Evaluation of Neutrophil Gelatinase-associated Lipocalin and Cystatin C as Early Markers of Diabetic Nephropathy

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Abstract

Introduction: Diabetes mellitus (DM) is a major cause of concern because of its increasing prevalence rate and related microvascular as well as macrovascular complications, including kidney disease. Microalbuminuria has been accepted as the earliest marker for diabetic nephropathy; however, a large proportion of renal impairment occurs in nonalbuminuric state. We planned to investigate the serum and urinary levels of the tubular damage markers (neutrophil gelatinase-associated lipocalin [NGAL] and cystatin C [Cys C]) in patients of type 2 diabetes to detect early kidney injury. Materials and Methods: This cross-sectional hospital-based study included 180 patients with type 2 DM according to the American Diabetes Association criteria. Serum NGAL (S.NGAL) and urine NGAL (U.NGAL) and Cys C were measured in all study participants and investigated for correlation with microalbuminuria. Results: Our results showed that U.NGAL and S.NGAL levels were significantly high in patients with microalbuminuria as compared to normoalbuminuric controls. Serum Cys C was also higher in microalbuminuric patients than who had normoalbuminuria. A positive correlation of urinary albumin excretion with S.NGAL and U.NGAL was noted. U.NGAL also showed positive correlation with duration of diabetes, glycated hemoglobin, and dyslipidemia. Receiver operating characteristic curve analysis showed that the area under the curve for U.NGAL and S.NGAL were 1 and 0.8, respectively, which indicates that they are sensitive markers for early renal damage. Conclusion: Urinary biomarkers were significantly elevated in normoalbuminuric type 2 diabetic patients compared with nondiabetic controls and could be used as markers of nephropathy at a very early stage even before the development of microalbuminuria, the current gold standard for early diagnosis. Despite the promise of these new biomarkers, further large, multicenter prospective studies are still needed.

Keywords: Albuminuria, diabetic nephropathy, neutrophil gelatinase-associated lipocalin, tubular markers

Résumé

Introduction: Le diabète sucré (DM) est une cause majeure de préoccupation en raison de son taux de prévalence croissant et de complications microvasculaires et macrovasculaires connexes, y compris des maladies rénales. La microalbuminurie a été acceptée comme le premier marqueur de la néphropathie diabétique; Cependant, une grande partie de l’insuffisance rénale se produit dans l’état non albuminurique. Nous avons prévu d’étudier les niveaux sériques et urinaires des marqueurs de dégâts tubulaires (lipocaline associée à la gelatinase neutrophile [NGAL] et cystatine C [Cys C]) chez les patients atteints de diabète de type 2 pour détecter une lésion rénale précoce. Matériaux et méthodes: cette étude transversale à l’hôpital comprenait 180 patients atteints de DM de type 2 selon les critères de l’American Diabetes Association. Le NGAL sérique (S.NGAL) et l’urine NGAL (U.NGAL) et Cys C ont été mesurés chez tous les participants à l’étude et ont été étudiés pour une corrélation avec la microalbuminurie. Résultats: Nos résultats ont montré que les niveaux d’U.NGAL et de S.NGAL étaient significativement élevés chez les patients atteints de microalbuminurie par rapport aux témoins normoalbuminuriques. Le sérum Cys C était également plus élevé chez les patients microalbuminuriques que ceux qui avaient une normoalbuminurie. Une corrélation positive de l’excrétion urinaire d’albumine avec S.NGAL et U.NGAL a été notée. U.NGAL a également montré une corrélation positive avec la durée du diabète, l’hémoglobine glyquée et la dyslipidémie. L’analyse de la courbe caractéristique d’exploitation du récepteur a montré que la zone sous...
INTRODUCTION

Diabetes mellitus (DM) is a major cause of concern because of its increasing prevalence rate globally that has led to consequent increase in the incidence of related microvascular as well as macrovascular complications, including kidney disease.[1]

Diabetes has been found to be the cause of end-stage renal disease (ESRD) in nearly 45% of patients undergoing dialysis.[2-3] In a study from India, 46% of the T2DM patients had chronic kidney disease (CKD), urinary albumin-creatinine ratio (UACR) ≥30 mg/g, and/or estimated glomerular filtration rate (eGFR) <60 mL/min/1.73 m².[4]

Development of CKD complicates the scenario by adding to the already elevated risk of morbidity and mortality with a significant impact on the health-care infrastructure.[5] Declining GFR limits oral antidiabetic agent options available for optimal glycemic control. Cardiovascular events have been found to increase by 19%-40% as the GFR declines from ≥90 mL/min/1.73 m² to <45 mL/min/1.73 m².[6]

Classically, albuminuria is regarded as the consequence of diabetes-induced glomerular damage. The onset of elevated levels of urinary albumin excretion is an early sign of diabetic nephropathy. Various studies have shown that in patients with diabetes, microalbuminuria predicts the occurrence of macroalbuminuria and renal function decline.[7] As a result, high albuminuria has become an established marker of CKD in these patients.[8]

More recently, it is increasingly appreciated that the renal tubulointerstitium plays a role in the pathogenesis of diabetic nephropathy with prolonged exposure to a variety of metabolic and hemodynamic injuring factors that are associated with sustained hyperglycemia as contributing factors.[9]

Markers of tubular damage are discovered and extensively investigated in predicting the occurrence of acute kidney injury after various nephrotoxic insults, such as ischemia during cardiac surgery, sepsis, and administration of contrast agents.[10-11] Little research has been done about their role as early markers in patients with CKD.[12-14]

In this study, we investigate the serum and urinary levels of the tubular damage markers (neutrophil gelatinase-associated lipocalin [NGAL] and cystatin C [Cys C]) in patients with type 2 diabetes to detect early kidney injury and correlate them with albuminuria.

MATERIALS AND METHODS

This is a cross-sectional study conducted at medical college hospital of North India between January and December 2016. We included 180 patients with type 2 DM according to the American Diabetes Association criteria[15] from medical wards and outpatient departments. A written informed consent was obtained from all study participants, and study was approved by the Institutional Ethics Committee.

All patients with hypertension, cancer, infections, inflammatory states, cardiovascular, pulmonary, other endocrinial diseases, and severe renal impairment (eGFR ≤ 30 mL/min), according to the Modification of Diet in Renal Disease equation, were excluded from the study to avoid potential confounding factors.[16] Patients were stratified according to UACR into three groups: normoalbuminuria (<30 mg/g Cr), microalbuminuria (30-300 mg/g Cr), and macroalbuminuria (>300 mg/g Cr).[17]

First morning urine specimens were obtained to measure urine creatinine, albumin, and NGAL. Microalbuminuria was measured by immunonephelometric method on Prospect Siemens (Siemens Healthcare Diagnostic Inc., Newark, USA). Serum Cys C (sCys C) was measured by nephelometry (reagents obtained from Siemens Marburg, Germany). The serum NGAL (S.NGAL) and urine NGAL (U.NGAL) were measured by ELISA (enzyme-linked immunosorbent assay), and antibodies were obtained from R and D Systems, Minneapolis, MN, USA.

Statistical analysis

Statistical analysis was done using the Statistical Package for Social Sciences (SPSS 15.0 version SPSS Inc., Chicago, IL, USA). The qualitative variables were described as frequencies and proportions while quantitative variables as mean and standard deviation. Logarithmtransformed values of urinary albumin and NGAL levels were used for analyses due to their skewed distribution. Student’s t-test was used for comparing the means of continuous variables. Numbers and percentages were compared by Chi-square test.

The associations between kidney injury markers and age, sex, glycemic status, duration of diabetes, and other parameters were examined by Spearman’s correlation coefficients. A coefficient was considered weak if <0.25, mild if between 0.25 and 0.49, moderate if between 0.5 and 0.74, and strong if >0.75. Receiver operating characteristic (ROC) curve analysis was done to identify the optimal cutoff of U.NGAL for detection of microalbuminuria. P < 0.05 was considered significant.
Results

Out of these 180 patients, 90 patients of type 2 diabetes with microalbuminuria were defined as cases and ninety patients who had normoalbuminuria were defined as controls. Out of 90 patients with microalbuminuria, 48 were males and 42 females, while among normoalbuminurics, 50 were males and 40 females. Table 1 is showing the baseline characteristics, clinical, and laboratory data of the study participants in two groups.

Our results showed that U.NGAL and S.NGAL levels were significantly high in cases as compare to controls [Figure 1]. sCys C was also higher in microalbuminuric patients than who had normoalbuminuria as depicted in Table 2.

Correlation analysis [Table 3] revealed positive correlation of urinary albumin excretion with S.NGAL and U.NGAL. U.NGAL showed stronger positive correlation with level of albuminuria than S.NGAL. U.NGAL was significantly higher in microalbuminuric in comparison with normoalbuminuric controls [Figure 2] and correlated positively with urine albumin-creatinine ratio. U.NGAL had a positive correlation with duration of diabetes, glycated hemoglobin (HbA1c), and dyslipidemia.

The ROC curve analysis for detection of microalbuminuria by U.NGAL is illustrated in the Figure 3. ROC curve analysis showed that the area under the curve for U.NGAL and S.NGAL were 1 and 0.8, respectively, which indicates that they are sensitive markers for early renal damage.

Discussion

In the present study, we assessed novel markers of acute kidney injury in patients with diabetic nephropathy which has not been adequately addressed in our patients. Burden of type 2 diabetes is a growing concern and so it is associated chronic complications.[18] Diabetic nephropathy occurs in 20%–40% patients of type 2 diabetes.[19] Although the presence of albuminuria is established marker of nephropathy,[20] renal dysfunction in diabetes does not always preceded or accompanied by albuminuria; several new biomarkers are being investigated in hope to detect nephropathy at the earliest.[21-25]

We included study participants who had normoalbuminuria and microalbuminuria with the aim to observe that the presence of tubulopathy that could have been preceded nephropathy.

Our patients with microalbuminuria had increased duration of diabetes and higher waist circumference than who had normoalbuminuria. They also had higher systolic and diastolic blood pressures with propensity to develop CKD than their normoalbuminuric counterparts in concordance with the natural history of diabetic nephropathy. In the present study, both S.NGAL and U.NGAL and Cys C as markers of tubulopathy were significantly higher in patients with microalbuminuria than normoalbuminuria. The patients who had normoalbuminuria were also having higher levels of these markers than normal controls, which means that these markers seem to increase in parallel with the severity of kidney

Table 1: Clinical and biochemical parameters of study participants

| Parameters            | Controls Patients with normoalbuminuria (n=90) | Cases Patients with microalbuminuria (n=90) | P  |
|-----------------------|-----------------------------------------------|---------------------------------------------|----|
| Age                   | 44.6±5.5                                       | 54.7±8.8                                    | 0.02|
| Male gender (%)       | 53                                             | 56                                          | 0.12|
| BMI (kg/m²)           | 26.16±2.02                                     | 27.33±2.49                                  | 0.05|
| Waist circumference (cm) | 93.88±4.85                                   | 97.04±5.4                                   | 0.04|
| Duration of diabetes (years) | 7.3±3.4                                       | 13.7±6.6                                    | 0.001|
| Systolic blood pressure (mmHg) | 120±6.32                                      | 137.9±12                                    | 0.01|
| Diastolic blood pressure (mmHg) | 80±6                                           | 88.02±10                                    | 0.03|
| HbA1c                 | 7.02±0.5                                       | 8.74±2.16                                   | 0.04|
| LDL cholesterol (mg/dL) | 112.6±11.5                                    | 132±20.97                                   | 0.001|
| HDL cholesterol (mg/dL) | 44±5.6                                        | 36±4.2                                      | 0.01|
| Triglyceride (mg/dL)  | 118.5±10.5                                     | 145.93±20.93                                | 0.03|

Data are expressed in mean±SD. SD=Standard deviation, LDL=Low-density lipoprotein, HDL=High-density lipoprotein, BMI=Body mass index, HbA1c=Glycated hemoglobin

Table 2: Renal injury markers in study participants

| Variable            | Cases Patients with normoalbuminuria (n=90) | Controls Patients with microalbuminuria (n=90) | P  |
|---------------------|----------------------------------------------|------------------------------------------------|----|
| Albuminuria (mg)    | 20.4±10.6                                     | 176.94±48.38                                  | 0.002|
| Urine albumin/creatinine (mg/g) | 11.08±1.4                                     | 55.25±41.2                                    | 0.01|
| Urine NGAL (ng/mL)  | 4.82±0.8                                      | 10.3±2.07                                     | 0.01|
| Serum NGAL (ng/mL)  | 126.68±12.33                                  | 155.06±30.15                                  | 0.04|
| Serum cystatin C (ng/mL) | 557.52±127.48                                | 1739.45±1395.23                              | 0.001|

Data are expressed in mean±SD. SD=Standard deviation, NGAL=Neutrophil gelatinase-associated lipocalin
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Figure 1: Box plots drawn between cases and controls show the increase levels of serum neutrophil gelatinase-associated lipocalin and serum cystatin C in cases

Figure 2: Box plots show increased level of urine albumin/creatinine ratio and urine neutrophil gelatinase-associated lipocalin in cases and controls

disease, reaching higher levels in patients with manifestation of diabetic nephropathy. ROC curve analysis showed that the area under the curve for U.NGAL and S.NGAL were 1 and 0.8, respectively, which indicated that they were sensitive markers for early renal damage.

A more recent study has reported that U.NGAL was significantly higher in type 2 diabetes patients than in controls and in micro- and macro-albuminuric than in normoalbuminuric patients and was positively correlated to urinary albumin excretion.[26]

| Variable       | Urine NGAL | Serum NGAL | Serum cystatin C |
|----------------|------------|------------|-----------------|
| Age            | 0.15       | 0.214      | −0.12           |
| Duration of diabetes | 0.380      | 0.380      | −0.54           |
| HbA1c          | −0.47      | −0.100     | 0.62            |
| Albuminuria    | 0.62       | 0.42       | 0.62            |
| Dyslipidemia   | 0.48       | 0.26       | 0.04            |

NGAL=Neutrophil gelatinase-associated lipocalin, HbA1c=Glycated hemoglobin

Table 3: Correlation analysis of renal injury markers with other parameters

In a study, Bolignano et al.[27] included 96 patients with CKD stages 2–4, among them 20% with diabetic nephropathy. All the patients were followed prospectively until the end of the observation period (20 months) or the primary study end point defined as doubling of baseline serum creatinine and/or the onset of ESRD. Baseline U.NGAL and S.NGAL levels predicted CKD progression in univariate and multivariate analysis independently of other potential confounders, including eGFR and age.

Fu et al. showed that U.NGAL was markedly increased in the type 2 diabetes patients compared with the controls and was significantly increased from the normoalbuminuria to macroalbuminuria group. U.NGAL showed stronger positive correlations with UACR and negative correlation with eGFR. The study suggested that tubular damage is common in short-term type 2 diabetes patients, and U.NGAL may be a promising early marker for monitoring renal impairment in these patients.[28]

A follow-up study by Yang et al. found an increasing tendency of U.NGAL in type 2 diabetics, from normoalbuminuria group to macroalbuminuria group, at both baseline and follow-up levels. U.NGAL was found to be correlated positively with urine Cys C, urea nitrogen, and serum creatinine (and inversely
with GFR, indicating that U.NGAL could be used to predict the progression of nephropathy in type-2 diabetic patients. Cys C, a cysteine protease inhibitor constantly produced by all nucleated cells, has been suggested as a marker of glomerular and tubular dysfunction for early diagnosis of diabetic nephropathy. In a study by Uslu et al., urine Cys C levels were significantly higher in microalbuminuria group compared to normoalbuminuria and were positively correlated with urine albumin-creatinine ratio in both diabetes and prediabetes. Garg et al. in their study on prediabetic patients had similar observations that urine levels of NGAL and Cys C were significantly higher in microalbuminuria group compared to normoalbuminuria.

Many other studies have reported that Cys C levels of urine might be a marker of early renal damage among patients with type 2 DM. Urinary levels of Cys C were significantly increased in patients with microalbuminuria without any other urinary abnormality and with normal serum creatinine. Another study from type 2 diabetes patients found that increased urinary Cys C was associated with decline in GFR, suggesting that higher urinary Cys C excretion was a better predictor of early nephropathy. All these data suggest that Cys C is a promising biomarker for detection of early nephropathy in type 2 diabetes.

In the present study, U.NGAL had a positive correlation with albumin-creatinine ratio, duration of diabetes, HbA1c, and dyslipidemia. Our results are in concordance to study by Hafez et al. who reported similar observations. The present study showed that U.NGAL and S.NGAL and sCys C levels were significantly increased in diabetic patients with microalbuminuria than those who had normoalbuminuria. This finding may support the hypothesis of a “tubular phase” of diabetic disease preceding overt diabetic nephropathy and hence the use of these markers, especially U.NGAL measurement for early evaluation of renal involvement even before appearance of microalbuminuria.

Our study has novelty in this respect that there are not many studies available on utility of acute kidney injury markers in diabetic nephropathy in our population; however, there are certain limitations of our study such as small sample size, its cross-sectional design, and less number of markers which could be assessed.

**Conclusion**

Microalbuminuria has been accepted and established as the earliest marker for diabetic nephropathy; however, a large proportion of renal impairment occurs in nonalbuminuric state. Urinary biomarkers were significantly elevated in normoalbuminuric type 2 diabetes patients compared with nondiabetic controls and could be used as markers of nephropathy at a very early stage even before the development of microalbuminuria, the current gold standard for early diagnosis. Despite the promise of these new biomarkers, further large, multicenter prospective studies are still needed to confirm their utility as a screening tool in clinical practice. It is important to implement different strategies for earlier detection of nephropathy aiming to prevent the long-term devastating outcomes of renal loss in diabetics.

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**Conflicts of interest**

There are no conflicts of interest.

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