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Comparative diagnostic utility of different urinary biomarkers during pre-albuminuric stages of non-hypertensive type 2 diabetic nephropathy

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***Background & objectives*: Activation of renin-angiotensin system and tubulointerstitial damage might be seen in pre-albuminuria stage of diabetic nephropathy (DN). Here, diagnostic utility of four urinary biomarkers [Angiotensinogen (Angio), Interleukin (IL)-18, Neutrophil Gelatinase-Associated Lipocalin (NGAL) and Cystatin] during pre-albuminuria stages of non-hypertensive type 2 diabetes patients was studied.**

***Methods*: A total of 952 type 2 diabetes mellitus (T2DM) patients were screened for nephropathy [estimated glomerular filtration rate (eGFR) ≥120 ml/min and albumin–creatinine ratio (ACR) ≥30], and 120 patients were followed up for one year. At one year, they were classified into hyperfiltration (43), normoalbuminuria (29) and microalbuminuria (48) groups. Another 63 T2DM patients without nephropathy were included as controls. Hypertension, patients on angiotensin-converting enzyme inhibitor/angiotensin receptor blocker, eGFR <60 ml/min/1.73 m^2 and all proteinuric conditions were excluded. All were subjected to testing for urine protein, ACR, HbA_1C, eGFR, along with urinary biomarkers (IL-18, cystatin-C, NGAL and AGT). Comparative analysis of all the diagnostic tests among different subgroups, correlation and logistic regression was done.**

***Results*: Urinary IL-18/Cr, cystatin/creatinine (Cr) and AGT/Cr levels were higher in groups of hyperfiltration (13.47, 12.11 and 8.43 mg/g), normoalbuminuria (9.24, 11.74 and 9.15 mg/g) and microalbuminuria (11.59, 14.48 and 10.24 mg/g) than controls (7.38, 8.39 and 1.26 mg/g), but NGAL/Cr was comparable. The area under receiver operating characteristic curve (AUC) and sensitivity of AGT to detect early CKD were higher than ACR and eGFR (0.91 and 90.4%, 0.6 and 40% and 0.6 and 37%, respectively). AUC values of other biomarkers, namely IL-18/Cr, cystatin/Cr and NGAL/Cr, were 0.65, 0.64 and 0.51, respectively. Angio/Cr and IL-18/Cr showed correlation with log albuminuria (r=0.3, P=0.00, and r=0.28, P=0.00, respectively). NGAL showed correlation with log eGFR (r=0.28 P=0.00). Multivariate logistic analysis showed that odds ratio of developing nephropathy was 7.5 times with higher values of log Angio/Cr.**

***Interpretation & conclusions*: Urinary AGT showed a higher diagnostic value than ACR and eGFR followed by IL-18 and cystatin to diagnose DN during pre-albuminuric stages.**

Key words Diagnostic tests - early diabetic nephropathy - pre-albuminuric stage - urinary angiotensinogen - urinary biomarkers

Diabetic nephropathy (DN) progresses through stages of hyperfiltration, microalbuminuria, macroalbuminuria and declining glomerular filtration rate (GFR) to reach end-stage kidney disease. At present, the most commonly used clinical index for evaluation and strong predictor of DN is albumin excretion rate or microalbumin which also reflects the decline in GFR[1]. However, microalbuminuria is diagnosed once significant glomerular damage has occurred and it does not necessarily lead to renal dysfunction; again, nephropathy sometimes occurs in the normoalbuminuric patients[2,3].

During pre-albuminuric stage, intraglomerular hypertension (HTN) and hypertrophy have been demonstrated as a cause of initial hyperfiltration. Furthermore, the role of tubulointerstitium has also been increasingly appreciated. It may be due to involvement of peritubular vessels induced by hypoxia or other antiangiogenic stimuli and production of pro-inflammatory cytokines by tubular epithelial cells. Involvement of renin–angiotensin system (RAS) might be activated before microalbuminuria and cause the development of tubulointerstitial fibrosis in the normoalbuminuric patients[4]. Angiotensin II also participates in cytokine- and chemokine-mediated recruitment of inflammatory cells into the kidney[1]. A study in 102 patients with type 2 diabetes mellitus (T2DM) and 18 healthy controls showed that urinary angiotensinogen (UAGT) might potentially serve as an early marker to determine intrarenal RAS activity and predict progressive kidney disease in T2DM patients without HTN[5]. Saito *et al*[6] have shown that urinary angiotensinogen (AGT) may function as an early marker of DN in patients with type 1 diabetes. Further, urinary neutrophil gelatinase-associated lipocalin (NGAL) has been found to be positively correlated with urinary IL-18 and AGT which supports involvement of RAS which might be a cause for the development of tubulointerstitial fibrosis[7]. Profile of urinary markers at early stage may reflect inflammatory process by activation of the intrarenal RAS and its progression. Hence, identifying nephropathy at pre-albuminuric stage by urinary biomarkers and their diagnostic utility is challenging.

In our previous study comprising 61 T2DM patients and 30 pre-diabetic patients, it was found that urinary NGAL and cystatin-C were significantly higher in microalbuminuria group compared to normoalbuminuria group; the area under receiver operating characteristic curve (AUC) of urinary NGAL/creatinine (Cr) was found to be better than urinary cystatin/Cr in estimating microalbuminuria[8]. Others have shown that cystatin-C, NGAL and kidney injury molecule-1 (KIM-1) were sensitive and specific in detecting early renal damage[9,10]. Satirapoj *et al*[11] found that the AUC of urine AGT (ng/mg Cr) was 0.62, 0.85 and 0.96 in established normoalbuminuric, microalbuminuric and macroalbuminuric T2DM patients, respectively. However, another study has shown that diagnostic accuracy of albumin creatinine ratio (ACR) is better than individual biomarker of DN when compared to both NGAL and IL-18[12]. The objective of this study was, therefore, to assess the diagnostic utility of four urinary biomarkers (Angio, IL-18, NGAL and cystatin) during pre-albuminuria stages of non-hypertensive T2DM patients.

Material & Methods

This one year prospective study (April 2017 to April 2018) was performed in the departments of Nephrology and Endocrinology, Atal Bihari Vajpayee Institute of Medical Sciences & Dr. Ram Manohar Lohia Hospital, New Delhi, India. The study was approved by the Institute Ethics Committee and written informed consent was obtained from each participant.

Screening and data collection: All consecutive T2DM patients of age above 30 yr attending the outpatient departments of Endocrinology, Internal Medicine and Nephrology, were screened for history and previous records. Inclusion criteria were (*i*) *e*-GFR ≥120 ml/min/1.73 m^2 with unrestricted ACR value, and (*ii*) estimated GFR (eGFR) 60-120 ml/min/1.73 m^2 and ACR 30-300 mg. Exclusion criteria were urinary tract infections, urinary stones, HTN, pregnancy, genital or any systemic infections, thyroid disease, nephrotoxic medications or steroids, patients on dialysis or post-renal transplant. Diabetes patients with 90-120 ml/min eGFR without microalbuminuria were considered as controls with exclusion criteria as above.

After performing urine ACR and eGFR (by MDRD4 formula), the patients were enrolled and were classified into four groups – glomerular hyperfiltration, normoalbuminuria, microalbuminuria and control groups as defined. All were subjected to routine biochemistry, namely; haemogram, kidney function test, liver function test, plasma glucose, HbA_{1c}, lipid profile after obtaining venous blood (7 ml) from each participant. Further, routine urine examination, urine for microalbumin

(spot urine for protein and Cr ratio), ultrasound abdomen, electrocardiogram, echocardiogram and fundus examination were also done. Standard definitions were used for defining DN, normoalbuminuria, micro - albuminuria and overt nephropathy[13-15].

These investigations were repeated at the third month for confirmation of nephropathy, thereafter at the 6th and 12th months. At the 12th month, extra urinary samples were collected in Eppendorf tubes and stored at −20°C for the estimation of urinary biomarkers. A total of 63 controls were enrolled from the same outpatient departments for clinical, biochemical and biomarker measurements for comparison.

Laboratory procedures: Urine routine examination was performed by urinary dipstick method by using Siemens Multistick® 10 SG Reagent Strips. For microalbumin estimation, Randox kits (Beckman Coulter AU 400, Danaher Corp, Brea, Calif) were used. Both microalbumin and spot urine creatinine were run on a fully automated clinical chemistry analyzer Olympus AU400 (Block Scientific, Bellport NY 11713, USA). Microalbumin-creatinine ratio was calculated manually which was expressed as mg of albumin per gram of creatinine. For NGAL, Human NGAL ELISA kit (96T; Epitope Diagnostic Inc, San Diego, CA 92130, USA) with detection range for plasma 0.48-3.9 ng/ml (normal urinary detection range is not available in the literature) was used. Cystatin was estimated using Cystatin-C-EIA-BEST kit (VECTOR best, Russia; detection range: 0-12 µg/ml). Angiotensinogen (AGT) and IL18 were estimated using Human Angiotensin ELISA kit (96T) Type 2 (Catalogue no. E13652264; dectection range: 2.74-200 ng/ml) and IL-18 ELISA kit type 2 (Catalogue no. E13651008; detection range: 13.7-1000 pg/ml), respectively (Sincere Biotech, China). Estimation of HbA_{1c} was done by using HPLC (high-performance liquid chromatography) method on Tosoh G8 analyzer (Ortho Clinical Diagnostics -Vitros 5.1, USA). Other tests were also done on a fully automated dry clinical chemistry analyzer.

Statistical analysis: All data were entered in Excel and analysis was done by using SPSS version 23 (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY, USA). Data normality and homogeneity of variance were assessed by using Levene's test. Arithmetic means and percentages were obtained for continuous and categorical data, respectively. Independent t test was used for comparing arithmetic means. Mann-Whitney U test was used for comparing median values of urine albumin, ACR and biomarkers. Diagnostic tests using receiver operating characteristic (ROC) curve for all biomarkers, urine albumin and ACR were individually carried out to identify their ability to classify individuals to cases or controls with a chosen cut-off value to attain acceptable sensitivity and specificity. The sensitivity and specificity, AUC and higher and lower confidence intervals were also calculated. The Pearson's correlation coefficients were calculated between log ACR, log urine albumin and log eGFR with different biomarkers for each stage. Logistic regression analysis was carried out to classify the individuals between cases and controls based on eGFR and ACR.

Results

A total of 952 pre-diabetic and T2DM patients were screened based on history and previous records. On six monthly follow up, at one year, 120 patients were available; 63 T2DM patients without nephropathy were also enrolled as controls (Fig. 1). The baseline characteristics and investigations including biomarkers of controls and patients groups are detailed in Table I. Patients groups and controls were found matching with respect to age, sex and body mass index (BMI). The glycaemic control was significantly better in controls than in microalbuminuric group. Median values of urine albumin, ACR, IL-18 and AGT, cystatin, IL-18/Cr and Angio/Cr were higher in hyperfiltration and microalbuminuric groups with respect to controls. AGT/Cr and urinary IL-18/Cr ratio were significantly higher in hyperfiltration and microalbuminuric subgroups compared to controls. High levels were significant in all except cystatin/Cr and IL-18/Cr in normoalbuminuric group. It was observed that all biomarkers except NGAL were high at hyperfiltration stage even before development of microalbuminuria (Table II and Fig. 2A-D).

ROC analysis of ACR, eGFR and different biomarkers with their urine Cr ratio at 12th month is shown in Table III. AGT/Cr and AGT had the highest AUC of 0.91 and 0.92. The cut-off value, sensitivity, specificity and AUC of AGT/Cr were 4.4 mg/g, 90.4 per cent, 86 per cent and 0.91, respectively. With the cut-off value of urine (ACR: 29.95 mg/g), sensitivity was poor at 40 per cent and specificity 100 per cent, whereas with the cut-off value of eGFR 119.5 ml/min, the sensitivity was 36 per cent and specificity 100 per cent.

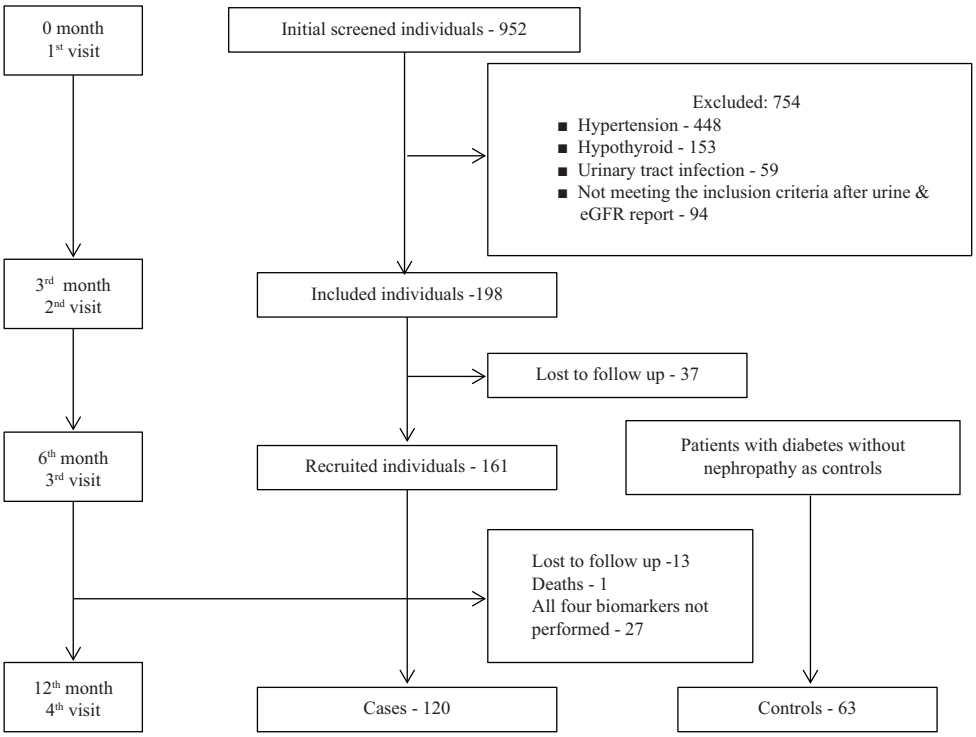

Fig. 1. Flowchart showing the total number of patients with diabetes with nephropathy (cases) and those without nephropathy (controls) during the study.

Following AGT, AUC of other biomarkers, namely IL-18, IL-18/Cr, cystatin, cystatin/Cr, NGAL and NGAL/Cr, were also in decreasing order. Both NAGL and NGAL/Cr had poor AUC (Fig. 3A-D).

Correlation analysis was performed between logarithmic transformation of ACR, urine albumin and eGFR with the four different biomarkers. Log urinary albumin showed a significant correlation with AGT/Cr (r=0.3, P=0.00) and IL-18/Cr (r=−0.28, P=0.01) at 12 months. There was a significant correlation of HbA_{1c} with log urine albumin (r=0.24, P=0.00) and log ACR (r=0.3, P=0.00). Log eGFR showed a significant correlation with NGAL (r=−0.28, P=0.00). All other biomarkers did not show any correlation.

Multivariate logistic regression analysis was carried out with independent variables age, BMI, mean blood pressure, cholesterol, HbA_{1c} and all ratios of log-transformed biomarkers with Cr. The odds of contracting DN was 7.5 and was 1.6 times greater in non-nephropathy patients having higher values of log AGT/Cr and IL-18/Cr, respectively, than non-nephropathy patients with low values (Table IV).

Discussion

The present study showed diagnostic test comparison of four different novel urinary biomarkers in hyperfiltration, normoalbuminuria and microalbuminuria stages of DN. Among all urinary biomarkers, AGT and IL-18 levels were higher in patients than controls and had a higher AUC and strong association with pre-albuminuria nephropathy. AGT showed a greater discriminatory value in terms of sensitivity and specificity compared to conventional ACR, urinary albumin and eGFR.

Being a hospital based study, it showed lower pre-diabetic prevalence (7.5%), uncontrolled diabetes status (HbA_{1c}: 8.4), 40 per cent prevalence of microalbuminuria and 24 per cent hyperfiltration. There were significantly higher uncontrolled blood glucose levels in microalbuminuria group, establishing the relation of nephropathy with the diabetic status. Similar to our study, Chowta *et al*[16] showed the prevalence of microalbuminuria of 37 per cent. There was no effect of BMI and sex on the prevalence of microalbuminuria similar to the present study, but there was a significant correlation of microalbuminuria with duration of diabetes[16]. Kundu *et al*[17] also found that uncontrolled glycaemic control and duration of diabetes were associated with significant elevations in urinary microalbumin levels.

Table 1. Baseline characteristics of patients with diabetic nephropathy completing follow ups (n=120)

Parameters	Mean±SD, n (%)
Age (yr)	49.2±9.39
Sex (male)	75 (62.5)
Married	115 (95.83)
Doing physical exercise	64 (53.33)
Vegetarian	45 (37.5)
Smoker	28 (23.33)
Alcoholic	28 (23.33)
Family income (million)	
<0.2	68 (56.66)
0.2-0.6	25 (20.83)
>0.6	27 (22.49)
Diabetes status	
Pre-diabetic	9 (7.5)
Diabetic	111 (92.5)
Duration of DM (yr)	
<1	38 (31.6)
1-5	27 (22.5)
>5	55 (45.83)
DM treatment	
No treatment	10 (8.33)
Diet	5 (4.16)
OHA	90 (75)
Insulin	12 (10)
Diabetes under control	49 (40.83)
Positive family history	52 (43.33)
Old history	
Renal stone disease	4 (3.33)
Taking pain killers	3 (2.5)
H/o of swelling	34 (28.33)
Investigations	
FBS (mg/dl)	153.58±72.67
PPBS (mg/dl)	225.97±95.45
HbA_1c (%)	8.41±2.22
ACR (mg/g)	101.17±232.54
Spot urine creatinine (mg/dl)	67.64±32.48
Spot urine albumin (mg/l)	60.62±108.51
Blood urea (mg/dl)	25.06±7.91
Serum creatinine (mg/dl)	0.71±0.17
Uric acid (mg/dl)	5.05±1.15
e-GFR (ml/min)	113.47±27.17
Cholesterol (mg/dl)	163.45±42.63
HDL (mg/dl)	44.44±11.56
LDL (mg/dl)	84.41±30.87
VLDL (mg/dl)	34.60±19.28
TG (mg/dl)	171.56±94.86
Serum albumin (g/dl)	4.51±0.32

Contd...

DM, diabetes mellitus; OHA, oral hypoglycaemic agent; FBS, fasting blood sugar; PPBS, post-prandial blood sugar; ACR, albumin-to-creatinine ratio; eGFR, estimated glomerular filtration rate; TG, triglyceride; LDL, low-density lipoprotein; HDL, high-density lipoprotein; VLDL, very-low-density lipoprotein

Compared to controls, both AGT and IL-18 were significantly increased in hyperfiltration stage but not NGAL or cystatin. ACR was in higher range than controls but did not significantly progress to the next stage similar to an earlier study where elevated GFR occurred without worsening of albuminuria[18]. At single time point, our study showed a significant difference of AGT and IL-18 in hyperfiltration, and microalbuminuria groups, but not cystatin and NGAL. Contrary to our observations, one-year follow up study showed that there was an increased tendency of urine NGAL, from normoalbuminuria group to macroalbuminuria group[19]. Some studies[20,21] showed an increased level of NGAL in diabetic normoalbuminuric patients than healthy controls, which was in contrast to our study where NGAL levels were not significantly different from non-nephropathy diabetic controls. Patients with T2DM with high levels of baseline urine tubular biomarkers (cystatin-C, AGT, KIM-1 and NGAL) had a greater incidence of end-stage renal disease and rapid GFR decline[22]. We observed that NGAL had a poor ROC of 0.51 with 65 per cent sensitivity and 35 per cent specificity in the present study. Sueud *et al*[12] similarly reported poor AUC of 0.54 with low specificity of 30 per cent for NGAL in their patients with DN. They concluded that urinary ACR was a better predictor of renal damage than NGAL. The diagnostic accuracy of ACR was better than individual biomarker of DN when compared to both NGAL and IL-18[12]. Contrary to our findings, Assal *et al*[23] reported an AUC of 0.75 for NGAL for diabetic patients with early renal disease; their control group included normal patients unlike our study where T2DM patients without nephropathy were included as controls. They indicated that urinary N-acetyl-beta-D-glucosaminidase (NAG) was the most sensitive marker for early renal damage in diabetic patients. However, for damage progress, serum cystatin-C was the most sensitive

Table II. Comparison of clinical and biochemical parameters between control and patients groups

Parameter	Control (n=63)	Case (groups) (n=120)			P^{**}	P^{***}
		Hyperfiltration, n=29 (24.2%)	Normoalbuminuria, n=43 (35.9%)	Microalbuminuria, n=48 (40%)		
Age (yr)	49.62±10.26	47.38±9.83	49.58±9.68	50.50±8.83	0.593	
BMI	27.65±15.32	26.54±4.66	25.06±3.87	25.75±4.23	0.550	
MBP (mmHg)	90.42±8.36	88.96±6.94	90.16±10.98	92.22±6.45	0.392	
FBS (mg/dl)	131.25±36.33	139.03±47.68	146.21±59.50	168.96±91.82	0.017[c]	0.011[c]
PPBS (mg/dl)	183.32±51.61	225.28±96.92	210.77±84.33	240±103.45	0.004[c]	0.003[c]
HbA_{1c} (%)	7.69±1.64	8.17±2.33	7.70±1.66	9.18±2.38	0.001[c, f]	0.001[c], 0.003[f]
Serum creatinine (mg/dl)	0.71±0.11	0.58±0.06	0.75±0.12	0.75±0.21	0.001[a, d, e]	0.001[a], 0.001[d], 0.001[e]
e-GFR (ml/min)	102.67±13.71	140.76±15.21	101.07±11.99	108.08±31.18	0.001[a, d, e]	0.001[a], 0.001[d], 0.001[e]
Uric acid (mg/dl)	5.35±1.31	5.08±1.03	5.12±1.04	4.96±1.31	0.394	
Serum albumin (g/dl)	4.53±0.36	4.58±0.29	4.50±0.30	4.46±0.35	0.453	
Cholesterol (mg/dl)	168.19±37.63	162.93±41.33	160.88±29.16	166.06±53.01	0.822	
TG (mg/dl)	165.09±108.13	165.17±84.05	164.93±87.36	181.35±107.64	0.815	
ACR (mg/g)[*]	9.90 (27.40)	11.60 (22.80)	10.30 (88.30)	115.50 (1743.30)	0.001[c, e, f]	0.001[c], 0.001[e], 0.001[f]
Spot urine albumin (mg/l)[*]	7.60 (84.8)	7.40 (21.3)	5.50 (43.1)	85.40 (454.6)	0.001[c, e, f]	0.001[c], 0.001[e], 0.001[f]
Spot urine Cr. (mg/dl)[*]	84.80 (198.30)	62.20 (128)	63.90 (135.60)	70.45 (112.70)	0.133	
Cystatin-C (ug/ml)[*]	0.06 (0.682)	0.06 (0.21)	0.07 (0.68)	0.09 (0.60)	0.096	
IL-18 (pg/ml)[*]	579.50 (1284.18)	702.08 (621.60)	701.45 (582.80)	705.41 (656.50)	0.012[a, c]	0.033[a], 0.005[c]
NGAL (ng/ml)[*]	10.10 (206.70)	7.15 (71.94)	7.78 (152.96)	7.37 (68.56)	0.801	
Angiotensinogen (ng/ml)[*]	7.32 (119.98)	58.72 (78.85)	60.75 (94.55)	60.45 (89.20)	0.001[a, b, c]	0.001[a], 0.001[b], 0.001[c]
Cystatin-C/Ur. creat. (mg/g)[*]	8.39 (78.09)	12.11 (78.39)	11.74 (340.63)	14.48 (211.82)	0.010[c]	0.002[c]
IL-18/Ur. creat. (mg/g)[*]	7.38 (55.16)	13.47 (69.94)	9.24 (35.69)	11.59 (47.61)	0.008[a, c]	0.021[a], 0.002[c]
NGAL/Ur. creat.(mg/g)[*]	1.54 (11.90)	1.44 (19.75)	1.62 (19.85)	1.74 (18.72)	0.796	
Angiotensinogen/Ur. creat. (mg/g)[*]	1.26 (20.51)	8.43 (88.77)	9.15 (33.39)	10.24 (42.68)	0.001[a, b, c]	0.001[a], 0.001[b], 0.001[c]

Statistical analysis of control and all patients groups was done by one-way ANOVA for continuous parametric data followed by *post hoc* and [*]Kruskal-Wallis test for non-parametric ordinal level data, [**]*P* values between control and all patients groups. Mann-Whitney U test was done to estimate *P* values of non-parametric data between two groups, [***]*P* values between control and patient groups: [a]Control *vs*. hyperfiltration, [b]Control *vs*. normoalbuminuria, [c]Control *vs*. microalbuminuria, [d]Hyperfiltration *vs*. normoalbuminuria, [e]Hyperfiltration *vs*. microalbuminuria, [f]Normoalbuminuria *vs*. microalbuminuria. Control: ACR <30 and eGFR: 60-120, patient groups: (*i*) Hyperfiltration: ACR <30 and eGFR ≥120, (*ii*) Normoalbuminuria: ACR <30 and e-GFR 60-120, (*iii*) Microalbuminuria: ACR ≥30 and eGFR 60-120. BMI, body mass index; MBP, mean blood pressure; FBS, fasting blood sugar; PPBS, post-prandial blood sugar; eGFR, estimated glomerular filtration rate; TG, triglyceride; ACR, albumin-to-creatinine ratio; NGAL, neutrophil gelatinase-associated lipocalin; IL-18, interleukin 18; Ur. Creat., urine creatinine; Cr., creatinine; alb, albumin

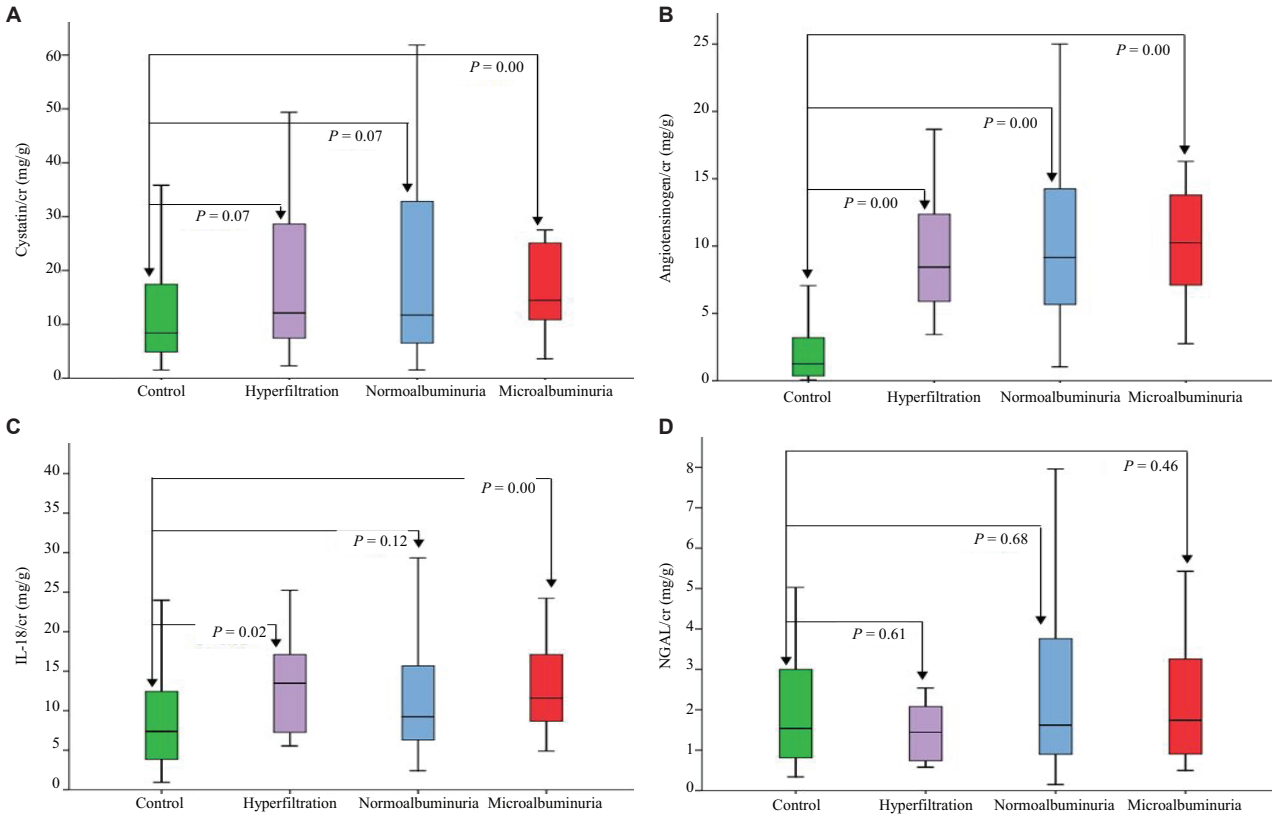

Fig. 2. (**A-D**) Box plots showing median values, range and significance of various biomarkers such as urine cystatin-C/urine creatinine, angiotensinogen/urine creatinine, interleukin-18/urine creatinine and neutrophil gelatinase-associated lipocalin/urine creatinine levels between controls (ACR <30 and eGFR <120) and patient groups: (*i*) Hyperfiltration: ACR <30 and eGFR ≥120, (*ii*) normoalbuminuria: ACR<30 and eGFR <120, and (*iii*) microalbuminuria: ACR ≥30 and eGFR >120. ACR, albumin-to-creatinine ratio; eGFR, estimated glomerular filtration rate; NGAL, neutrophil gelatinase-associated lipocalin; IL-18, interleukin 18.

Table III. Performance of different biomarkers/urine creatinine in diagnosing diabetic nephropathy at 12th month in ROC analysis

Biomarkers	Different cut-off value	Sensitivity (%)	Specificity (%)	*P*	AUC	Range (95 per cent CI)	
						Lower	Upper
Angiotensinogen/Ur. Creat. (mg/g)	4.4	90.4	86	0.00	0.91	0.85	0.96
Cystatin/Ur. Creat. (mg/g)	9.35	70	56	0.00	0.64	0.55	0.73
IL-18/Ur. Creat. (mg/g)	7.8	75	56	0.00	0.65	0.56	0.75
NGAL/Ur. Creat. (mg/g)	1.19	65	34.3	0.71	0.51	0.42	0.61
ACR (mg/g)	29.95	40	100	0.00	0.69	0.62	0.76
e-GFR (ml/min)	119.5	37	100	0.02	0.60	0.52	0.68
Cystatin-C (ug/ml)	0.06	67	51	0.05	0.59	0.49	0.68
IL-18 (pg/ml)	625.50	76	60	0.00	0.65	0.55	0.75
NGAL (ng/ml)	6.76	65	40	0.47	0.46	0.37	0.56
Angiotensinogen (ng/ml)	34.19	92	88	0.00	0.92	0.87	0.98

AUC, area under the curve; Ur. creat, urine creatinine; NGAL, neutrophil gelatinase-associated lipocalin; IL-18, interleukin 18; CI, confidence interval; ACR, albumin-to-creatinine ratio; eGFR, estimated glomerular filtration rate; ROC, receiver operating characteristic

and specific marker for follow up and monitoring renal dysfunction[23]. Cystatin had an ROC of 0.64 with 70 per cent sensitivity and 56 per cent specificity. These results were different from our previous study, where

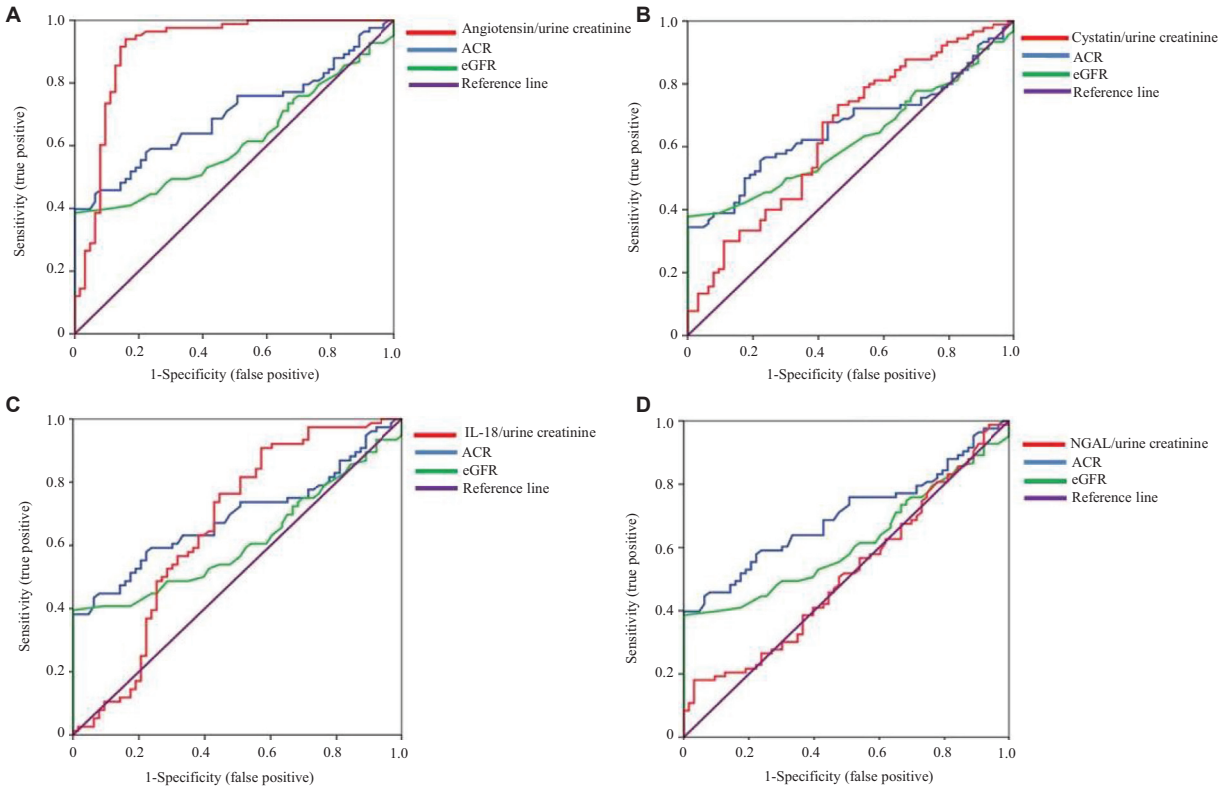

Fig. 3. (**A-D**) Receiver operating characteristic (ROC) curves of all four biomarkers/urine creatinine considering control reference (albumin-to-creatinine ratio <30 and estimated glomerular filtration rate <120) to determine the discriminatory power of biomarkers for the diagnosis of diabetic with nephropathy. (**A**) Angiotensinogen/urine creatinine, (**B**) cystatin-C/urine creatinine, (**C**) IL-18/urine creatinine and (**D**) NGAL/urine creatinine. ACR, albumin-to-creatinine ratio; eGFR, estimated glomerular filtration rate; NGAL, neutrophil gelatinase-associated lipocalin; IL-18, interleukin 18.

Table IV. Univariate and multivariate logistic regression analysis outcome

Parameters	Univariate				Multivariate			
	Odds ratio	*P*	95 per cent CI for Exp (B)		Odds ratio	*P*	95 per cent CI for Exp (B)	
			Lower	Upper			Lower	Upper
Age	0.998	0.893	0.967	1.030	0.973	0.341	0.919	1.030
BMI	0.977	0.286	0.936	1.020	0.998	0.947	0.947	1.053
MBP (mmHg)	1.004	0.834	0.968	1.041	1.019	0.675	0.934	1.111
HbA_{1c} (%)	1.210	0.027	1.021	1.433	1.039	0.833	0.727	1.485
Cholesterol (mg/dl)	0.997	0.457	0.990	1.005	0.990	0.161	0.976	1.004
Log cystatin-C/Ur. creat. (mg/g)	1.777	0.002	1.236	2.557	0.512	0.086	0.239	1.099
Log IL-18/Ur. creat. (mg/g)	2.063	0.003	1.279	3.327	1.628	0.281	0.671	3.948
Log NGAL/Ur. creat. (mg/g)	1.148	0.424	0.818	1.610	0.982	0.954	0.540	1.788
Log angiotensinogen/Ur. creatinine (mg/g)	7.770	0.000	3.844	15.706	7.502	0.000	3.238	17.381

BMI, body mass index; MBP, mean blood pressure; Ur. creat., urine creatinine; NGAL, neutrophil gelatinase-associated lipocalin; IL-18, interleukin 18; CI, confidence interval

both cystatin and NGAL had a higher AUC of 0.86 and 0.95, respectively[8]. Further, controls in our previous study had a lower BMI (23 kg/m^2 previous study *vs.* 27 kg/m^2 in the present study) and cases had a higher ACR (more marked albuminuria) compared to cases in the present study (112.1+68 mg/g *vs.* 20 mg/g) in

the present study. Our previous study[8] showed a higher AUC of urinary NGAL/Cr than urinary cystatin/Cr for estimating microalbuminuria. Assal *et al*[23] reported a AUC of 0.72 for cystatin; however, their control group was normal individuals unlike our controls. Sueud *et al*[12] had showed that diagnostic accuracy of ACR was better than individual biomarker of DN when compared to both NGAL and IL-18.

Satirapoj *et al*[11] observed that the AUC of urinary AGT-Cr were 0.85 and 0.96 in their patients with T2DM with microalbuminuria and macroalbuminuria, respectively. They reported a high sensitivity of 80-90 per cent, but a lower specificity of 75-80 per cent for the diagnosis of microalbuminuria and macroalbuminuria, respectively. It was observed that AGT with urine albumin creatinine (ACR) ratio was the significant biomarker to identify DN at early stage. However, unlike our study, they included patients on renin angiotensinogen system (RAS) blockers which could have confounded their results[11]. Ba Aqeel *et al*[24] observed that AGT/Cr had a high AUC of 0.92 and AUC of ACR of 0.94 in T2DM patients with CKD. The better discriminatory value of ACR in comparison to AGT/Cr ratio could be because of definition of their cases, which was CKD stage 3 or higher. Sueud *et al*[12] observed that urinary levels of IL-18 predicted the presence of nephropathy with a 72 per cent sensitivity and 53.33 per cent specificity with AUC of 0.59. Non-haemodynamic effects of angiotensin II may contribute to the development of tubulointerstitial fibrosis, which may be the reason for early-stage higher AGT and its higher AUC. Non-haemodynamic effects of angiotensin II appear to contribute to the development of tubulointerstitial fibrosis, which may be the reason for early-stage higher AGT and its higher AUC as seen in our cases[25,26].

We have observed a significant correlation of urinary albumin with IL-18–Cr ratio and AGT-Cr ratio. Other researchers have also observed a significant correlation of AGT and albuminuria[11,27]. However, there was no significant correlation of eGFR or ACR with any of the three biomarkers, except NGAL which correlated with eGFR. Sueud *et al*[12] observed no correlation between NGAL and IL-18 with ACR, whereas Vijay *et al*[28] showed a positive correlation of urine NGAL and cystatin-C levels with urine ACR. A meta-analysis of 28 studies observed a significant negative correlation of eGFR with urinary NGAL (r=−0.34)[29] as found in our study.

There was a significant association of HbA_{1c}, log AGT, IL-18 and cystatin with CKD as the dependent variable in our study. In multivariate regression analysis, AGT was the only parameter associated with the presence of CKD. Some previous studies on type 1 as well as type 2 diabetes observed that increase in AGT preceded the albumin excretion, suggesting that urinary AGT may function as an early marker of DN[6,11]. In contract to our study, a review including 42 studies showed high levels and association of NGAL and cystatin-C with early DN compared with non-diabetic controls[30]. The use of diabetic controls might be the reason to show high AGT and IL-18 but not NGAL and cystatin in the present study.

The present findings are preliminary in nature and cannot be translated into the routine diagnostic situations. Larger studies with a longer follow up of both cases and diabetes controls (at least 1:2 ratios) will be required for validation of the present study.

Among all four biomarkers, AGT/Cr ratio showed a greater diagnostic value in terms of sensitivity and specificity than ACR for diagnosing early diabetic nephropathy (EDN). Urinary AGT and IL-18 may be used as biomarker to diagnose EDN at pre-albuminuric stage. These biomarkers may also have the potential to identify patients at high risk of progression in non-proteinuric DN which would have been missed by conventional ACR.

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