Serum Cystatin C and Tubular Urinary Enzymes as Biomarkers of Renal Dysfunction in Type 2 Diabetes Mellitus

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Abstract: Renal tubulointerstitium plays an important role in the development and progression of diabetic nephropathy. The aim of this study was to assess serum cystatin C and 2 renal tubular enzymes, neutrophil gelatinase associated lipocalin (NGAL) and N-acetyl-beta-D-glucosaminidase (NAG), as screening markers for early renal dysfunction in patients with type 2 diabetes mellitus (T2DM). ROC curve analysis showed that urinary NAG is the most sensitive marker of microalbuminuria and early renal damage with sensitivity of 83.3%, while serum cystatin C was the most sensitive and specific marker of macroalbuminuria and damage progress with sensitivity of 70.8% and specificity of 83.3% versus 70.6% and 83.3% for uNGAL; and 64.7% and 66.7% for NAG, respectively. Our data indicate that urinary NAG is the most sensitive marker for early renal damage in diabetic patients. However, for damage progress, serum cystatin C is the most sensitive and specific marker for follow-up and monitoring renal dysfunction.

Keywords: diabetic nephropathy, neutrophil gelatinase-associated lipocalin, N-acetyl-beta-D-glucosaminidase, cystatin C
Introduction

The appearance of pathological levels of urinary albumin excretion (UAE) represents the most common clinical sign of early renal involvement in patients affected by diabetes mellitus (DM). Pathological albuminuria and proteinuria represent the consequence of diffuse diabetes-induced glomerular damage. However, renal tubulointerstitial also seems to play an equally important role in the pathogenesis of diabetic nephropathy as a consequence of persistent exposure to a metabolic and hemodynamic injuring factors associated with sustained diabetic disease. Persistent diabetic proteinuria constitutes another important cause of tubular injury, as the sustained passage of plasmatic proteins within the tubular lumen is harmful to the epithelial cells, due to progressive intratubular complement cascade activation. This last condition can lead first to tubular inflammation and then to tubulointerstitial fibrosis, which ultimately signals the appearance of an irreversible renal impairment, leading to chronic kidney disease.

Serum cystatin (CysC), a cysteine protease inhibitor, is freely filtered through the glomerulus and almost completely reabsorbed and catalyzed by tubular cells. Therefore, serum CysC levels correlate with glomerular filtration rate (GFR) and have been proposed to be a superior marker for the evaluation of renal function, in comparison to other markers such as serum creatinine (S-Cr) or creatinine clearance (Ccr). It is found to be a more sensitive and accurate for the estimation of GFR than S-Cr, and less complicated than Ccr.

Previous studies have focused on the role of the tubular tract in the pathogenesis and progression of renal damage in diabetes and reported increased levels of several ‘tubular factors’. These include cathepsin B, N-acetyl-beta-D-glucosaminidase (NAG) and monocyte chemoattractant protein-1 (MCP-1). The levels of these substances have been found to be correlated to the severity of nephropathy, confirming the recent hypothesis that the renal damage progress and the long-term outcome in diabetic patients is more related to the degree of renal tubulointerstitial impairment than to the severity of glomerular lesions.

Neutrophil gelatinase-associated lipocalin (NGAL) has emerged as one of the most promising tubular biomarkers in the diagnostic field of acute and chronic renal diseases. In patients undergoing treatments potentially detrimental to the kidney, including contrast medium administration, cardiac surgery and unstable nephropathies, increased NGAL levels predict the onset of acute kidney injury, which can advance upcoming increases in serum creatinine levels. Also, in chronic renal diseases, NGAL levels reflect the severity of the active damage associated with autoimmune diseases, glomerular diseases and autosomal dominant polycystic kidney disease.

The aim of this study is to evaluate serum CysC and tubular enzymes NGAL and NAG in T2DM patients with different stages of nephropathy in order to assess their significance as markers of early renal dysfunction.

Subjects and Methods

Study population

The study included a small cohort of 70 patients with T2DM according to the American Diabetes Association criteria. All patients were insulin-requiring and hypertensive under treatment with angiotensin-converting enzyme inhibitors or angiotensin II receptor antagonists. Patients with cancer, infections, inflammatory states, alterations in leukocyte count and severe renal impairment GFR < 30 mL/min, according to the Modification of Diet in Renal Disease (MDRD) equation, were excluded from the study to avoid potential confounding factors. 20 healthy volunteers were included as a control group. Exclusion criteria were the presence of any medical treatment or history of arterial hypertension, diabetes, neoplastic, cardiovascular, inflammatory, renal, pulmonary and endocrinal diseases. The study was approved by the local ethical committee and informed consent was obtained from every subject.

Patient’s history was carefully recorded. Clinical examination and assessment of body mass index (BMI) were performed. Blood pressure was measured 3 times, and the average value was considered for data analysis.

Laboratory methodology

Samples collection

Serum and early morning urine samples were collected, centrifuged, aliquoted and frozen at −20 °C. Samples were thawed and mixed thoroughly just prior to the assay to avoid erroneous results of repeated freeze/thaw cycles.
Assay of biochemical markers

Fasting plasma glucose (FBG) and 2-hour postprandial plasma glucose (PPBG) were measured using glucose oxidase enzymatic assay (Bio Merieux). Glycosylated Haemoglobin (HbA₁c) was quantitatively assayed by ion exchange chromatography (Stanbio Laboratory). Blood urea, serum creatinine (SCr), creatinine in urine and lipid profile (total cholesterol, triglycerides, HDL-C, and LDL-C) were measured by enzymatic colorimetry using Olympus AU 400 auto analyzer (Olympus Diagnostics, GmbH, Germany).

Albumin in urine was estimated by immunoturbidimetric method using Boehringer reagents (Germany). To compensate for variations in urine concentration in spot urine samples, we compared the albumin in the sample against its creatinine concentration and albumin/creatinine ratio was calculated. Positive urine samples for albuminuria were repeated 3 to 6 months after the first positive test.

As serum creatinine was measured by enzymatic method, the glomerular filtration rate (GFR) was calculated based on the following isotope dilution mass spectrometry (IDMS)-traceable MDRD study equation: GFR (mL/min/1.73 m²) = 186 × SCr⁻¹.154 × age⁻⁰.²⁰³ × (1.212 if black) × (0.742 if female).16

Assay of serum Cystatin C, uNGAL and uNGAL

Serum cystatin C was measured by latex particle-enhanced turbidimetric immunoassay (PETIA) on Hitachi 7600 auto-analyzer (Hitachi Co., Tokyo, Japan) using HiSense kit (HBI Co., Anyang, Korea). NAG in urine was estimated kinetically using Sigma reagents (USA). Urinary NGAL was measured by Enzyme-linked immunosorbent assay using Human NGAL ELISA kit supplied by BioVendor Laboratory Medicine, Inc.; Cat. No. RD191102200R.

Statistical analysis

Data were statistically described in terms of mean ± standard deviation. Comparison of more than 2 variables was done using an ANOVA test. A Pearson correlation between various variables was conducted and significant correlations were presented using graphs. A receiver operating characteristics (ROC) analysis was employed to calculate the area under the curve (AUC) to find the best cutoff values providing the highest diagnostic specificity followed by the best sensitivity. A probability value (P) less than 0.05 was considered statistically significant. All statistical calculations were done using the following computer programs: Microsoft Excel version 7 (Microsoft Corporation, NY, USA) and SPSS version 16 (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA).

Results

According to the albumin/creatinine ratio, patients were classified into normal albuminuric (n = 20) with albumin/creatinine ratio <30 µg/mg, microalbuminuric (n = 25) with albumin/creatinine ratio from 30 to 300 µg/mg (for males) and 400 µg/mg (for females), and macro-albuminuric (n = 25) with albumin/creatinine ratio ≥300 µg/mg (for males) and ≥400 µg/mg (for females). Demographic and laboratory data of patients and controls are summarized in Table 1.

Our results showed that serum Cystatin C, uNGAL, and NAG were significantly high in diabetic patients compared with control subjects (Table 1). Correlation studies revealed that the 3 studied parameters showed significant positive correlations with urinary albumin excretion (UAE; P < 0.001), serum creatinine (P < 0.001 for Cystatin and uNGAL; P = 0.002 for NAG), disease duration (P < 0.001 for NAG and uNGAL; P = 0.002 for Cystatin), and with each other (P < 0.001). Both uNGAL and NAG showed positive correlations with poor glycemic control (HbA₁c) with P = 0.03 and 0.02, respectively, and with disease duration (P < 0.001) (Table 2).

Figure 1 shows the ROC curve analysis of serum cystatin, uNGAL and NAG for prediction of microalbuminuria in patients with diabetes. For cystatin C, the area under the curve (AUC) was 0.727 with an optimal cutoff value of 1423.0 ng/mL, sensitivity = 83.3% and specificity = 61.1%. For uNGAL, the AUC was 0.759, the cutoff value was 8.8 ng/mL, sensitivity = 66.7% and specificity = 88.9%. Meanwhile, for NAG, the AUC was 0.890, the cutoff value was 15.5 ng/mL, sensitivity = 83.3%, and specificity = 77.8%.

Figure 2 shows the ROC curve analysis of the 3 studied parameters for prediction of macroalbuminuria. For cystatin C, the AUC was 0.797, the cutoff value was 2450.0 ng/mL, sensitivity = 70.8% and specificity = 83.3%. For uNGAL, the AUC was 0.848, the cutoff was 13.5 ng/mL, sensitivity = 70.6% and
NGAL is a 25-kDa molecule known to be hyper produced in kidney tubules within few hours after damaging experimental stimuli. Tubular secretion of NGAL correlates with the severity of chronic renal impairment; for instance, in autosomal dominant polycystic kidney disease or glomerulonephritis.\textsuperscript{28} In this study, uNGAL was significantly higher in patients than in controls, and in micro- and macroalbuminuric than in normalalbuminuric patients. Also, uNGAL positively correlated to the disease duration, urinary albumin, and poor glycemic control (HBA\textsubscript{1c}). In accordance with our findings, NGAL was 5–10-fold higher in normo- or micro-albuminuric patients compared to controls, indicating that tubular injury (proximal and distal) occurs early and perhaps before albumin excretion in patients with emerging diabetic nephropathy. In addition, NGAL increased 2–4-fold further when the injury progressed to become diabetic nephropathy; ie, serum NGAL showed an almost identical relationship with progression of diabetic nephropathy.\textsuperscript{29}

### Discussion

In diabetic nephropathy, tubular involvement may precede glomerular involvement, as several tubular proteins and enzymes are detectable even before the appearance of microalbuminuria or rising in serum creatinine.\textsuperscript{22}

Cystatin C has been identified as a promising marker of renal failure\textsuperscript{23} and has been demonstrated to be an early renal marker in patients with diabetes.\textsuperscript{24} In our study, serum cystatin C was significantly high in diabetic patients and correlated positively with serum creatinine, NAG, uNGAL, urinary albumin, and disease duration. Our findings agree with results from previous studies.\textsuperscript{25,26} These findings are in contrast to one previous study, in which increased serum cystatin level was found to be significant in overt nephropathy but not in early nephropathy.\textsuperscript{27}
In a cohort of 56 patients with type 2 DM, Lu-Yang and coworkers 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 non-invasive tool for the evaluation of renal involvement in diabetes, accelerating the early diagnosis of diabetic nephropathy. Nielsen and colleagues reported that elevated uNGAL in Type 1 diabetes patients with or without albuminuria indicates tubular damage at an early stage. Furthermore, NGAL showed a good correlation with GFR in diabetic patients and was a sensitive and accurate marker of early diagnosis of nephropathy.

The urinary enzyme NAG is found in the lysosomes of the proximal tubule epithelial cells. A high NAG activity in urine during the course of illness or toxic insult is a consequence of tubule cell damage in renal diseases, or can be due to nephrotoxic effects (drugs, proteinuria, diabetes and pregnancy) and represents an early sign of tubule disorder. According to our results, urinary NAG was significantly higher in all patient groups than in controls and in microalbuminuric than in normoalbuminuric patients. Also, NAG correlated posi-
tively with albuminuria, duration of diabetes, poor glycemic control, serum cystatin, and uNGAL. Similar findings were reported by Manal et al. In a previous study of type 2 diabetes, NGAL showed positive correlations with cystatin-C, blood urea nitrogen (BUN), serum creatinine and albuminuria, and negatively with GFR; our results confirmed these findings.

The catalytic activity of urinary NAG in type 2 diabetes patients has been reported as the most sensitive parameter for the early discovery of the tubular damage. The enzyme activity in diabetes patients without microalbuminuria was increased 1.5-fold and 3-fold in patients with microalbuminuria. Meanwhile, in macroalbuminuric patients, there was a 2-fold increase. These findings point to the great importance of NAG in discovering the renal tubule cell damage, especially at the early stage before the appearance of microalbuminuria. Nauta et al. reported increased urine NAG by 9-fold in normoalbuminuric diabetic patients compared to controls, correlating with the development and progress of microalbuminuria. In another study of type 1 diabetic patients, regression of microalbuminuria was associated with a highly significant reduction in urine NAG excretion.

ROC analysis showed that urinary NAG was as sensitive as serum Cystatin for the prediction of microalbuminuria (83.3%), but had much higher specificity (77.8% versus 61.1%, respectively). Meanwhile, uNGAL showed poor sensitivity (66.7%) albeit higher specificity (88.9%). However, for macroalbuminuria and diabetic nephropathy progress, serum cystatin C was the most sensitive and specific marker (70.8% and 83.3%, respectively) versus uNGAL (70.6% and 83.3%, respectively) or NAG (64.7% and 66.7%, respectively).

In conclusion, in diabetic nephropathy, urinary NAG is the most sensitive marker for early renal damage superior to cystatin C and uNGAL. However, for damage progress, serum cystatin C is the most sensitive and specific marker for follow up and clinical monitoring of renal dysfunction.

Author Contributions
Conceived and designed the experiments: HSA, ST. Analyzed the data: HSA, ST, EAR. Wrote the first draft of the manuscript: HSA, EAR. Contributed to the writing of the manuscript: DE. Agree with manuscript results and conclusions: HSA, ST, EAR, DE, EHT. Jointly developed the structure and arguments for the paper: HSA, ST, EAR, DE, EHT. Made critical revisions and approved final version: SA, ST, EAR, DE, EHT. All authors reviewed and approved of the final manuscript.

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