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Exploring urinary biomarkers for the diagnosis of diabetic and hypertensive chronic kidney disease: A promising pilot study

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In the current clinical setting, conventional serum biomarkers such as serum creatinine (Scr) and estimated glomerular filtration rate (eGFR) have several lapses in chronic kidney disease (CKD) diagnosis. Diagnosing CKD using non-invasive methods is crucial for implementing prompt therapeutic interventions and preventing disease progression. This study aims to identify the potential diagnostic urinary biomarkers and their correlation with existing renal markers, Scr, eGFR, and proteinuria in diabetic and hypertensive CKD. RNA was extracted from eighty-two urine samples of CKD patients and healthy controls (HC) and reverse transcribed for gene expression analysis using quantitative polymerase chain reactions. The expression of *NGAL*, *MMP9*, *ANXA3*, *OLFM4*, *PI3*, and *PRMT3* genes was analyzed relative to the reference gene, *B2M*. Fold changes (FC) in gene expression in diabetic nephropathy (DN), and hypertensive nephropathy (HT) were calculated against HC. Log_2 normalized FC was used to determine significance levels and correlation with existing serum markers. *NGAL*, *ANXA3*, and *OLFM4* exhibited the highest upregulations in DN with mean Log_2FC 1.42, 2.66, and 5.87, respectively. A two-fold increase in *NGAL* FC was observed in early DN than in late DN, suggesting its potential as an early urinary biomarker for DN. *PI3* and *MMP9* were upregulated in HT patients with higher FC values. *PRMT3* showed a significant negative correlation ($P<0.05$) in HT patients with Scr ($r=-0.738$) and proteinuria ($r=-0.906$). The gene panels including *ANXA3*, *OLFM4*, and *NGAL*, and *PI3*, *PRMT3*, and *MMP9*, could have potential diagnostic value in DN and HT, respectively.

Keywords: Gene expression analysis, Non-invasive diagnosis, RT-qPCR (Reverse transcription quantitative polymerase chain reaction), Serum biomarker

Chronic kidney disease (CKD) is an abnormal structure or function of the kidney that persists for over three months[1]. CKD accounted for 1.2 million global deaths in 2017 and is expected to rise to 4.0 million by 2040[2]. Diabetes mellitus (DM) and hypertension are major risk factors for CKD. More than 40% of type 2 DM patients develop diabetic nephropathy (DN) worldwide, initially characterized by microalbuminuria followed by overt proteinuria[3]. The kidney size progressively increases than its original following the onset of diabetes, indicating the early sign of nephropathy[4]. While glomerular alterations are of greater importance in the pathogenesis of DN, tubular changes in the kidney also play a critical role in DN[5].

Hypertension accounts for approximately 29% incidence rate in annual renal transplantation. Classically, hypertensive nephropathy (HT) diagnosis remains unclear and made by excluding CKD of unknown cause with long-standing hypertension before the onset of CKD. The definitive diagnosis of HT can only be made by renal biopsy examinations[6]. Serum creatinine (Scr) and estimated glomerular filtration rate (eGFR) are critical indicators to assess kidney function. However, these measures can be influenced by non-renal factors[7]. Therefore, it is worth considering the use of urine to study non-invasive biomarkers in urogenital diseases.

This study hypothesizes that a gene panel has the potential to screen for diabetic and hypertensive nephropathy. The expression patterns of the selected genes, Neutrophil gelatinase-associated lipocalin (*NGAL*), Matrix metalloproteinase 9 (*MMP9*), Annexin A3 (*ANXA3*), Olfactomedin 4 (*OLFM4*),

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Peptidase inhibitor 3 (*PI3*) and Protein methyl transferase 3 (*PRMT3*) were analyzed in urine samples obtained from patients with diabetic and hypertensive nephropathy and compared with other CKD categories. Both *NGAL* and *MMP9* genes are involved in DN pathogenesis[8]. *MMP9* degrades the extracellular matrix protein in the glomerular basement membrane, inducing epithelial-mesenchymal transition of renal tubular cells[9]. *OLFM4* expression was found in various tissues including pancreatic β cells and involved in glucose homeostasis[10]. *ANXA3* gene regulates many cancers, including renal carcinoma[11]. *PRMT3* inhibits renal fibrosis through asymmetric dimethyl-arginine (ADMA) synthesis[12]. The *PI3* gene encodes the elafin protein and its expression increases in response to inflammation[13,14]. However, the direct involvement of *PRMT3*, *ANXA3*, and *PI3* in CKD requires further understanding.

Materials and Methods

Study population

The study population (*n*=82) was recruited as diabetic nephropathy (DN; *n*=17), hypertensive nephropathy (HT; *n*=31), CKD patients with both diabetes and hypertension (HD *n*=11), CKD caused by other etiological causes (O; *n*=13), and healthy controls (HC; *n*=10). CKD patients for this study were recruited from the Nephrology clinic at Vavuniya Hospital from November 2020 to April 2022. The diagnosis of DN and HT was established through clinical assessments, laboratory investigations, and renal imaging techniques during the clinic visit. A renal biopsy examination was performed to confirm the diagnosis of DN. The presence of retinopathy and/or neuropathy in type 2 diabetes patients also supported the confirmation of DN. Notably, DN patients with hypertension resulting from CKD were excluded to ensure consistency in gene expression analysis[15]. HT was diagnosed based on blood pressure levels ≥160/100 mmHg without antihypertensive treatment or ≥140/90 mmHg with at least two antihypertensive drugs, along with evidence of CKD. The existing clinical diagnostic tools were used to confirm HT. Secondary hypertension resulting from CKD after the onset of renal impairment was not considered for HT[16]. CKD patients with pre-existing diabetes and hypertension before the onset of CKD, and without confirmed DN and HT, were categorized into the HD study group. The O study group included CKD patients with other etiological causes such as glomerular nephritis, IgA nephropathy, systemic lupus erythematosus (SLE), polycystic kidney disease, snake bites, prolonged usage of the nephrotoxic drug (e.g., Nonsteroidal anti-inflammatory drugs), chronic urinary tract infections, and other known causes of CKD[17].

Inclusion and exclusion criteria

The study included CKD patients aged above 40 years, diagnosed by a consultant nephrologist according to the clinical practice guideline for chronic kidney disease[1]. Age-matched healthy volunteers residing in the Kandy district, who did not show any symptoms of diseases or have a past medical history of any chronic illness, were included as controls. We excluded CKD patients with an unknown etiology (CKDu) and any participants from CKDu-endemic areas. The quantity and quality of RNA were the major determining factors for including samples in the gene expression analysis. Degraded RNA and RNA concentrations < 5 ng/μL were excluded from the study.

Urine sample collection

The second-morning urine samples were collected using the clean catch mid-stream urine sample collection technique and kept on gel ice packs immediately after collection and transported to the National Institute of Fundamental Studies (NIFS), Kandy. About 10–90 mL urine samples (depending on the output of CKD patients) were collected from each study participant. The urine samples were stored immediately at -80°C until RNA extraction.

Urine RNA extraction

Urine samples were transferred into conical centrifuge tubes and centrifuged at 6,500 *g* (Eppendorf® 5,430, Germany) for 20 min at 4°C. About 100–150 μL of the urine pellet was resuspended in 500 μL of lysis buffer containing guanidinium thiocyanate. Total RNA was extracted from urine samples using a modified phenol-chloroform RNA extraction method[18]. The extracted RNA was used for complementary DNA (cDNA) synthesis after checking its integrity using 1% native agarose gel electrophoresis.

Serum and urine biochemical tests

Serum creatinine level (Scr) was measured in the serum samples of patients during their clinic visit at the hospital laboratory. The test was done using

Table 1 — Details of primer sequences used for the study

Gene	Accession No	Sequence 5'–3' (F-forward, R- reverse)	Size (bp)
ANXA3	NM_005139.3	F: CCACCGCGCTTTGGATTAG R: TCAGCATCCACTGATGGGCT	126
NGAL	NM_005564.5	F: CACCTCCGTCCTGTTTAGGAAA R: CACCACTCGGACGAGGTAAC	136
OLFM4	NM_006418.5	F: CAAAACACCCCTGTCGTCCA R: TGATGTTCCACCACACCACCA	71
MMP9	NM_004994.3	F: CTTTGAGTCCGGTGGACGAT R: TCGCCAGTACTTCCCATCCT	101
PI3	NM_002638.4	F: TTTCGTTCCCCAGTGAGAGGG R: TTAGGACCAGATGGGGCCTG	79
PRMT3	NM_001145167.2	F: GTCAGGCGCTACCGGTTATT R: CCCAAGGCACTGGGTTGTAT	196
B2M[41]	NM_004048.2	F: TGCCGTGTGAACCATGTGA R: CCAAATGCGGCATCTTCAA	98

VITROS® 5600 biochemistry analyzer using a commercial kit for enzymatic method (CREA ENZ 200) according to the manufacturer protocol. CKD-EPI formula was used to calculate eGFR. According to the calculated eGFR, CKD was classified into early (Stage 1-3) and late stage (stage 4 and 5)[1]. Proteinuria was tested using Mission® Urinalysis reagent strips (USA) according to the manufacturer protocol.

Complementary DNA (cDNA) synthesis and qPCR

According to the manufacturer's protocol, reverse transcription was done using the Go-Script Reverse transcription kit (Promega). Approximately 100 ng of RNA was quantified using QuantiFluor™ RNA System (Promega) for cDNA synthesis. Using the Rotor Gene-Q PCR machine (Qiagen), the synthesized cDNA was used for quantitative real-time PCR reactions.

The 25 µL PCR master mixture consisted of 1X PCR buffer, 0.8 mM $MgCl_2$, 0.3 µM of each forward and reverse primer, 0.1 mM of each dNTP, 0.1× SYBR green, 0.625 unit of *Taq* DNA polymerase (Promega) and 2 ng of cDNA. The primers were designed using NCBI primer blast software using exon-exon spanning. The primer details are summarized in Table 1. The qPCR conditions were optimized individually for each gene used in this study (Supplementary table 1). The specificity of each amplification reaction was confirmed by melt curve analysis (Supplementary fig. 2).

Statistical analysis

Quantification cycle (Cq) values of each amplification were used to calculate individual fold changes (FC) using Equation 1[19]. Log_2 normalized FC (Log_2FC) values were used to calculate the significance levels. One-way ANOVA using Tukey and Games Howell Post-hoc analysis was employed to assess the significance level of gene expression among study groups. Pearson correlation with a two-tailed significance test was used to analyze the correlation between Scr, eGFR, proteinuria, and the Log_2FC of individual subjects.

Results

Study population

Eighty-two study subjects, including CKD and HC, were included in this study. The mean age of CKD and HC was 55.18 ± 1.27 and 58.70 ± 2.95 years, respectively. The Scr levels of early (n = 27) and late-stage of CKD (n = 45) were 1.53 ± 0.07 and 4.08 ± 0.33 mg/dL, respectively. The mean urine volume obtained from CKD was 51.32 ± 21.68 mL, and HC was 81.67 ± 10.80 mL. The yield of total RNA was 718 ± 164 ng in CKD urine and 790 ± 231 ng in HC urine. The sociodemographic and clinical characteristics of each study group including age, gender, geographical location of study participants, blood pressure levels, and serum and urine parameters of the study population are summarized in Table 2.

Gene expression analysis

The gene expression of the selected genes was separately analyzed in all study groups. Outliers were determined based on the Log_2FC values, and any median value exceeding 150% of the interquartile range was excluded from the calculations. The FC of the genes of interest and the statistical significance of the Log_2FC level compared to the HC group are

Table 2 — Characteristics of study population

	HC (*n*=10)	DN (*n*=17)	HT (*n*=31)	HD (*n*=11)	O (*n*=13)
Age (years)	55.18±1.27	54.47±2.13	58.16±1.53	57.55±2.05	47.00±4.52
Gender (*n*)					
Male	4	10	20	6	6
Female	6	7	11	5	7
Geographical location					
Urban	06	03	06	02	03
Rural	04	14	25	09	10
Blood pressure (mmHg)					
Systolic pressure	-	112.64 ± 7.93	136.45 ± 17.04	125 ± 7.41	113.07 ± 7.21
Diastolic pressure		75.29 ± 7.17	85.16 ± 10.60	77.27 ± 4.67	70.38 ± 4.99
Scr (mg/dL)					
Early stage	-	1.58 ± 0.35	1.58 ± 0.28	1.56 ± 0.63	1.33 ± 0.24
Late stage		5.31 ± 2.05	4.02 ± 2.54	4.07 ± 2.11	3.24 ± 0.68
eGFR (mL/min/1.73 m2)					
Early stage	-	45.82 ± 16.28	47.43 ± 11.03	44.33 ± 11.59	60.17 ± 16.29
Late stage		12.67 ± 8.19	17.75 ± 7.36	16.25 ± 5.36	18.57 ± 4.92
Proteinuria (g/L)					
Negative/ ND	10	1	9	5	5
0.01 – 0.15 g/L	-	5	5	1	2
0.16 – 0.3 g/L	-	5	5	1	4
0.31 – 1.00 g/L	-	2	10	2	2
1.01 – 3.0 g/L	-	3	1	2	0
>3.0 g/L	-	1	1	0	0
Total RNA yield (ng)	790 ± 231	714 ± 390	742 ± 234	837 ± 579	602 ± 308

[HC: healthy controls, DN: diabetic nephropathy, HT: hypertensive nephropathy, HD: CKD with diabetes and hypertension, O: CKD caused by other than diabetes and hypertension, Scr: serum creatinine, eGFR: estimated glomerular filtration rate, ND: proteinuria not detected]

summarized in Table 3. Fig. 1 shows the graphical summary of Log_2FC of study genes. The genes such as *NGAL* (log_2FC = 1.423), *ANXA3* (log_2FC = 2.661), *OLFM4* (log_2FC = 5.868), and *PI3* (log_2FC = 1.827) were upregulated in DN patients and, shows the statistically significant difference for *ANXA3* (*P*= 0.000) and *OLFM4* (*P*=0.008) compared with HC. The Post hoc Tukey test results in one-way ANOVA analysis revealed that *NGAL* expression is statistically significant in DN patients compared to the HT (*P* =0.012) and HD (*P*= 0.000) study groups. The upregulation of *ANXA3* was observed in all four study groups (DN, HT, HD and O). However, FC was not statistically significant in the HT, HD and O study groups. Significant differences were found between the DN group and HT group (*P*=0.000) and the DN group and O group (*P*=0.000).

In the case of *OLFM4*, the highest upregulation was observed in DN patients. The FC and Log_2FC were calculated as 548.51 (*P*=0.000) and 5.868-fold (*P*=0.008) respectively. A statistically significant difference in *OLFM4* gene expression was found between the early (Log_2FC = 4.10 ± 1.03) and the late stage (Log_2FC =3.37 ± 0.66) (*P*=0.035) of CKD, irrespective to the aetiology. In HT patients, the genes *PI3*, *MMP9* and *PRMT3* showed upregulation with mean FC of 49.78-fold, 11.178-fold and 21.94-fold, respectively. Statistically significant results were observed for *PI3* (*P*=0.013) and *MMP9* (*P*=0.017) genes.

Notably, there was no expression of *MMP9* and *PRMT3* genes in most CKD patients, excluding those with HT. Among the HT study groups, 93.5% (29/31) of patients expressed the *PI3* gene, while 67.7% (21/31) expressed the *MMP9* gene. Furthermore, the study revealed a two-fold difference in *NGAL* expression between early (FC = 3.96-fold; log_2FC = 1.842) and late-stage (FC = 2.15-fold; log_2FC = 0.836) DN patients. However, no significant difference was observed between the early and late stages of CKD in the other CKD study groups. In contrast to *NGAL*, the *ANXA3* gene was highly upregulated with disease progression in DN and HT patients. The results show that the mean FC of *ANXA3* in early and late DN were 845.23-fold and 2042.73-fold, respectively. The effect of *PRMT3* on

Table 3 — Gene expression fold changes of genes in different study groups

Gene	Fold changes (Median)	Significance (*P*- value)
NGAL	DN= 2.143	0.838
N=56	HT= 0.443	0.344
NE=24	HD= 0.027	0.004[b]
O=2	O= 0.152	0.412
	HC= 1.388	-
ANXA3	DN= 689.784	0.000[c]
N=77	HT= 27.552	0.000[c]
NE=3	HD= 238.856	0.020[a]
O=2	O= 6.453	0.155
	HC= 0.585	-
OLFM4	DN=657.114	0.008[b]
N=77	HT=8.974	0.301
NE=5	HD=5.589	0.866
O=0	O=82.385	0.080
	HC=0.560	-
PI3[d]	DN=4.605	0.265
N=71	HT=8.440	0.013[a]
NE=6	HD=0.557	0.374
O=4	O=0.145	0.020[a]
	HC=2.188	-
MMP9	DN=0.494	1.000
N=35	HT=14.557	0.017[a]
NE=45	HD=1.260	0.483
O=2	O=0.0215	0.669
	HC=1.428	-
PRMT3	DN=2.301	0.964
N=25	HT=1.623	0.864
NE=54	HD=3.099	0.998
O=3	O=0.102	0.921
	HC=0.438	-

[Statistically significant at [a]$P<0.05$, [b]$P<0.01$ and [c]$P<0.001$ compared to the healthy controls. [d]total sample size less than 82 due to insufficient RNA. N: number of samples had the particular gene in its transcript, NE: number of samples did not express the gene, O: number of outliers in the data set]

disease progression was not analyzed, as 73.61% of CKD patients did not express this gene. The study findings indicate a non-significant increase in *PI3* gene expression during the late stages of both DN and HT study groups compared to the early stages.

Correlation of the gene expression with serum creatinine, eGFR, and proteinuria

The correlation of the log_2FC of the studied genes with Scr, eGFR and proteinuria was separately analyzed for DN (Fig. 2) and HT (Fig. 3) study groups. The Pearson correlation coefficients and two-tailed significances are summarized in Table 4. No significant correlation ($P>0.05$) was found between the Log_2FC and Scr or eGFR values for the genes such as *NGAL*, *ANXA3*, *OLFM4*, and *PI3* in both DN and HT study groups. The correlations of *MMP9* and *PRMT3* were not analyzed in the DN study group, as

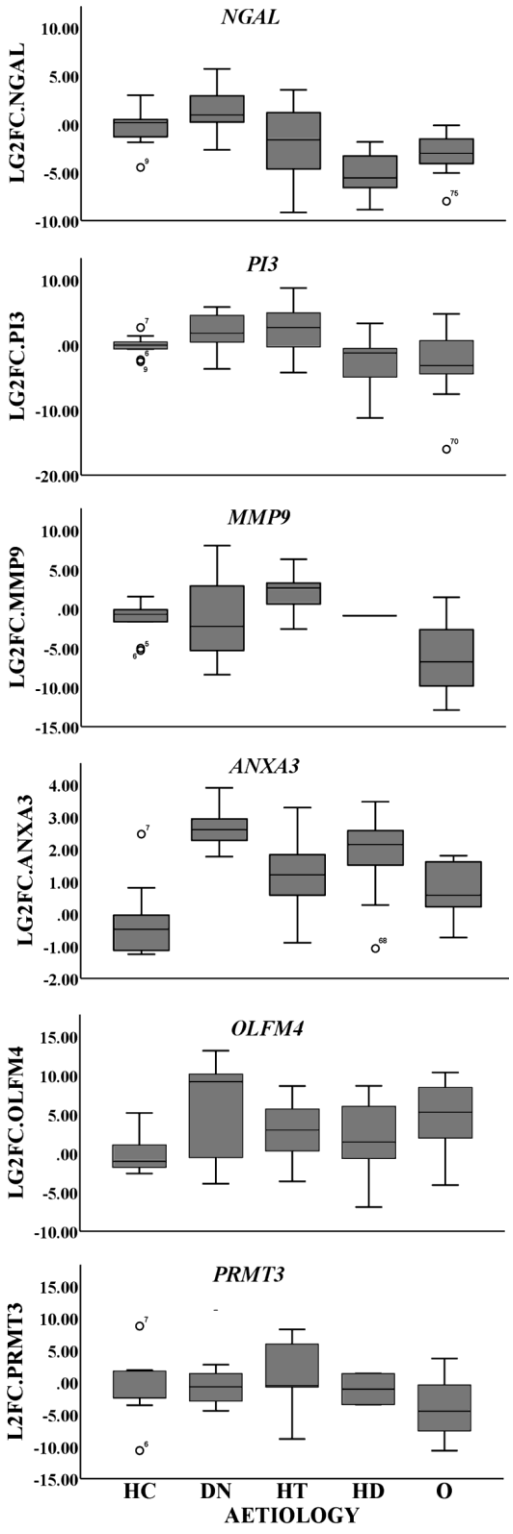

Fig. 1 — Box plots showing median values and range of log_2 normalized fold changes of genes in the four groups. D: diabetic nephropathy, HT: hypertensive nephropathy, HD: CKD with both diabetes and hypertension, and O: CKD caused by other that diabetes and hypertension. Outliers in each group are depicted by (∘) for values exceeding 150% IQR

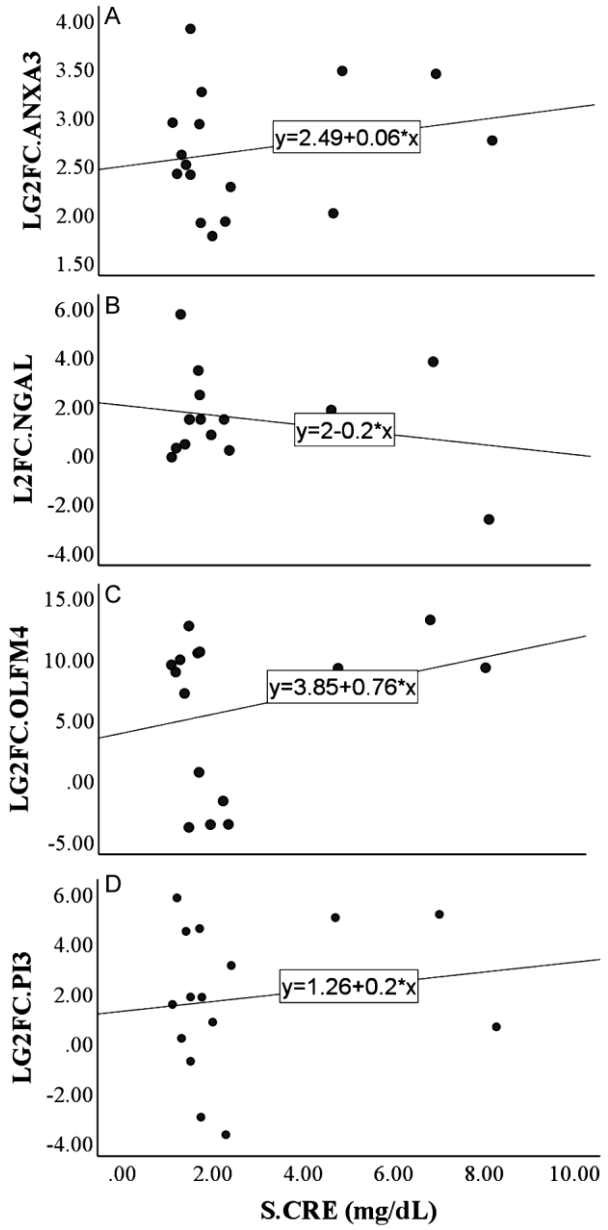

Fig. 2 — Scatter plot of correlation graph of serum creatinine and $\log_2$ normalized foldchanges of (A): *NGAL*, (B): *ANXA3*, (C): *OLFM4* and (D): *PI3* in diabetic nephropathy patients. (n = 17).

82.4% and 70.6% of study populations did not express these genes, respectively. In the HT study group, a statistically significant negative correlation was observed between the expression of the *PRMT3* gene and both Scr (r=−0.738; P=0.023) and proteinuria (r=−0.906; P=0.013).

Discussion

This pilot study explores differentially expressed genes within a proposed gene panel to establish their potential as biomarkers for distinguishing diabetic and

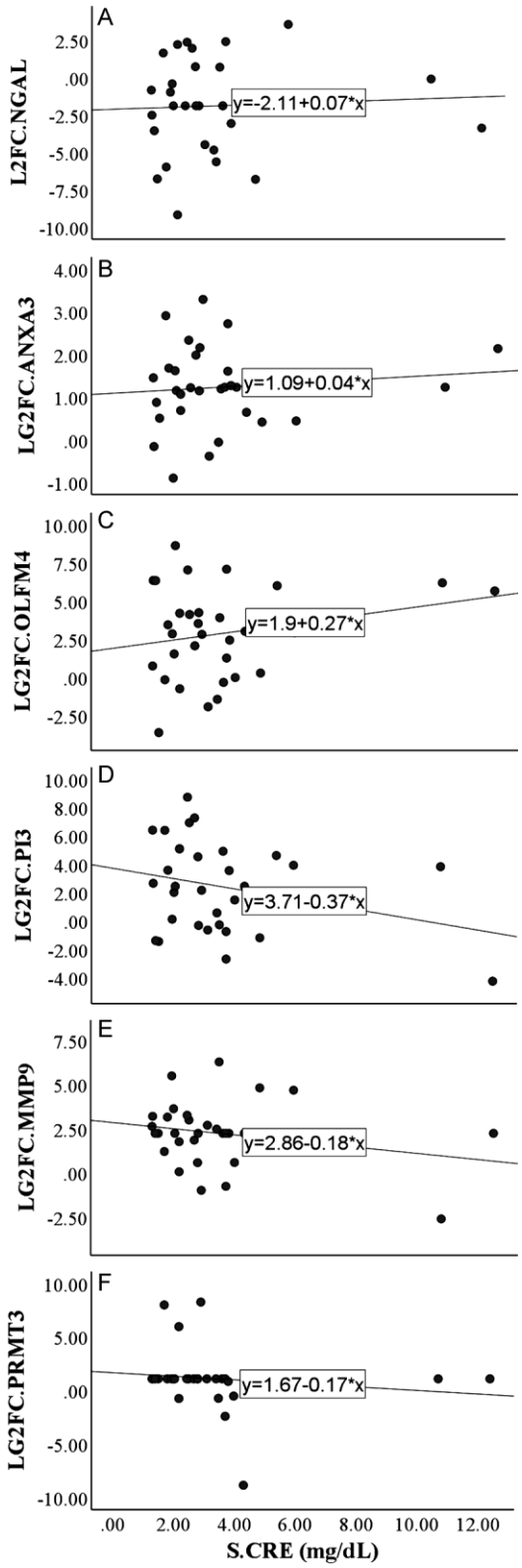

Fig. 3 — Scatter plot of correlation graph of serum creatinine and $\log_2$ normalized foldchanges of (A): *NGAL*, (B): *ANXA3*, (C): *OLFM4*, (D): *PI3*, (E): *MMP9* and (F): *PRMT3* in hypertensive nephropathy patients. (n = 31).

Table 4 — Correlation of $\log_2$ normalized fold changes with serum creatinine, eGFR and proteinuria of study subjects

		Diabetic Nephropathy				Hypertensive Nephropathy					
		NGAL	*ANXA3*	*OLFM4*	*PI3*	*NGAL*	*ANXA3*	*OLFM4*	*PI3*	*MMP9*	*PRMT3*
S.Cr	Pearson correlation	−0.237	0.214	0.264	0.158	0.053	0.106	0.226	−0.281	−0.238	−0.738[a]
	Significance (two tailed)	0.0359	0.409	0.341	0.545	0.776	0.569	0.222	0.126	0.197	0.023[a]
eGFR	Pearson correlation	0.135	−0.028	−0.06	−0.210	−0.119	−0.101	0.017	0.131	0.138	0.658
	Significance (two tailed)	0.605	0.915	0.831	0.419	0.523	0.589	0.928	0.481	0.459	0.054
Proteinuria	Pearson correlation	−0.297	0.551[a]	0.503	−0.015	−0.059	0.202	−0.066	−0.084	−0.267	−0.906[a]
	Significance (two tailed)	0.348	0.022[a]	0.056	0.959	0.806	0.355	0.759	0.704	0.336	0.013[a]

[a]Statistically significant at P <0.05; Scr: serum creatinine, eGFR: estimated glomerular filtration rate.

hypertensive nephropathy from other causes of CKD. In current clinical practice, diagnosis of CKD often depends on serum creatinine and albuminuria. While these measures serve as valuable diagnostic tools, the differentiation of CKD based on its underlying causes relies on histological examinations[20]. Although kidney biopsies are considered a gold standard method for CKD diagnosis, there is a need to develop non-invasive diagnostic tools for CKD subtyping. Urine emerges as a promising biological sample for the development of CKD biomarkers. In contrast to serum or plasma, urine presents itself as a non-invasive and easily accessible source of clinical information for renal diseases[21]. Therefore, we chose urine as the preferred biological sample for the development of CKD biomarkers. A study conducted by Ju *et al*. in 2015, demonstrated that tissue transcriptomes derived from urine can substantially diminish the necessity for invasive renal biopsies. This underscores the potential of urine as a valuable reservoir of information, offering direct insight into kidney function[22]. Additionally, the richness of transcripts in the glomerular filtrate provides more reliable information related to systemic and metabolic diseases[23]. Our study cohort included more HT patients in comparison with DN. A study conducted by Arambewela *et al*. in Sri Lanka in 2017 reported that 77.6% of Type 2 diabetes patients had a history of hypertension[24]. Notably, 60-90% of CKD patients develop hypertension after the onset of CKD[25]. Therefore, it was challenging for us to recruit individuals with DN who did not have accompanying CKD risk factors, primarily hypertension.

Tubular injury plays a significant role in diabetic kidney injury. *NGAL* expression prominently elevated in proximal kidney tubules with increased urinary NGAL protein excretion even before the occurrence of albuminuria. Liu *et al*. revealed a 5.95-fold increase in *NGAL* expression in the DN group compared to HC[26]. These findings were further reinforced in the present study, resulting in a 3.3-fold *NGAL* mRNA expression in DN patients. Furthermore, a 2-fold upregulation of *NGAL* was observed in early DN than in late DN, indicating that *NGAL* could serve as a better predictive biomarker for early DN. In a recent study of Greco *et al*, they demonstrated that urinary NGAL levels did not differ significantly in individuals with type 2 diabetes mellitus who showed no evidence of nephropathy[27]. Consistent with the aforementioned literature, our findings also indicate no considerable dysregulation of *NGAL* mRNA expression in urine of CKD patients with diabetes who do not exhibit evidence of DN. A significant difference between DN and HD study groups underscores the potential of the *NGAL* gene as a valuable and predictive biomarker, enabling the differentiation of diabetic kidney disease from chronic kidney disease in individuals with diabetes.

NGAL-MMP9 complex prevents the degradation of MMP9 and prolongs its activity[28]. Yang *et al.* demonstrated that increased *MMP9* expression in albuminuria and is associated with renal injury[8]. Moreover, studies suggested that increased *MMP9* excretion in normoalbuminuric individuals with diabetes could be a more effective diagnostic indicator for the early detection of DN[9]. However, the present study found that only 3 out of 17 DN patients expressed the *MMP9* gene. Therefore, the validity of the *MMP9* gene as a molecular biomarker for DN, particularly in combination with the *NGAL* gene

needs further understanding. The anti-diabetic and antioxidant activity of gliclazide has been shown to inhibit endothelial *MMP9* expression[29]. Similarly, paricalcitol, a compound with anti-inflammatory activity, has also been shown to downregulate *MMP9* expression. Paricalcitol is a synthetic form of vitamin D used to treat certain kidney disorders[30]. In the current study, the lack of *MMP9* expression in 82.4% of DN patients suggests the possibility of null mutations due to oral anti-diabetic therapy. A recent study by Rodríguez-Sánchez *et al*. demonstrated that the functional level of MMP9 was significantly increased in hypertensive patients with renal impairment, particularly in mid-stage (eGFR between 30-60 mL/min/1.73 m^2), compared to hypertensive patients without renal damage. However, no correlation was observed between those patients' total MMP9 level and eGFR[31]. Consistent with these findings, our present study also revealed no significant correlation between the decline in kidney function (eGFR) and urinary *MMP9* expression (r=0.138; P>0.05).

Insulin produced by pancreatic β cells exerts a crucial role in the maintenance of glucose homeostasis. The overexpression of the *OLFM4* gene in pancreatic β cells has been identified to impair insulin secretion, particularly under conditions of glucose intolerance[32]. A recent study done by Chen *et al*. in 2023, revealed that fatty acid overload induces *OLFM4* gene expression and leads to developing hepatic steatosis and insulin resistance[33]. In the present study, significant upregulations of *OLFM4* gene exclusively in DN study group provides a new insight for biomarker marker development in DN. However, despite the diagnostic characteristics of the *OLFM4* gene in DN, Liu *et al*. found that the deletion of the *OLFM4* gene would significantly improve insulin secretion and could serve as a potential therapeutic target in type 2 DM to prevent the morbidity of diabetic nephropathy[32]. In a separate study by Dorr *et al*., the *OLFM4* gene exhibited a 10.73-fold upregulation in post-transplanted patients, with this expression gradually diminishing after one week of transplantation[34]. However, the molecular pathology of this gene in both post-transplantation needs further studies. Further, a comparison of *OLFM4* gene expression between the early and late stages of CKD, regardless of the etiology, revealed a significant decrease (P<0.05) in *OLFM4* expression with disease progression.

The 95.83% of the CKD population shows the *ANXA3* gene expression, which indicates a good sign in this study for the biomarker development. Even though its expression in CKD demonstrates the upregulation of *ANXA3* irrespective of the aetiology of CKD, the statistically significant, highest fold change in DN would add more value in its diagnosis. Though the mechanism of the *ANXA3* gene in DN is not fully understood, previous studies reported that *ANXA3* is upregulated in patients with acute renal failure[35]. Additionally, proteomic analysis has shown an increase in urine ANXA3 excretion in individuals with collapsing focal segmental glomerular sclerosis[36]. Furthermore, *ANXA3* gene is also involved in the prostaglandin synthesis and regulation pathway. *ANXA3* binds to cell membrane phospholipids in the presence of calcium, leading to the inhibition of phospholipase A2 (PLA2) activity in the arachidonic acid metabolism[37]. Prostaglandins mainly regulates haemodynamics of the kidney via binding with prostaglandin E2 receptors which are widely distributed in various structures of the kidney including mesangial cells, renal tubules, collecting duct, renal interstitial cells and vascular smooth muscles. They regulate intracellular calcium ion homeostasis, blood pressure, cAMP activity and renal fibrosis[38]. However, there is no evidence yet to uncover *ANXA3* gene expression in chronic kidney disease patients to choose it as a biomarker and therapeutic target for disease intervention. This is the first study to elucidate the involvement of the urinary *ANXA3* gene and its potential role in renal biomarker development in CKD.

The *PI3* gene encodes the elafin protein, expressed mainly in the neutrophils and phagocytes. However, its role in CKD is not fully understood. The study by Bronze-da-Rocha and Santos-Silva in 2018 provided evidence supporting the therapeutic potential of the *PI3* gene in CKD[39]. Nevertheless, the diagnostic potential of *PI3* gene requires further investigations to develop biomarkers for CKD. The present study revealed a significant upregulation of *PI3* gene in HT study group, uncovering its potential as a diagnostic biomarker for hypertensive CKD. Moreover, a non-significant increase in its expression in DN group necessitates further validation with a larger sample size.

Furthermore, in their study, Musante *et al*. explored the expression of *PI3* in urinary extracellular vesicles of patients with DN. They reported a decrease in *PI3*

expression with albuminuria in DN[14]. In contrast to their findings, our study demonstrated a non-significant increase in *PI3* gene expression in DN as the disease progressed. Importantly, this trend was also explicitly observed in hypertensive CKD. These results highlight the potential of *PI3* as a biomarker for disease progression and underscore the importance of further investigation into its role in CKD subtyping.

The direct involvement of *PRMT3* in CKD has not been elucidated yet. The *PRMT3* gene is involved in the regulation of asymmetric dimethylarginine (ADMA) synthesis. Wang *et al*. conducted a study that revealed a significant role for the *PRMT3* gene in renal tubulointerstitial fibrosis. They found that *PRMT3* expression stimulates the production of renal ADMA, a molecule that plays a protective role in preventing renal fibrosis[12]. In the present study, 26.39% of CKD study groups exhibited the *PRMT3* gene indicating tubulointerstitial fibrosis in CKD, while 90% of healthy controls showed its expression. However, decreased expression in healthy controls compared to CKD, suggesting its protective role in preventing renal fibrosis in healthy controls.

Matsuguma *et al*. demonstrated a positive correlation between plasma ADMA levels and blood pressure level in CKD subjects. It suggests that an increased expression of *PRMT3* leads to elevated plasma ADMA levels, contributing to blood pressure regulation in CKD. Additionally, insulin resistance can also influence plasma ADMA levels. However, the direct involvement of either ADMA or *PRMT3* in DN has not yet been elucidated[40]. In the present study, among the CKD groups, DN, HT and HD study groups showed a non-significant upregulation compared to HC. However, the correlation of *PRMT3* expression to serum creatinine and eGFR provides significant insights into its potential clinical relevance in kidney function. In hypertensive nephropathy individuals, a robust negative correlation between *PRMT3* expression and serum creatinine and proteinuria, indicating that higher *PRMT3* expression is associated with improved kidney function. This suggests that *PRMT3* may serve as a valuable marker for assessing and monitoring kidney health in hypertensive patients. These findings underscore the potential clinical implications of *PRMT3* in assessing and managing kidney health only in HT patients. Further research is necessary to fully understand the mechanisms involved and validate *PRMT3* as a biomarker or therapeutic target.

In this study, gene expression analysis provided the necessary groundwork for developing non-invasive biomarkers for diabetic and hypertensive nephropathy. However, expanding the sample size ensures a more comprehensive validation of the observed gene expression patterns, strengthening the study's reliability. It is strongly recommended to validate the identified mRNA expression patterns of the selected genes, serving as diagnostic biomarkers, through comprehensive proteomic analysis. This validation step will enhance the reliability and robustness of the study's findings and bridge the gap between mRNA expression and protein production. Establishing a longitudinal study focusing on the same CKD patients to monitor changes in gene expression patterns over time as the disease progresses. This approach allows for a more comprehensive understanding of patient-specific molecular signatures associated with CKD progression.

Conclusions

The upregulation of *NGAL* and *OLFM4* genes in diabetic nephropathy patients suggests their potential as diagnostic biomarkers. Conversely, the significant upregulation of *MMP9* and *PI3* genes in hypertensive nephropathy indicates their potential as indicative biomarkers for this condition. Though the *ANXA3* gene upregulated in all CKD conditions associated both diabetic and hypertensive groups, the exceptionally high fold change particularly in diabetic nephropathy, underscores its potential for distinguishing this condition from other CKD subtypes. The two-fold increase in *NGAL* expression, in early diabetic nephropathy, suggests its utility as an early diagnostic biomarker. Conversely, the exceptionally high fold-change of *ANXA3* in the late stage of diabetic nephropathy implies its potential as a biomarker for disease monitoring. Correlations observed between *PRMT3* gene expressions and clinical parameters such as serum creatinine and proteinuria in hypertensive nephropathy suggest their potential clinical implications. However, these findings necessitate further validation with larger sample sizes before considering their implementation in clinical practice.

Ethical statement

The study was approved by the committee for ethical clearance of the Postgraduate Institute of

Science (CEC-PGIS), University of Peradeniya, Sri Lanka (CES-PGIS approval No: CEC_PGIS_ 2020_08). Informed written consent was obtained from each study participant before recruiting them into this study.

Conflict of interest

The authors declare that they have no conflict of interest.

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