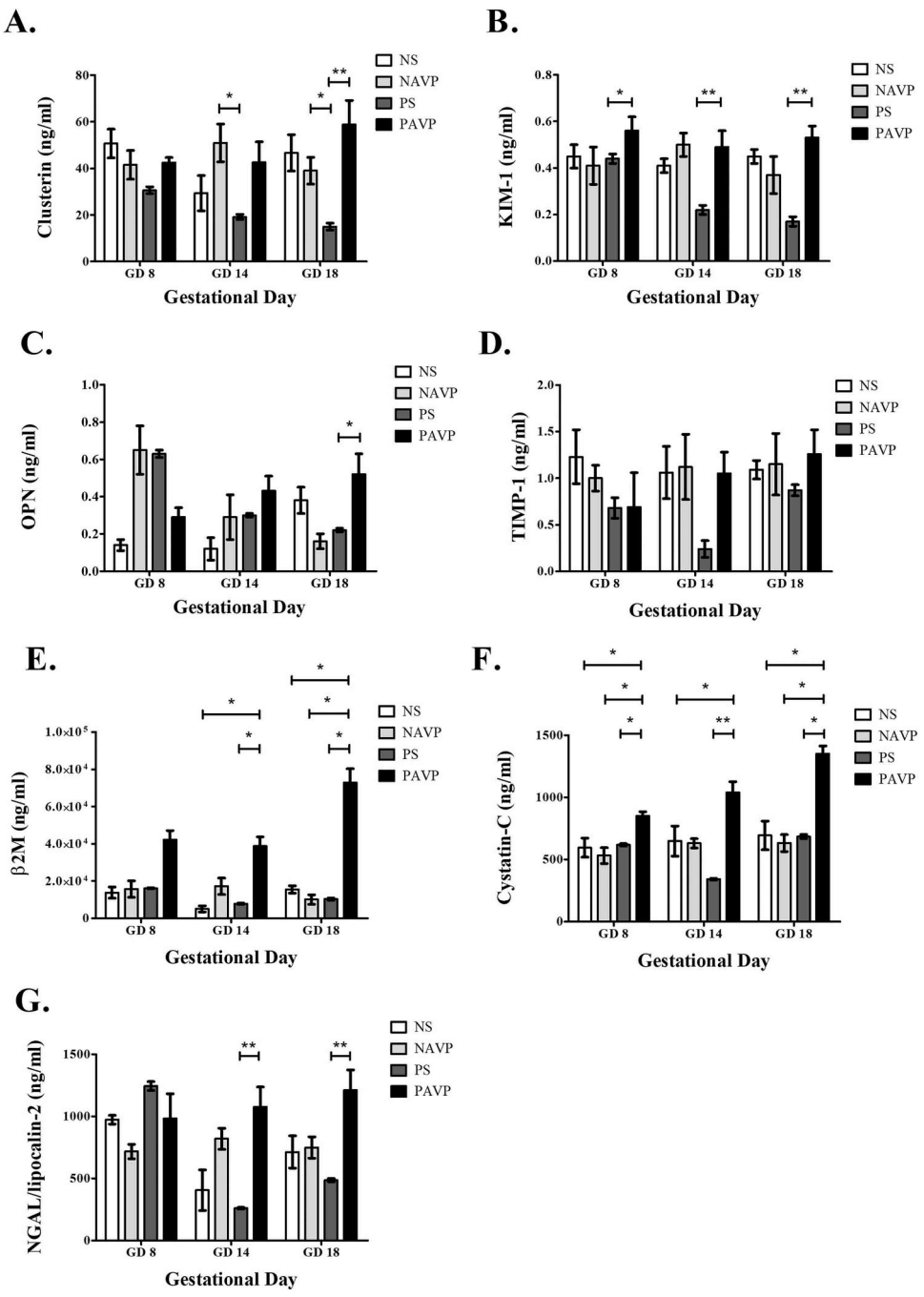


**Fig. 3.** Tubular injury demonstrated by biomarker concentrations on GD 8, 14 and 18 of (A) Clusterin, (B) KIM-1, (C) OPN, (D) TIMP-1, (E) β2M, (F) Cystatin C and (G) NGAL/lipocalin-2. Repeated measures ANOVA and Boniferroni's post-hoc test was conducted between pairs of group means to determine significance. Data is shown as mean ± SD. NS: non-pregnant Saline; NAVP: non-pregnant AVP; PS: pregnant saline; PAVP: pregnant AVP, $*p < 0.05$; $**p < 0.001$; (n = 6 per group).

between albumin and cystatin C, NGAL/Lipocalin-2, OPN and KIM-1 in the AVP treated pregnant rats (Table 2). Likewise, cystatin C was positively associated with NGAL/Lipocalin-2; and clusterin was positively associated with OPN on GD18 in PAVP rats. In the treated pregnant groups, urinary output was inversely associated with cystatin C and NGAL/Lipocalin-2, while fetal number was inversely correlated with TIMP-1 levels on GD18. A strong negative association was also noted on GD18, between KIM-1 and systolic and diastolic blood pressure.

## 4. Discussion

As expected, systolic and diastolic blood pressures were significantly elevated in pregnant AVP-treated rats as previously reported [32]. A notable increase in diastolic pressure was observed in the NAVP group compared to the NS and PS groups. However, variations in baseline blood pressure recording in non-pregnant female Sprague dawley rats exist between studies [34,35]. Higher systolic (117 ± 2 mmHg) and diastolic (70 ± 2 mmHg) measurements were reported by Ferrario's team [34] compared to Kitpipatkun and Sukwan (2022), who reported lower systolic (108 ± 23 mmHg) and diastolic pressures (62 ± 13 mmHg) respectively [35]. Elevations in diastolic pressure observed in the NAVP in comparison to the saline groups may be attributed to the vasoconstrictive function of AVP and activation of the renin-angiotensin aldosterone system. AVP also significantly reduces

**Table 1**
Clinical characteristics of the study groups on gestational day 18.

| | Non-pregnant Saline | Non-pregnant AVP | Pregnant Saline | Pregnant AVP |
|---|---|---|---|---|
| Systolic Blood Pressure (mmHg) | 122.00 (120.50-125.78) | 125.33 (123.00-129.00) | 122.00 (120.00-124.00) | 135.33 (133.67-138.67)[b,e,f] |
| Diastolic Blood Pressure (mmHg) | 82.00 (80.83-84.50) | 92.34 (91.00-97.33)[a,d] | 81.00 (80.00-87.00) | 103.50 (100.33-106.88)[c,g] |
| Urinary output (ml) | 20.50 (20.00-23.50) | 9.50 (8.00-15.00)[a,d] | 25.50 (25.00-29.00) | 17.00 (12.00-20.00)[f] |
| Fetal number | | | 9.00 (8.00-10.00) | 12.00 (11.00-12.00)[f] |
| Individual fetal weight (g) | | | 1.79 (1.70–1.85) | 1.57 (1.45–1.67)[f] |
| Individual placenta weight (g) | | | 0.59 (0.55-0.63) | 0.39 (0.31-0.46)[f] |

[a]$p < 0.05$: NS *vs* NAVP; [b]$p < 0.05$ and [c]$p < 0.001$: NS *vs* PAVP; [d]$p < 0.001$: NAVP *vs* PS; [e]$p < 0.05$: NAVP *vs* PAVP; [f]$p < 0.05$ and [g]$p < 0.001$: PS *vs* PAVP.
NS: non-pregnant Saline; NAVP: non-pregnant AVP; PS: pregnant saline; PAVP: pregnant AVP (n = 6 per group)
Note: data is expressed as median (25th percentile – 75th percentile).
- Kruskal-Wallis and Dunn's *post hoc* tests were used to determine if there were statistically significant differences in blood pressure and urinary output
- Mann-Whitney test was used to determine if there were statistically significant differences in fetal and placental parameters

**Table 2**
Pearson correlation analyses between selected clinical factors and kidney injury biomarkers in pregnant AVP-treated rats on gestational day 18.

| Kidney injury biomarkers | Clinical Factors / Kidney injury biomarkers | *r* value | *p* value |
|---|---|---|---|
| KIM-1 | Systolic blood pressure | -0.895 | 0.040* |
| | Diastolic blood pressure | -0.968 | 0.007* |
| | Urea | 0.853 | 0.031* |
| β2M | Systolic blood pressure | 0.813 | 0.049* |
| Albumin | Cystatin C | 0.946 | 0.004* |
| | NGAL/Lipocalin-2 | 0.893 | 0.017* |
| | OPN | 0.844 | 0.035* |
| | KIM-1 | 0.828 | 0.042* |
| | Chloride | 0.912 | 0.011* |
| | Sodium | 0.961 | 0.002* |
| | Potassium | 0.826 | 0.043* |
| | Urea | 0.992 | < 0.001** |
| Cystatin C | NGAL/Lipocalin-2 | 0.952 | 0.003* |
| | Urinary output | -0.870 | 0.024* |
| | Urea | 0.933 | 0.007* |
| OPN | Clusterin | 0.897 | 0.015* |
| | Calcium | 0.868 | 0.025* |
| | Chloride | 0.940 | 0.005* |
| | Sodium | 0.867 | 0.025* |
| | Urea | 0.847 | 0.034* |
| NGAL/Lipocalin-2 | Urinary output | -0.914 | 0.011* |
| | Urea | 0.895 | 0.016* |
| TIMP-1 | Fetal number | -0.824 | 0.044* |

β2M: beta-2-microglobulin; KIM-1: kidney injury molecule-1; OPN: osteopontin; TIMP-1: tissue inhibitor of metalloproteinases-1
*$p < 0.05$; **$p < 0.001$

urine output in the PAVP and NAVP in comparison to the saline treated groups. This may be attributed to the antidiuretic actions of AVP which stimulates the renal reabsorption of water [36]. An inverse relationship is observed between AVP levels and renal water handling as higher circulating levels of AVP leads to a decrease in urine output. Elevations in urinary protein were accompanied by a concomitant reduction in urinary output in pregnant AVP-treated rats [32].

Our findings demonstrate significantly higher levels of urinary injury markers in the AVP treated pregnant groups compared with untreated groups. Glomerular injury is confirmed by the significant elevations in albumin and the concomitant reduction in VEGF-A throughout gestation. Albumin levels are significantly higher in pregnant AVP treated rats as early as GD14 in comparison to saline treated rats. Albumin excretion rather than total protein excretion has gained support as an indicator of glomerular damage and systemic endothelial cell dysfunction [13]. The elevated albumin levels in the pregnant AVP-treated rats noted in our study confirm glomerular injury. This 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 [17]. 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 [37]. 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 [17]. The significant positive correlations between albumin and NGAL/Lipocalin-2 and cystatin C observed in our study suggest that those with albuminuria may exhibit high levels of NGAL/Lipocalin-2 and cystatin C as a consequence of albumin competing with these proteins 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 [38]. Hence, kidney injury may be attributed to an imbalance in glomerular VEGF-A expression [39]. Moreover, circulating levels of VEGF-A are significantly higher in PE compared to normotensive pregnancies [20], as well as in preeclamptic women giving birth to small gestational aged infants [40]. The reduced VEGF-A levels observed in pregnant AVP-treated rats in our study, suggests an angiogenic imbalance, and are associated with elevations in soluble fms-like tyrosine kinase (sflt-1) [41,42]. The administration of sFlt-1 to diabetic mice is reported to reverse kidney damage and albuminuria, and subsequently reduces mesangial proliferation [43]. Patients with diabetic nephropathy exhibit lower levels of renal VEGF-A which correlate with disease progression [44].

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 [10]. We report significant elevations for clusterin, cystatin C, β2M, NGAL/lipocalin-2, KIM-1 and OPN concentration in AVP treated pregnant rats compared to untreated rats, which may be indicative of tubular injury. Kidney disease is associated with increased tubular expression of clusterin which is subsequently released into the lumen [16,45]. Elevated circulating levels of clusterin often result from tissue specific injury and tissue remodeling [46]. 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 remodeling and inadequate placental development emanating from the shallow trophoblastic invasion in PE [47]. Our findings are corroborated by significant differences in clusterin levels between PE and normotensive groups in both urine [18] and serum [48].

The proximal tubular injury markers NGAL/Lipocalin-2, KIM-1, cystatin C, β2M and OPN were 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 [49]. 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 [50]. 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 are reported to be associated with acute kidney injury [14,51] and ischemic renal injury [52]. Our results are similar to the high urinary NGAL/Lipocalin-2 and KIM-1 levels reported in women with or without severe features of PE compared to normotensive pregnancies complicated by chronic hypertension [53]. 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 GD18 once again suggest that AVP induces elevations in BP which consequently stimulates KIM-1 release from the tubular cells, and consequent tubular injury.

It is possible that the elevations in clusterin (GD8–18), KIM-1 (GD14–18) and NGAL/Lipocalin-2 (GD14–18) levels in the NAVP group may be a consequence of elevations in renal plasma flow and glomerular filtration rate, arising from the prolonged activation of AVP receptors induced by chronic infusion of AVP [54]. Cystatin C and β2M are freely filtered by the glomerulus and are reabsorbed and metabolized by the proximal tubules, [21,55]. They are degraded and not reabsorbed into circulation [56], 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 [12]. In our study, urinary β2M was positively associated with systolic blood pressure on GD18. 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 [57] and their subsequent catabolism and loss in the urine [58]. Urinary levels of cystatin C are significantly higher in PE compared to normotensive pregnancies [16], and is closely correlated with impaired glomerular filtration and acute kidney injury [17]. Of 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 [59,60].

Osteopontin (OPN) is widely expressed in bone, immune, smooth muscle, epithelial and endothelial cells [61], as well as human and murine placental tissue [62]. 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 [22], whereas urinary OPN levels are significantly increased in diabetic patients with renal injury [63]. It was recently established that elevated urinary OPN is associated with proteinuria, reduced creatinine clearance, fibrosis and macrophage and T-cell infiltration [64]. Osteopontin and β2M are also implicated in modulating the immune response, thereby enhancing the T helper cell 1 ($T_H1$)-mediated response [64]. 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 [65]. Scroggins et al., (2018) also demonstrated an increased pro-inflammatory $T_H1$- associated interferon gamma (IFN-ϒ) response in a PE mouse model subcutaneously infused with AVP throughout gestation [25].

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 [66]. 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 [67]. Furthermore, the kidney also maintains chloride homeostasis, through reabsorption thereby regulating extracellular fluid volume [68]. 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, albeit non-significant, in the PAVP group as a consequence of AVP infusion. The elevated TIMP-1 levels may prevent extracellular matrix remodeling in the kidney by inhibiting the action of matrix metalloproteinases (MMP) [69]. Elevations in TIMP-1 is also associated with chronic renal disease [69]. In PE, significant elevations in TIMP-1 expression was observed in the placenta [70] as well as in serum [19,71,72]. 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, [69].

Our previous histological report confirm that AVP is implicated in kidney injury associated with PE development [33]. The findings from this study provides additional evidence of the potential applications of this model in studying the mechanisms underlying renal damage in PE, as shown by the elevations in the urinary levels of the kidney injury markers.

## 5. Conclusion

This study confirms a significant upregulation in the urinary levels of albumin, clusterin, NGAL/Lipocalin-2, KIM-1, OPN, β2M and cystatin C with a reduction in VEGF-A in the AVP treated pregnant group compared to untreated pregnant group. Our findings suggests that AVP has a potential role in inducing substantial renal injury, characteristic of PE development, as well as demonstrates their preclinical value as early predictors for PE development. Additionally, both KIM-1 and cystatin C are elevated as early as GD8 and remain so throughout gestation. These proteins also correlate significantly with systolic and diastolic blood pressures in pregnant rats treated with AVP, further demonstrating their potential clinical utility. 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.

## Animal studies

All institutional and national guidelines for the care and use of laboratory animals were followed.

## Disclosure statement

The authors declare that they have no conflicts of interest.

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## CRediT authorship contribution statement

Protocol/project development: NG, SB. Data collection: SR. Data analysis NG, SB, SR. Manuscript writing/editing: NG, TN, SB, SR.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Nalini Govender reports financial support was provided by National Research Foundation.

## Data Availability

Data availability is not applicable as all data pertaining to this study have been published.

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