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NEUTROPHIL GELATINASE ASSOCIATED LIPOCALIN (NGAL) AS AN EARLY BIOMARKER OF NEPHROPATHY IN TYPE II DIABETIC PATIENTS

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ABSTRACT Diabetic nephropathy is the most important microvascular complication of diabetes. Detection of microalbuminuria plays a crucial role in the diagnosis of diabetic nephropathy. Recent studies reveal that diabetes not only affect glomerulus but also tubules (diabetic tubulopathy). N-GAL is the member of lipocalin protein family. It is produced after 2-4 hours of renal injury, even before the albumin appears in urine. It leads to assumption whether tubular injury precedes glomerular injury in diabetic nephropathy. A case control study, included 100 cases and 50 controls, composed of both genders and age range included was between 18 and 60 years, which divided into three groups by using albumin/creatinine ratio. The serum NGAL(sNGAL), urine NGAL (uNGAL), and uNGAL/urine creatinine were significantly higher in diabetic individuals than in the control populations with significant difference in between the groups ($P < 0.05$). Difference of above values between control value and normoalbuminuria was also statistically significant ($P < 0.05$). Again, sNGAL and uNGAL correlate positively with albuminuria ($P < 0.05$). Tubular injury may precede glomerular injury in diabetic individuals, and NGAL can be used as a biomarker to diagnose DN even earlier to incipient nephropathy. Both sNGAL and uNGAL can predict albuminuria and be used as a noninvasive tool for diagnosis, staging, and progression of DN.

KEYWORDS : Diabetic kidney disease, neutrophil gelatinase−associated lipocalin,type 2 diabetes

INTRODUCTION

Diabetes is a global disease with multiple complications which affect both micro and macrovasculature. Diabetic nephropathy is one of the most serious microvascular com- plications of diabetes. It is characterized by elevated urinary albumin excretion rate, increase in blood pressure, and decline in renal function leading to end-stage renal disease. Diabetic nephropathy (DN) constitutes around 40% of patients with type 2-diabetes Mellitus (T2DM). Its progression to end stage renal disease affecting both mortality and morbidity. Efficient DN treatment includes glycemic management and lowering blood pressure. It is very much important to identify DN within early stages as brief treatment can reduce the medical and economic burden of this sickness and death of T2DM patients.

The pathophysiologic changes in DN linked to renal function decline are associated with cellular and extracellular derangements in both the glomerular and tubular compartments. Several studies have reported that nonalbuminuric subjects with long-standing diabetes, often have glomerular basement membrane (GBM) thickening, mesangial expansion and significant glomerulopathy. Currently microalbuminuria is the most explored biomarker of Diabetic nephropathy but its significance in the early period of Diabetic nephropathy is constrained since renal tubular injury occurs before glomerular proteinuria.

Pathological albuminuria and proteinuria represent the consequence of diffuse diabetes induced glomerular damage. Renal tubular interstitium also seems to play an equally pivotal role in the genesis of diabetic nephropathy due to persistent exposure to a variety of metabolic and hemodynamic injuring factors related with sustained diabetic disease.

Glomerular and renal tubular interstitial injury plays a role in the pathogenesis of DN and various tubular markers have been assessed in the early detection of Diabetic Nephropathy. Recent studies have confirmed the important role of the tubular tract in the pathogenesis and progression of renal damage in diabetic disease, reporting increased levels of several 'tubular factors' in diabetic patients, such as cathepsin B, N-acetyl-D-glucosaminidase and monocyte chemoattractant protein-1 (MCP-1),Retinol-binding protein 4 (RBP4),Beta-trace protein (β-TP) .Furthermore, levels of these substances were found to be well associated with the severity of nephropathy, confirming the recent hypothesis that in diabetic patients the rate of worsening in renal function, and the overall renal long-term outcome, is related better to the degree of renal tubulointerstitial impairment than to the severity of glomerular lesions.

In recent years, neutrophil gelatinase associated lipocalin (NGAL) has emerged in clinical and experimental nephrology as one of the most promising tubular biomarkers in the diagnostic field of acute and chronic renal diseases. Neutrophil gelatinase-associated lipocalin (NGAL), first purified and identified in 1993 by Kjeldsen et al., seems to be a promising biomarker. NGAL is a 178-amino acid 25kDa protein that belongs to the lipocalin protein family. It is primarily produced in renal tubules in response to structural kidney injury. In contrast to conventional markers, such as serum creatinine, blood urea nitrogen, or serum cystatin C (CysC), NGAL is not considered as a marker of renal function, but rather reflects structural damage of renal tubular cells. In previous studies, NGAL was reported as effective in the early diagnosis of acute kidney injury (AKI) in several clinical settings and was also validated as a significant prognostic factor in cardiovascular morbidity. The association between the early tubular lesions in nonalbuminuric patients with T2DM and NGAL was further supported by recently published studies.

OBJECTIVE

The aim of the study is to evaluate serum levels and urinary excretion of NGAL in a small cohort of type 2 diabetic patients with different stages of nephropathy, in order to assess the potential relationships between this tubular biomarker and the severity of renal involvement.

MATERIALS AND METHODS

101 patients with type 2 diabetes mellitus were recruited. The control group consisted of 51 healthy volunteers without a history of arterial hypertension, diabetes and neoplastic, cardio- vascular, inflammatory, renal, lung or endocrine diseases. None of these subjects was under medical treatment.

INCLUSION CRITERIA

Age between 18 and 60 years, estimated GFR (eGFR) (>60 mL/min/1.73 m2), serum creatinine <1.2 mg/dl with stable renal function for at least 1 year (i.e., variation <0.3 mg% from baseline serum creatinine).

EXCLUSION CRITERIA

Patients with a history of intake of renin–angiotensin system inhibitors, patients those with infection, inflammatory disorders, uncontrolled hypertension, history of intake of nonsteroidal anti inflammatory drug (NSAID), nephrotoxic medications, immunosuppressant, history of non-Diabetic Kidney Disease, coronary artery disease (CAD), stroke, Peripheral vascular disease, malignancy, thyroid disorders, liver dysfunction, pregnancy.

The study protocol was approved by the local Ethics Committee and every participant gave a fully informed approval to take part in the study.

Collection of Blood and Urine

Blood samples were taken in the morning before any food intake, and the second micturition of the day was also collected. The blood samples were immediately placed into chilled vacutainer tubes containing potassium ethylenediamine tetraacetate, and the plasma was promptly separated in a refrigerated centrifuge. The samples were then stored at –80°C until assayed. Ten milliliters of fresh urine were mixed with 1 ml of 10 mM Tris buffer, pH 8.6 with 0.05% Tween 20 and 0.01% of NaN3 containing protease inhibitors (10 mM benzamidine, 10 mM aminocaproic acid, 20 mM ethylenediamine tetra acetate and aprotinin). This mixture was centrifuged at 3,000 rpm for 8 min and stored at –80°C until assayed. A first morning urine sample was collected from each subject into a sterile container and used for the determination of microalbumin and urinary ACR. All the urine and blood specimens were used for the study within 3 months after collection. Common biochemical parameters were measured according to standard methods in the routine clinical laboratory.

Randomized plasma glucose concentration was determined by using glucose oxidase method. Lipid profile parameters were measured with commercial kits based on different techniques. Serum urea and creatinine levels were determined using an enzymatic colorimetric method. GFR was estimated using the modification of diet in renal disease (MDRD) abbreviated equation: [GFR = 186 × (serum creatinine)-1.154 × (age)-0.203 × (0.742 if female)].

NGAL ELISA Assay

NGAL was measured in the blood and urine using ELISA commercial available kit. All specimens were often diluted to obtain concentration for the optimal density according to ELISA kit instructions. The enzymatic reactions were quantified in an automatic microplate photometer. All measurements were done in a triplicate and blinded manner. Serum (sNGAL) and urinary NGAL (uNGAL) levels were expressed as ng/ml.

STASTICALANALYSIS

Results were expressed as mean ± SD. Differences between the groups were analyzed by one-way analysis of variance (ANOVA). The correlations between various variables were calculated using the Pearson correlation coefficient. A receiver operating characteristic (ROC) analysis was employed to calculate the area under the curve (AUC) and find the best cutoff values to maximize diagnostic specificity and, secondarily, sensitivity. P-values < 0.05 were accepted as statistically significant. All statistical calculations were done using Statistical Package for the Social Sciences version.

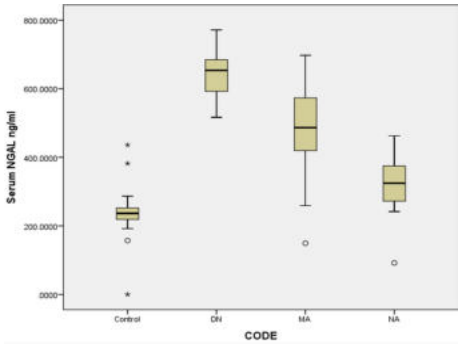
RESULTS

100 Diabetic patients were categorized into three groups, depending on their ACR(mg/g). 23 patients (9 males, 14 females; mean age 53.74 ± 10.04 years) were in the **Normoalbuminuria** group (ratio persistently below 30). In **Microalbuminuria** group (ratio persistently between 30 and 300) included 55 patients (14 males, 41 females; mean age 55.35 ± 9.84 years). The third group included 22 patients (10 males, 12 females; mean age 54 ± 12.93 years) with **Macroalbuminuria or Diabetic Nephropathy** (ratio persistently over 300). The three groups were well-matched with regard to age, sex, R.sugar (mg/Dl),Cholesterol (Mg/Dl), TGL, and HDL. 50 healthy controls (23 males, 27 females; mean age 33.92 ± 14.73 years) were well-matched with diabetic patients with regard to age, sex, random sugar (mg/Dl), Cholesterol (Mg/Dl), TGL, HDL. The main characteristics of patients and controls are detailed in **Table 1** .

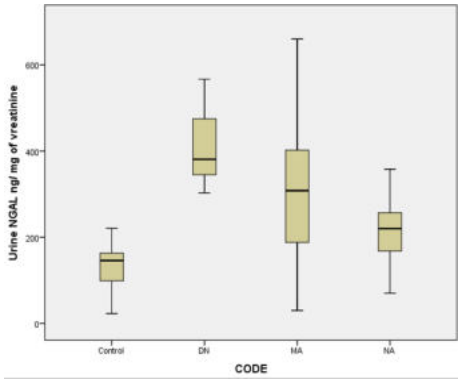
Table 1. Main Parameters of Healthy Subjects and Diabetic Patients

	Control	Normoalbuminuria	Microalbuminuria	Diabetic Nephropathy
Number	50	23	55	22
Age	33.92 ± 14.73	53.74 ± 10.04	55.35 ± 9.84	54 ± 12.93
Sex (Male/Female)	23/27	9/14	14/41	10/12
R.Sugar (Mg/Dl)	106.42 ± 28.21	235.15 ± 65.35	271.69 ± 84.43	306.559 ± 61.13
T.CHO (Mg/Dl)	151.02± 39.02	199.61 ± 32.10	216.81 ± 31.23	232.53 ± 22.86
TGL(Mg/Dl)	177.6±94.13	205.93 ± 26.60	251.57 ± 58.24	264.76 ± 34.79
HDL (Mg/Dl)	35.48±18.89	50± 7.89	44.05 ± 11.43	42.18 ± 8.89
Serum NGAL Ng/Ml	236.4(221-251)	323.2 (291-356)	488.09 (458-518)	646.24(615-677)
Urine NGAL Ng/ Mg of Creatinine	127.9(114-142)	209.30 (172-241)	321.2 (280-363)	401.55 (369–435)
ACR(Mg/G)	134.14 (106-162)	19.51 (17.8-21.2)	201.22 (186-216)	618.49 (598-639)

Healthy subjects showed serum NGAL (sNGAL) levels of 236.4 mg/ml (221-251).The urinary NGAL (uNGAL) levels were 127.9 ng/ml (114–142).Normoalbuminuric patients showed increased sNGAL [323.2 ng/ml (291–356)] and uNGAL levels [209.3 ng/ml (172–241)] compared with controls (P < 0.000 for both comparisons). In **Microalbuminuric** patients also, sNGAL values [488.09 ng/ml (458-518)] were increased compared to controls (P< 0.000). In uNGAL values [321.2ng/ml (280-363)] were significantly increased compared with controls (P< 0.000) and also with normoalbuminuric subjects (P<0.000). Finally, patients affected by over diabetic nephropathy showed sNGAL [646.24 ng/ml (615–677)] and uNGAL levels [101.55 ng/ml (369–435)] which were statistically higher compared with all the other groups. NGAL levels and comparisons between groups are summed up in table 1 and Figure 1 and 2.



In Figure1. sNGAL in control subjects and patient groups with normoalbuminuria (NA), microalbuminuria (MA) and diabetic nephropathy (DN).P< 0.000 for NA, MA, DN vs. controls.



In Figure2. uNGAL in control subjects and patient groups with normoalbuminuria (NA), microalbuminuria (MA) and diabetic nephropathy (DN).P< 0.000 for NA, MA, DN vs. controls.

ROC Analysis of sNGAL and uNGAL

ROC analyses were performed in order to define the diagnostic profile of sNGAL and uNGAL in identifying diabetic patients among all subjects with normal albumin excretion (controls + diabetic normoalbuminuric patients). To this end, sNGAL showed a good diagnostic profile, describing an AUC of 0.889 (CI: 0.791– 0.986) with a best cutoff value of 265.7 ng/ml (sensitivity 82.6%; specificity 94.0%; see table 3; fig. 3). Also uNGAL evidenced a good diagnostic profile, showing an AUC of 0.813 (CI: 0.687–0.939) and a best cutoff value of 177.4 ng/ml (sensitivity 73.9%; specificity 90%; see table 4 ; fig.4).

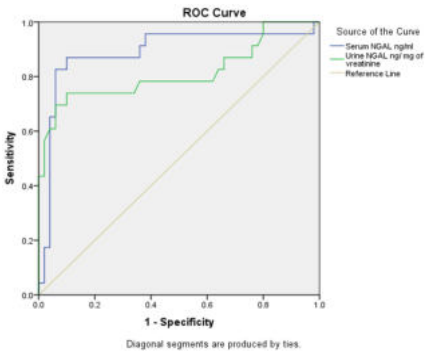
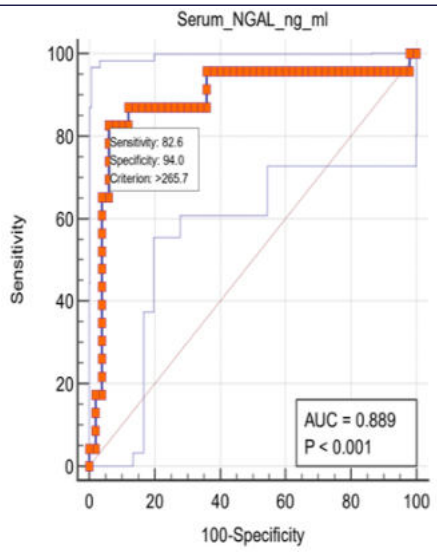


Figure 3



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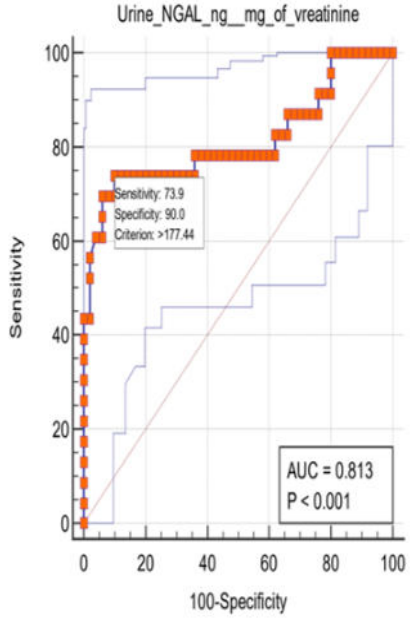

Figure: 5

ROC analysis of sNGAL value

Best cut-off value for the identification of diabetic patients among all subjects with normal albumin excretion (controls and diabetic normoalbuminuric patients): >265.7* ng/ml. Area under the ROC curve = 0.889; standard error = 0.0503; 95% confidence interval = 0.793 - 0.950. +LR = Positive likelihood ratio; –LR = negative likelihood ratio. *Best sNGAL cut-off value.

Table 2. ROC analysis of uNGAL values

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	95% CI	-LR	95% CI
>0.42	100	85.2 - 100.0	2	0.05 - 10.6	1.02	1.0 - 1.1	0	-
>241.73	91.3	72.0 - 98.9	64	49.2 - 77.1	2.54	1.7 - 3.7	0.14	0.04 - 0.5
>265.7*	82.61	61.2 - 95.0	94	83.5 - 98.7	13.77	4.5 - 41.9	0.19	0.08 - 0.5
>435.66	4.35	0.1 - 21.9	100	92.9 - 100.0	-	-	0.96	0.9 - 1.0
>462.5	0	0.0 - 14.8	100	92.9 - 100.0	-	-	1	1.0 - 1.0

ROC analysis of uNGAL values

Best cutoff value for the identification of diabetic patients among all subjects with normal albumin excretion (controls and diabetic normoalbuminuric patients): >177.4 ng/ml. Area under the ROC curve = 0.813; standard error = 0.0655; 95% confidence interval = 0.704 - 0.894. +LR = Positive likelihood ratio; –LR= negative likelihood ratio. * Best uNGAL cutoff values.

DISCUSSION

Most of the patient with the diabetic illness will acquire renal infection. Around half of the people with T2DM and 33% of individuals with that is presently the main source of CKD and ESRD world.

NGAL is a small protein belonging to the superfamily of 'lipocalins'. It is a 25-kDa molecule associated with purified gelatinase obtained from the supernatant of activated neutrophils. In kidney tubules, in particular, NGAL is hyperproduced within a few hours after damaging experimental stimuli such as ischemia-reperfusion and cisplatin administration, suggesting that this protein belongs to that limited panel of stress-induced renal biomarkers involved in the pathophysiology of acute renal damage. NGAL measurement has emerged as a useful diagnostic weapon in the early prediction of acute kidney injury (AKI) after the treatments that are potentially detrimental to kidney such as contrast medium administration, cardiac surgery and even renal transplantation.

Recent studies showed that NGAL also be involved in the pathophysiology of chronic renal diseases, as the biological levels of this protein have been well correlating with the severity of renal impairment, seems expressing the degree of active damage in the chronic renal diseases.

With the present study we aimed at evaluating sNGAL and uNGAL levels in a small cohort of patients affected by type 2 diabetes mellitus, categorized into three groups depending on their different clinical degree of kidney damage (normoalbuminuria, microalbuminuria and diabetic nephropathy).

Results showed that all diabetic patients presented elevated NGAL values compared with a well matched control group. Furthermore, a characteristic trend was observed among the three groups of patients as both sNGAL and uNGAL values increased in parallel with the severity of renal involvement, reaching higher levels in patients with diabetic nephropathy.

These results are in perfect accordance with recent studies which have reported similar tendencies for other biomarkers of tubulointerstitial damage, such as cathepsin B, N-acetyl-D-glucosaminidase and MCP-1, those levels were found to be strictly proportional to the degree of urinary albumin excretion.

In a cohort of 56 patients with T2DM, Jiao et al demonstrated increased levels of NGAL in both serum and urine, which correlated with the severity of renal damage. Being elevated in serum and urine, even before albumin appears in urine, NGAL has been reported as a useful noninvasive tool for the evaluation of renal involvement in diabetes, accelerating the early diagnosis of DN. Nielsen et al reported that elevated urine neutrophil gelatinase-associated lipocalin (uNGAL) in type 1 diabetic patients with or without albuminuria indicates tubular damage at an early stage.NGAL showed a good correlation with GFR in diabetic patients. According to our results, serum NGAL showed positive correlation with albuminuria and negative correlation with GFR. Similar findings were reported by Yang et al.

In this study, serum NGAL was significantly higher in microalbuminuric and macroalbuminuric diabetic groups compared to the control and normoalbuminuric diabetic groups. Also, serum NGAL positively correlated to the ACR and blood glucose levels.In our findings, NGAL was higher in diabetic patients with nephropathy compared to controls, indicating that tubular injury occurs early and perhaps before albumin excretion in patients with DN. In addition, NGAL increased further when the injury progressed to become DN. Nauta et al demonstrated that NGAL is 1.5-fold significantly elevated in diabetic patients with normoalbuminuria compared to nondiabeic control group. Lacquaniti et al. stated that NGAL increases in patients with type 1 diabetes even before diagnosis of microalbuminuria representing an early biomarker of normoalbuminuric DN. NGAL measurement could be useful for the evaluation of early renal involvement in the course of diabetes. Zachwieja et al. showed that serum and urine NGAL were elevated in diabetic children without albuminuria, and normoalbuminuria does not exclude DN that is defined as increased serum and urine NGAL concentration. NGAL measurement can be more sensitive than microalbumin and may become a useful tool for evaluating renal involvement in diabetic children.

In our study, serum NGAL showed a significant positive correlation with the duration of diabetes, HbA1c, and urinary ACR, while it showed a significant negative correlation with GFR. Fu et al. found a

significant inverse correlation between serum NGAL and GFR, and a positive correlation with albuminuria, which is consistent with our findings. Furthermore, Woo et al.70 found an inverse correlation between serum NGAL and GFR.

CONCLUSION

We found that NGAL values are increased even before the appearance of pathological albuminuria, the earlier measurable sign of renal diabetic involvement. This interesting finding supports the growing hypothesis of a 'tubular phase' of diabetic disease that precedes the manifestation of classic glomerular lesions. Increase in NGAL values may express the degree of subclinical tubular impairment, thus representing an earlier measurable index of renal suffering compared with classic glomerular injury indicators ; further studies are needed to assess the diagnostic and therapeutic Value of NGAL for diagnosing diabetic nephropathy earlier.

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