



ORIGINAL ARTICLE

Serum and Urine Neutrophil Gelatinase- Associated Lipocalin: as an Indicator for Early Diabetic Nephropathy in Type 2 Diabetes Mellitus Patients

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ABSTRACT

Background: Diabetic Kidney Disease (DKD) represents the major cause Chronic Kidney Disease (CKD) where 5- 40% of patients with Type 2 Diabetes Mellitus (T2DM) ultimately develop DKD. CKD diagnosis is reliable on levels of blood urea nitrogen and serum creatinine, however, serum creatinine has a low predictive value in early detection of renal impairment. So, new biomarkers for early diagnosis of CKD is of interest. Our objective is to elucidate the significance of Neutrophil Gelatinase- Associated Lipocalin (NGAL) as an early predictor of diabetic nephropathy in comparison with albuminuria in (T2DM). Methods: Prospective, case control study, carried out on patients with T2DM with a duration of disease ranged from (5-15) years, presented to outpatient clinics of nephrology and endocrinology units of internal medicine department in Zagazig University hospitals from August 2018 to February 2019. The study included (100) participants divided into: group (A) 25 healthy control, group (B) 75 diabetic type 2 patients stratified into three subgroups according to albumin / creatinine ratio: subgroup (1): 25 normoalbuminuric, subgroup (2): 25 microalbuminuric, subgroup (3): 25 macroalbuminuric. Results: There was statistically significant difference between healthy individuals, normo, micro and macroalbuminuria diabetic patients regarding serum NGAL (sNGAL) & urinary NGAL (uNGAL), where their levels incremented parallel to the degree of albuminuria. Conclusion: tubular injury may precede glomerular injury in diabetic patients and As NGAL is a tubular marker, So NGAL is superior to albumin / Creatinine ratio (ACR) as an early predictor of DKD among T2DM patients.

Key words: Chronic kidney disease; Body mass index; Acute kidney injury.

INTRODUCTION

Diabetic nephropathy (DN) is a major microvascular complication of diabetes mellitus accounting 20%- 40% of population requiring renal replacement therapy [1]. Whether tubular, epithelial and interstitial injury in diabetic patient is primary, that precedes glomerular endothelial injury or secondary to albuminuria, is a subject of debate [2]. Chronological age of appearance of glomerular basement membrane and tubular basement membrane thickening on histopathology of type 1 diabetes suggests

that tubular injury is independent of glomerular injury as both appears after similar duration of disease, As with other renal diseases , outcome of diabetic kidney disease (DKD) is better determined by tubulointerstitial changes than glomerular changes [3]. Classically, albuminuria which is considered as a hallmark of diabetic nephropathy is a marker of glomerular injury (glomerular phase). However, many other researchers suggested that tubular injury can occur even at stage that precedes microalbuminuria (tubular phase) [4]. Chronic

hyperglycemia leads to increased production of oxidative free radicals, advanced glycosylated end products (AGEs), vasoactive amines (Angiotensin II), and cytokines (transforming growth factor B), which result in functional followed by structural damage to tubulointerstitium [5]. NGAL is a 25 KDa (KiloDalton) glycoprotein with 178 amino acid belonging to lipocalin superfamily [6]. NGAL is involved in antimicrobial defense mechanism and upregulated in systemic bacterial infection [7]. NGAL plays a protective role in epithelial injury by its anti-apoptosis effect [8]. NGAL is supposed to be a marker of active injury, as it is not produced by burnt out nephron and represent mass of salvageable nephrons [9].

METHODS

This study will be conducted in Zagazig university internal medicine department hospital. The study included one hundred (100) participants Group (A): **Twenty five (25)** age matched non-diabetic healthy subjects (selected as volunteers) referred as healthy **control group** which includes 10 (40%) males and 15 (60%) female; their ages range from 42-65 years with mean age of 52.44 ± 6.23 years. Group (B): Seventy five (75) diabetic type 2 patients stratified into three groups according to albumin / creatinine ratio: Subgroup 1: It includes 25 type 2 diabetic patient with albumin / creatinine ratio ($<30\text{mg/g}$ creatinine) called normo-albuminuric , 10 (40%) males , 15(60%) females and their ages range from 39-65 years with mean age of 54.08 ± 6.9 years. Subgroup 2: It includes 25 diabetic type 2 patient with albumin / creatinine ratio (30-299 mg /g creatinine) called micro-albuminuric (moderately increased albuminuria), 15 (60%) males, 10 (40%) females and their ages range from 39-62 years with mean age of 52 ± 7.26 years. Subgroup 3: It includes 25 diabetic types 2 patients with albumin / creatinine ratio ($\geq 300\text{mg} / \text{g}$ creatinine) called macro-albuminuric (severely increased albuminuria), 12 (48%) males, 13 (52%) females and their ages range from 50 – 63 years with mean of 56.28 ± 3.81 years.

Inclusion criteria: Age group between

(30-65) years old of both sex males and females, Diagnosed with type 2 DM with a period of disease (5-15 years), Estimated GFR (e GFR) by (CKD - epidemiology collaboration Based on serum creatinine, Age, Race, Gender) $> 60\text{ml/min}/1.73\text{m}^2$, Serum creatinine $< 1.2 \text{ mg/dl}$, with stable renal function for at least 1 year, (Variation $< 0.3 \text{ mg} \%$ from Base line serum creatinine), Patients on diabetic diet without protein restrict and had stable blood pressure and glycemic control for at least 30 days. Exclusion criteria: Patients with history of using Angiotensin Receptor Blocker (ARBS) or Angiotensin Converting Enzyme Inhibitor (ACEI), Patients with infection and inflammatory disorders, Patients with uncontrolled hypertension, Patients with history of using Non-Steroidal Anti Inflammatory Drugs (NSAIDs), nephrotoxic drugs, immunosuppressive agents, Patients with coronary artery disease, stroke, peripheral vascular disease, malignancy, thyroid disorder and liver dysfunction, Pregnant patients or History of non-diabetic kidney disease.

Detailed past and present medical history taking include Name, age, sex, special habits, marital status, residence, Co-morbidity as (History of diabetes mellitus and duration of disease /history of HTN (hypertension) and antihypertensive drugs). History of medications e.g. antihypertensive drugs, antihyperlipidemic drugs. Full general and local examination including: Pulse examination, Blood pressure measurement by mercury sphygmomanometer with patient recumbent in bed, Weight in (kg) and Height by (Cm), Body mass index (Quetelet's index (Kg/m^2), Funds examination by ophthalmoscope at ophthalmology outpatient clinic of Zagazig University Hospitals. Routine investigation in the form of: Lipid profile (total cholesterol, triglycerides, LDL (low density lipoprotein), HDL (high density lipoprotein)) (mg/dl), Serum albumin (g/dl), Urinary albumin/creatinine ratio (mg albumin/g creatinine), Serum creatinine (mg/dl) and urine creatinine (mg/ml), Glycated Hemoglobin A1C (HbA1c)%, Fasting blood sugar (FBs) (mg/dl), Blood

urea (mg/dl), urine analysis (midstream urine sample at morning), serum uric acid (mg/dl), Complete blood count (CBC), Electro cardio gram (ECG), Special investigation in the form of: Serum and urine human neutrophil gelatinase-associated lipocalin (NGAL) ELISA kit purchased from SunRed Biotechnology Company.

Written informed consent was obtained from all participants and the study was approved by the research ethical committee of Faculty of Medicine, Zagazig University. The work has been carried out in accordance with the code of ethics of the world medical association (Declaration of Helsinki) for studies involving humans.

Statistical analysis

Data analysis was performed using the software SPSS (Statistical Package for the Social Sciences) version 20 for windows (SPSSINC, Chicago, IL, USA) and medcalc 13 for windows (medcalc software bvba, Ostend, Belgium). Quantitative variables were described using their means and standard deviations. Categorical variables were described using their absolute frequencies and to compare the proportion of categorical data, chi square test was used when appropriate. Kolmogorov-Smirnov (distribution-type) and Levene (homogeneity of variances) tests were used to verify assumptions for use in parametric tests. To compare means of more than two groups, one way ANOVA test was used when appropriate. Nonparametric test (Kruskal Wallis test) was used to compare medians of more than two groups. Spearman correlation coefficient was used to measure correlation between two continuous variables. To measure strength of association between dependent continuous variables and other independent ones, ROC curve was used to determine best cutoff of the studied parameters in diagnosis of certain health problem. The level statistical significance was set at 5% ($P < 0.05$). Highly significant difference was present if $p \leq 0.001$

RESULTS

Serum and urine NGAL were significantly associated with Urine NGAL/urine creatinine level among the studied patients (unstandardized β 0.0001,

$p < 0.001$) for each of them (table 3).

There was statistically significant difference between healthy individuals, non-albuminuric, micro-albuminuric and macro-albuminuria diabetic patients regarding serum and urine NGAL levels, where their levels incremented parallel to the degree of albuminuria. Also, there was statistically significant difference between control group and the three subgroups of T2DM patients which mean that normo-albuminuric diabetic patients had sNGAL & uNGAL levels higher than controls, so sNGAL & uNGAL are useful markers of onset of DKD even when serum creatinine is in normal range and can be used to predict progression of DKD (table 1, 2).

There is statistically significant difference between the studied groups regarding serum albumin. On LSD comparison, the difference is significant between the group of diabetic patients with macroalbuminuria and each other group ($p < 0.001$) regarding serum albumin. There is statistically significant difference between the studied groups regarding serum creatinine. On pairwise comparison, the difference is significant between diabetic non-albuminuric and diabetic macroalbuminuric groups ($p = 0.028$). There is statistically significant difference between the studied groups regarding albumin/creatinine ratio. On pairwise comparison, the difference is significant between diabetic micro-albuminuric, diabetic macroalbuminuric groups and each other group ($p < 0.001$) and also, the difference is significant between them ($p = 0.014$). There is statistically significant difference between the studied groups regarding serum uric acid. On pairwise comparison, the difference is significant between control and diabetic macroalbuminuric groups ($p = 0.005$). There is statistically significant difference between the studied groups regarding urine creatinine. On pairwise comparison, the difference is significant between diabetic macroalbuminuric group and both control and diabetic non-albuminuric groups ($p < 0.001$). Similarly, the difference is significant between diabetic microalbuminuric group and

both control and diabetic non-albuminuric groups ($p < 0.001$). There is significant difference between the studied groups regarding eGFR. On pairwise comparison, the difference is significant between diabetic macroalbuminuric and diabetic non-albuminuric groups ($p = 0.033$) (table 1).

Cutoff values of normal NGAL: sNGAL (142 ng/ml) and uNGAL (78ng/ml). sNGAL with a cutoff value of ≥ 197.55 (ng/dl) can diagnose early non- albuminuric diabetic

nephropathy with sensitivity of 100%, and specificity of 88%, positive predictive value of 89.3%, and negative predictive value of 100%. uNGAL with a cutoff value of ≥ 681.095 (ng/dl) can diagnose early non-albuminuric diabetic nephropathy with sensitivity of 100%, and specificity of 92 %, positive predictive value of 92.6%, and negative predictive value of 100%.

Table 1. Comparison between the studied groups regarding their serum albumin and kidney function test:

	Healthy control group		Type 2 diabetic patients						F	p
			With no albuminuria		With microalbuminuria		With macroalbuminuria			
	Mean \pm SD		Mean \pm SD		Mean \pm SD		Mean \pm SD			
Serum albumin (g/dl)	4.4 \pm 0.35 ∞		4.24 \pm 0.23		4.17 \pm 0.16		3.4 \pm 0.3 ∞ (a), (b),(C)		68.4	<0.001**
	Mean \pm SD	Median	Mean \pm SD	Median	Mean \pm SD	Median	Mean \pm SD	Median	KW	P
Serum creatinine (mg/dl)	0.65 \pm 0.32	0.6	0.56 \pm 0.27	0.51	0.75 \pm 0.2	0.8	0.78 \pm 0.23	0.83	9.32	0.025*
Albumin/creatinine ratio (mg/dl)	9.84 \pm 7.39	8	12.22 \pm 8.	10	151.8 \pm 49.6	145	566.8 \pm 165.49	501	83.82	<0.001**
Serum uric acid(mg/dl)	3.62 \pm 1.06	3.5	4.04 \pm 0.72	4	4.01 \pm 1.18	4.2	4.93 \pm 1.58	5	11.82	0.008*
Blood urea (mg/dl)	16.36 \pm 6.28	16	31.52 \pm 6.35	30	30.62 \pm 10.19	33	33.48 \pm 7.43	35	42.72	<0.001**
eGFR	110.32 \pm 36.97	103	115.1 \pm 30.6	117	101.6 \pm 21.33	98	92.56 \pm 21.94	95	8.08	0.044*
Urine creatinine (mg/dl)	10628 \pm 3052.1	9600	12264 \pm 4346	9500	7248 \pm 820.6	7200	6100 \pm 1562.1	6000	65.46	<0.001**

Table 2. Comparison between the studied groups regarding their NGAL levels:

NGAL	Healthy control group		Type 2 diabetic patients						KW	P
			With no albuminuria		With microalbuminuria		With macroalbuminuria			
	Mean \pm SD	Median	Mean \pm SD	Median	Mean \pm SD	Median	Mean \pm SD	Median		
Urine NGAL (ng/dl)	421.4 \pm 218.5	444.14	836.3 \pm 45.5	851.4	942.1 \pm 173.3	981.92	1173.6 \pm 84	1146.8	81.11	<0.001**
Serum NGAL (ng/dl)	118.8 \pm 62.25	125.21	301.4 \pm 59.8	293.01	602.1 \pm 188.8	557.9	1651 \pm 669.7	1316.5	92.82	<0.001**
uNGAL/uCr (ng/mg)	0.04 \pm 0.034	0.035	0.09 \pm 0.09	0.084	0.13 \pm 0.02	0.13	0.21 \pm 0.8	0.19	77.83	<0.001**

Table 3. Linear stepwise regression analysis of the factors independently associated with serum NGAL in the studied patients:

	Unstandardized Coefficients		Standardized Coefficients	t	p
	β	Standard error	β		
Serum NGAL	0.0001	0	0.548	6.661	<0.001**
Urine NGAL	0.0001	0	0.331	4.015	<0.001**

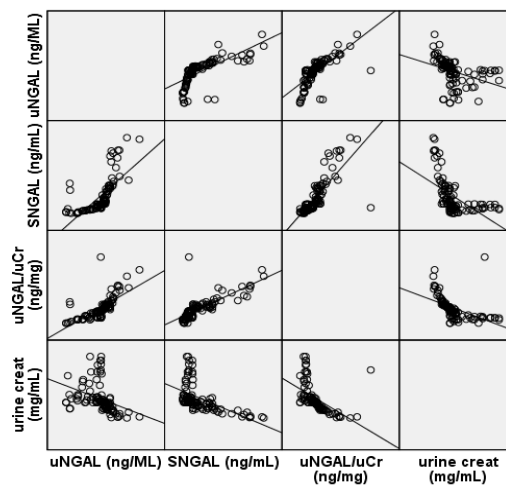


Figure 1. Matrix scatter graph showing negative correlation between each of the three markers and urine creatinine:

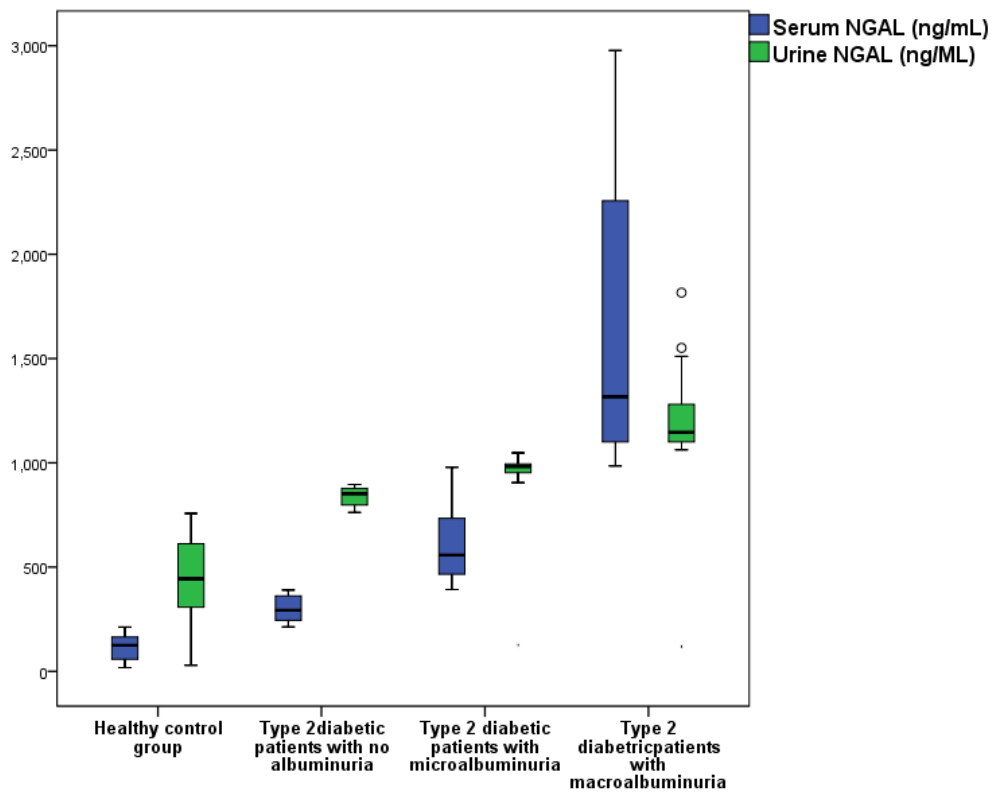


Figure 2. Boxplot showing serum and urine NGAL among the studied groups:

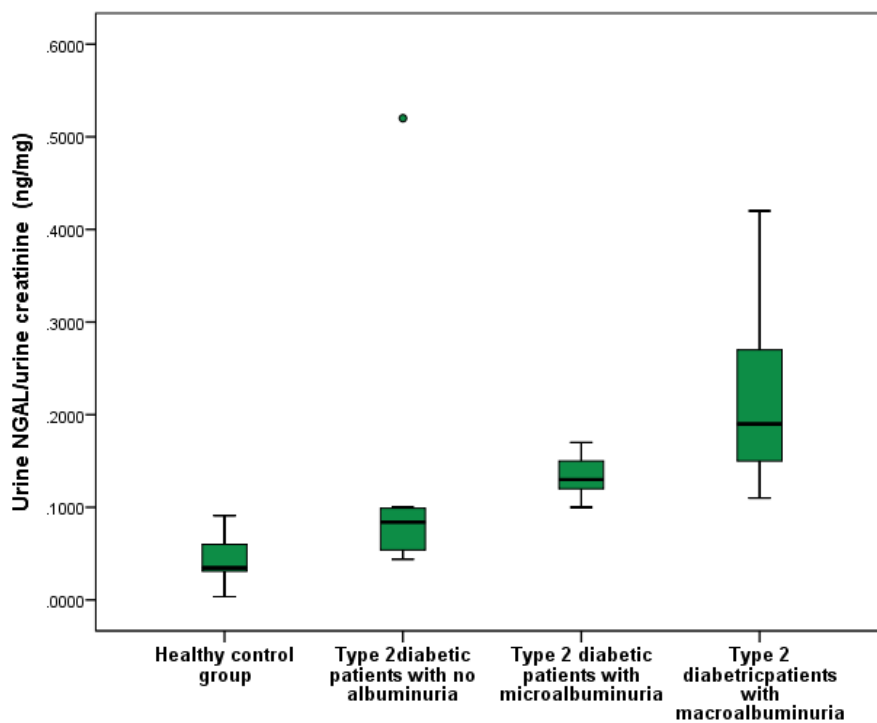


Figure 3. Boxplot showing urine NGAL/ urine creatinine among the studied groups:

DISCUSSION

Diabetic kidney disease (DKD) usually occurs in patients with T2DM without long-term adequate glycemic control [10]. DKD is defined according to the changes in renal structure and function. Major renal structural alterations in DKD include mesangial expansion, glomerular and tubular basement membrane thickening, and glomerular sclerosis. DKD usually manifests a clinical syndrome including persistent albuminuria, incremented blood pressure, sustained decline in glomerular filtration rate, increased cardiovascular events and cardiovascular event-associated mortality [11]. In CKD clinical diagnosis is dependent on levels of blood urea nitrogen and serum creatinine. Serum creatinine, however, has shortcomings because of its low predictive value. Therefore, next generation that is candidate biomarkers are being identified for early diagnosis of CKD. Biomarkers are proteins and act as a signaling molecule in the diseased state. So, attention must be focused on intracellular signaling biomarkers associated with the onset of kidney impairment [12]. NGAL, first purified and identified in 1993 by Kjeldsen, is a 25 kDa protein that belongs to the lipocalin protein family [13].

In our study, we elucidate the significance of NGAL as an early predictor of DKD in comparison with albuminuria in T2DM patients and discover NGAL's diagnostic role in non albuminuric early DKD. Our prospective, case control study included one hundred participant divided into: 25 non diabetic healthy subjects as control group and 75 diabetic type 2. Our results revealed marked decline in HDL cholesterol in DKD patients when compared to controls because DKD patients are suffering from decreased activity of lecithin- cholesterol acyltransferase (LCAT) [14] .which esterifies the accepted free cholesterol to allow more efficient packaging of the cholesterol for transport [15] . In our results, there was significant difference between the diabetic patients with macroalbuminuria, microalbuminuria, normo-albuminuria and control group regarding hemoglobin, MCH (mean corpuscular hemoglobin), MCV (mean

corpuscular volume) and hematocrit level. This attributed to relative erythropoietin deficiency, absolute and functional iron deficiencies, short survival of RBC (red blood cells), and uremic environment that inhibit erythropoiesis among DKD patients [16]. Regarding serum creatinine, uCr and eGFR, our study showed significant difference between diabetic non-albuminuric and diabetic macro-albuminuric patients. This constant with [17] .who stated that the development of microalbuminuria and the progression to overt proteinuria are the most common clinical features of DKD.

Regarding serum and urine NGAL levels, our results revealed that there was statistically significant difference between healthy individuals, non-albuminuric diabetic patients, micro-albuminuric diabetic patients and macro-albuminuria diabetic patients. Also, there was statistically significant difference between each two groups. This in agreement with [18] .whose results showed that normoalbuminuric patients had increased sNGAL and uNGAL levels compared with controls. uNGAL values, also in microalbuminuric patients were significantly increased compared with controls and with normoalbuminuric subjects. Finally, patients affected by overt diabetic nephropathy showed sNGAL and uNGAL levels which were statistically higher compared with all the other groups.

In our study, there was statistically significant difference between controls and both diabetic microalbuminuric and diabetic macroalbuminuric patients regarding uNGAL/uCr ratio. Similarly, there was statistically significant difference between diabetic non-albuminuric patients and both diabetic microalbuminuric and diabetic macroalbuminuric patients regarding uNGAL/uCr ratio [19]. Results were constant with ours where there was a significant difference among the control, normoalbuminuria, microalbuminuria and macroalbuminuria individuals regarding uNGAL/uCr ratio, also while comparison of each 2 groups separately showed that there was significant difference between the control group with each group separately. However,

in contrast to our study results [19] did not show significant difference among the diabetic groups themselves.

There is significant positive correlation between each of sNGAL, uNGAL, uNGAL/uCr ratio and all of LDL, total cholesterol, serum triglycerides, HbA1c and fasting blood sugar. This in agreement with [20] who found that sNGAL was found to be directly correlated with (HbA1c). Also this agrees with [21] who showed that uNGAL positively correlated to the disease duration, urinary albumin, and poor glycemic control (HbA1c). patients stratified into three groups according to albumin / creatinine ratio, Group1 included 25 normoalbuminuric patient with albumin / creatinine ratio (<30mg/g creatinine), Group2 included 25 microalbuminuric patient with albumin / creatinine ratio (30-299 mg /g creatinine) and Group3 included 25 macroalbuminuric patient with albumin / creatinine ratio (≥ 300 mg / g creatinine). Our results showed statistically non-significant difference between healthy individuals and T2DM patients (with or without albuminuria) regarding age or gender, so the different group participants were matched.

In our study, there was significant difference between the diabetic patients with and without albuminuria regarding results of fundus examination. Also, there was statistically significant difference between the studied groups regarding systolic blood pressure and BMI (body mass index). Results of [22] were constant with ours where a significant difference was found as regard systolic blood pressure, diastolic blood pressure, HbA1c, and diabetic retinopathy. In our study, uNGAL/uCr ratio was significantly associated with uNGAL level among the studied patients, followed by HbA1c, then serum albumin, and hematocrit level. Results of [19] stated that linear regression analysis demonstrated that independent predictors of log uNGAL/uCr ratio were HbA1c, uCr and eGFR. It means that uNGAL as a marker of tubular damage is highly associated with uncontrolled diabetes and decline of kidney function detected by decreased uCr and eGFR.

CONCLUSION

Tubular injury may precede glomerular injury in diabetic patients and as NGAL is a tubular marker NGAL is superior to albumin / Creatinine ratio (ACR) as an early predictor of DKD among T2DM patients as there was significant difference between non- albuminuric diabetic patient and non-albuminuric healthy individuals. sNGAL uNGAL and uNGAL/Urinary creatinine (Ucr) ratio can be used to predict and follow up progression of DKD as they correlate with DKD severity. Poor glycemic control has a significant correlation with progression of DKD, proven by presence of significant positive correlation between NGAL and hemoglobin A1c (HbA1c).

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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