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Urinary kidney injury molecule-1 and neutrophil gelatinase-associated lipocalin as indicators of tubular damage in normoalbuminuric patients with type 2 diabetes

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ABSTRACT

Objectives: Renal dysfunction has been reported in normoalbuminuric patients, demonstrating the necessity to improve the diagnostic and prognostic tools for diabetic kidney disease (DKD) investigation. Therefore, the aim of this study was to investigate whether the urinary levels of neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1) are increased in type 2 diabetes mellitus (DM) patients with normal or mildly increased albuminuria.

Design and methods: In this study, 117 type 2 DM patients classified into three groups according to urinary albumin/creatinine ratio (uACR): uACR < 10 mg/g creatinine, uACR 10–30 mg/g creatinine and uACR > 30 mg/g creatinine were enrolled. Urinary concentrations of KIM-1 (uKIM-1) and NGAL (uNGAL) were measured.

Results: uKIM-1 levels increased progressively from uACR < 10 mg/g creatinine (69.0 ± 20.8 pg/ml) to uACR 10–30 mg/g creatinine (106.1 ± 41.2 pg/ml) and to uACR > 30 mg/g creatinine (166.0 ± 31.9 pg/ml) ($P < 0.001$). In addition, uNGAL levels increased progressively from uACR < 10 mg/g creatinine (29.5 ± 8.8 ng/ml) to uACR 10–30 mg/g creatinine (51.7 ± 10.9 ng/ml) and to uACR > 30 mg/g creatinine (71.0 ± 9.6 ng/ml) ($P < 0.001$) patients. Similarly, both uKIM-1 and uNGAL adjusted by urinary creatinine were increased in patients with uACR 10–30 mg/g creatinine. Significant and positive correlations were observed between uACR, uKIM-1 and uNGAL.

Conclusions: uKIM-1 and uNGAL were increased in type 2 DM patients with normal or mildly increased albuminuria, which indicates that tubular and glomerular injuries may be occurring even at the earliest stage of DKD.

1. Introduction

Diabetic kidney disease (DKD), the most common microvascular complication of diabetes mellitus (DM), is the leading cause of mortality and morbidity in DM patients [1,2]. DKD is a clinical syndrome defined by persistent albuminuria (micro- or macroalbuminuria) on at least two occasions separated by three or six months [3,4]. The renal injury in DKD is caused by a complex series of pathophysiological changes initiated by disturbed glucose homeostasis and is similar in both type 1 and type 2 DM patients [5]. Urinary albumin (uAlb) is generally considered the earliest non-invasive marker for the development of DKD [6] and a well-established independent risk factor of cardiovascular disease [7]. Although most attention has focused on glomerular changes, less than one-third of microalbuminuria cases in diabetic patients actually have a histologic glomerulopathy [8]. In this context, it is now increasingly recognized that tubular damage plays an important role in the pathogenesis of DKD [9,10], and some authors partially attribute the onset of albuminuria in patients with DM to tubular damage [11]. Some of the proteins and tubular enzymes associated with proximal tubular

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injury are N-acetyl-β-D-glucosaminidase, gamma-glutamyltransferase, neutrophil gelatinase-associated lipocalin (NGAL), and kidney injury molecule-1 (KIM-1) [10].

KIM-1 is a type 1 membrane protein expressed on the apical membrane of proximal tubule cells. Its ectodomain is cleaved and released into the lumen of the tubule and finally appears in the urine, which is stable [12,13]. This biomarker is not detectable if the kidneys are normal [14]. Thus, it is a specific and sensitive biomarker for proximal tubule damage and may be a predictive biomarker for renal diseases [15–17]. NGAL, a small protein that belongs to the lipocalin protein family, is produced in epithelial cells and neutrophils in most tissues and is a marker of renal tubular injury [18]. Interestingly, these urinary tubular markers are independently associated with uAlb in the early stage of DKD in type 2 DM [19].

Although there is considerable evidence that the levels of urinary KIM-1 and NGAL are changed in diabetes [19–22], it is uncertain whether these biomarkers are increased in type 2 DM patients in the stage of normoalbuminuria, which usually precedes the DKD. Therefore, the aim of this study was to investigate whether the urinary levels of NGAL and KIM-1 are increased in type 2 DM patients with normal or mildly increased albuminuria.

2. Materials and methods

2.1. Study population

Participants were recruited between March and October 2013 in the diabetic clinic at the University Hospital of Santa Maria located in Santa Maria, Rio Grande do Sul, Brazil. The study involved 117 type 2 DM patients classified into three groups according to the urinary albumin/creatinine ratio (uACR) [23,24]: normal (uACR < 10 mg/g creatinine, n = 64, 21 males and 43 females), mildly increased (uACR 10–30 mg/g creatinine, n = 31, 12 males and 19 females), and moderately/severely increased (uACR > 30 mg/g creatinine, n = 22, 9 males and 13 females). Additionally, the diagnosis of DKD was defined according to American Diabetes Association (ADA) recommendations [25] and based on the detection of uACR in two of three specimens collected within a three- to six-month period; uACR ≥ 30 mg/g of creatinine was considered an increased urinary albumin excretion. Exclusion criteria consisted of urinary tract diseases, prior renal disease other than DKD, neoplastic disorders, uncontrolled thyroid disorders, infectious and liver diseases, active or chronic persistent infection or inflammatory disorders, pregnancy, renal transplantation, and the use of nephrotoxic drugs. All eligible patients provided informed written consent, and all studies were conducted in accordance with guidelines approved by the Institutional Ethics Review Board for human studies (number 12303113.0.0000.5346).

2.2. Laboratory analysis

Blood samples were collected from all patients after an overnight fast by venous puncture into Vacutainer® (BD Diagnostics, Plymouth, UK) tubes with sodium fluoride plus EDTA, with EDTA and no anticoagulants. Each patient also provided a urine sample for biochemical analysis. Blood samples were routinely centrifuged at 2500 ×*g* for 15 min, and urine specimens were centrifuged at 1000 ×*g* for 5 min. Plasma with sodium fluoride plus EDTA was used to measure the levels of fasting glucose, and whole blood in EDTA was used to measure the levels of glycated hemoglobin (HbA_{1c}) and hemoglobin. The serum was used to assess the levels of insulin, uric acid, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, high-sensitive C-reactive protein (hsCRP), creatinine, aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Levels of urinary creatinine, uAlb, urinary NGAL (uNGAL) and urinary KIM-1 (uKIM-1) were assessed. The results of uAlb were expressed as milligrams of albumin per gram of creatinine as a tool to match the levels of albumin in accordance with the concentration of urine [26]. Measurements of the glucose, uric acid, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, hsCRP, creatinine (serum and urine), AST, ALT and uAlb were performed using standard methods on a Dimension RXL MAX® (Siemens Healthcare Diagnostics Inc., Newark, DE, USA) automated analyzer. HbA_{1c} was measured using chromatographic method with a D10® automated analyzer (Bio-Rad, California, USA). Insulin was determined using a Cobas 6000® automated analyzer (Roche Diagnostics, Mannheim, Germany). Hemoglobin was measured using a Sysmex XE-5000® automated hematology system (Sysmex, Kobe, Japan). uNGAL and uKIM-1 were measured using ELISA kits designed to analyze random spot urine samples (KIM-1: R&D systems, Minneapolis, MN, USA; NGAL: HYB211-05, Antibody Shop, Gentofte, Denmark). All assays were conducted according to the manufacturer's instructions. The inter-assay and intra-assay precision for the uKIM-1 were 6.0–7.8% and 3.9–4.4%, and 5.6–7.9% and 3.1–4.4% for NGAL. The detection limits of the assays were 0.009 ng/ml for KIM-1 and 0.012 ng/ml for NGAL. The estimated glomerular filtration rate (eGFR) was assessed by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [27].

2.3. Statistical analysis

Distributions of continuous variables were examined for skewness and kurtosis by the Kolmogorov–Smirnov omnibus normality test. Data are presented as mean ± standard deviation (SD) for parametric variables and median and interquartile range (IQR) for non-parametric variables. Categorical data are summarized as percentages, and comparisons between groups were performed with the χ^2 test. A comparison of more than two groups was performed using one-way analysis of variance (ANOVA) followed by the Tukey post hoc test or was performed using Kruskal–Wallis test. Spearman's correlation coefficient was calculated between uACR, uKIM-1 and uNGAL. Logistic regression analysis and ordinal logit regression were performed to investigate whether some factors interfere with urinary tubular biomarkers. Statistical significance was assumed at $P < 0.05$. Data were analyzed using GraphPad Prism® version 4.00 for Windows (GraphPad Software, San Diego, CA), Statistical Package for Social Sciences®, version 17.0 (SPSS Inc., Chicago, IL, USA) and MedCalc® version 13.2.2 (MedCalc Software, Ostend, Belgium).

Table 1
Baseline characteristics of study participants.

	uACR < 10 mg/g	uACR 10–30 mg/g	uACR > 30 mg/g	*P*-value
N	64	31	22	–
Age (years)	58.0 ± 14.0	62.0 ± 11.0	57.0 ± 12.0	0.288
Gender (male, %)	32.8	38.7	40.9	0.737
BMI (kg/m^2)	29.6 (26.1–35.2)	29.8 (27.1–36.2)	30.6 (26.6–43.8)	0.506
Hypertension (%)	59.4	87.1	77.3	0.082
Smokers (%)	7.8	6.4	9.1	0.937
Duration of type 2 DM (years)	12.0 (6.0–18.0)	12.0 (8.0–20.0)	10.0 (6.0–20.0)	0.718
Insulin (%)	26.6	54.8	36.4	0.027
Oral hypoglycemic agents (%)	86.4	90.3	68.8	0.023
ACE inhibitors (%)	70.3	70.9	72.3	0.977

ACE inhibitors, angiotensin-converting-enzyme inhibitors; BMI, body mass index; uACR, urinary albumin/creatinine ratio.

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