Association of neutrophil-gelatinase-associated lipocalin with microvascular complications in patients with type 2 diabetes: a cross-sectional study

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Introduction Diabetic nephropathy and diabetic retinopathy are serious microvascular complications of diabetes mellitus. Recent studies have demonstrated that neutrophil-gelatinase-associated lipocalin (NGAL) may be accompanied by these complications during and before the appearance of microalbuminuria. In this study, we set out to research the role of NGAL in patients with diabetic nephropathy and diabetic retinopathy.

Material and methods Eighty-two patients with type 2 diabetes were enrolled in our study. Urinary microalbumine and NGAL levels were measured in urine samples over 24 hours. We also studied NGAL levels in serum. All patients went through an ophthalmologic examination. The results were evaluated based on the presence of microalbuminuria and retinopathy.

Results There were no significant differences in serum and urine NGAL levels between normoalbuminuric ($n=66$) and microalbuminuric ($n=16$) patients. We also did not find any significant difference in patients with retinopathy ($n=16$) or without retinopathy ($n=66$).

Conclusion There are controversial findings about the role of NGAL in diabetic patients in medical literature. Standard values of urine and serum NGAL levels have yet to be determined. Our study suggests that NGAL is not a useful marker to differentiate microalbuminuric patients from normoalbuminuric subjects. We also did not find a relationship between NGAL levels and the presence of retinopathy. Additional studies with larger sample sizes will be required to confirm or refute these findings. *Cardiovasc Endocrinol Metab* 8:82–87 Copyright © 2019 Wolters Kluwer Health, Inc. All rights reserved.

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Introduction

Diabetes mellitus and its resulting complications present a major healthcare problem. Diabetic nephropathy and diabetic retinopathy are well known microvascular complications. Diabetic nephropathy is associated with an increased risk of all-cause mortality, cardiovascular disease, and end-stage renal disease [1]; whereas diabetic retinopathy may lead to blindness [2].

Diagnostic markers to detect the complications of diabetes mellitus at an early stage are important clinical management tools. Once diagnosed, early intervention can help preserve kidney function and reduce disease progression. The appearance of albumin in urine, known as ‘microalbuminuria’, has been regarded as the gold standard for diagnosing diabetic nephropathy. On the other hand, according to current studies, renal impairment occurs before microalbuminuria appears in urine [3] and diabetic kidney disease is not, in some cases, detected by testing microalbuminuria [4]. It has been reported that 29.1–61.6% of subjects with type 2 diabetes mellitus could have renal impairment before the detection of microalbuminuria [5,6]. New biomarkers to

detect diabetic nephropathy in patients with diabetes could also prove useful as renal impairment may begin in the prediabetic period. Studies have indicated that microalbuminuria only exists in 10% of patients with prediabetes [7].

Microalbuminuria may spontaneously resolve in 40% of diabetic patients. About 30–40% of the cases with diabetic nephropathy microalbuminuria do not progress to macroalbuminuria over the ensuing 5–10 years [8].

Microalbuminuria is not associated solely with patients with diabetes. There are other contributing factors, such as hypertension, exercise, urinary tract infection, and cardiac failure. The existence of microalbuminuria in nondiabetic subjects indicates the nonspecificity of microalbuminuria as an accurate predictor of diabetic nephropathy [9].

Pathological albuminuria and proteinuria are results of diabetes-induced glomerular damage. However, recent studies suggest that renal tubulointerstitium also seems to play an important role in the pathogenesis of diabetic nephropathy [10]. Some studies demonstrate that

impairment in renal function and the overall outcome are more strongly associated with the degree of renal tubulointerstitial damage than with the severity of glomerular lesions [11]. Therefore, changes in albuminuria may be regarded as a complementary manifestation of diabetic nephropathy rather than an obligatory one [12].

New biomarkers may have a useful role for further clinical management in order to predict the complications of diabetes mellitus at a very early stage before the appearance of microalbuminuria. Recent studies have presented that new biomarkers may be significantly elevated in patients with diabetes, compared with those of control subjects. These markers may be useful for earlier, specific and accurate prediction of diabetic nephropathy [13]. Neutrophil-gelatinase-associated lipocalin (NGAL) has been proposed as one of these markers. It has been reported that NGAL elevates both serum and urine, before the presence of microalbuminuria in patients with diabetes [14].

Human NGAL is a ubiquitous protein of approximately 25 kDa molecular mass [15]. It is produced and secreted into the urine as a response to ischemic kidney damage. The appearance of NGAL in the urine of subjects with diabetes may indicate early glomerular injury, and this has been demonstrated at an earlier stage before the appearance of microalbuminuria. Kuwabara *et al.* [16] studied NGAL existence in the urine of subjects with type 1 diabetes by injecting NGAL exogenously, assuming that tubular reabsorption impairment may play a role in diabetic nephropathy. NGAL is expressed in lower concentrations in human tissues other than the kidney, such as tissues in the trachea, lungs, stomach, and colon [17]. Recent studies presented that NGAL might be associated with obesity, insulin resistance, hyperglycemia [18], and retinal inflammation [19]. During the course of acute bacterial infections, NGAL is expressed by the injured epithelia [20]. The proliferating epithelial cells in proximal tubules secrete NGAL in the postischemic kidney [21].

NGAL might have a role as well in re-epithelization. The identification of NGAL as a regulator of epithelial morphogenesis in cultured kidney tubule cells [22] and as an iron-transporting protein during nephrogenesis supports this hypothesis [23,24]. Delivery of iron into cells is essential for cell growth and development and may have an important role for renal regeneration after ischemic injury. Moreover, findings indicate that exogenously administered NGAL favors injured tubule cells toward survival after ischemic acute renal injury [25]. Supavekin *et al.* [26] studied the genetic response in animal models after renal ischemic injury. They identified NGAL expression as a result of upregulated genes (HUGO-approved gene name LCN2) [26].

In our study, we intended to investigate urine and serum levels of NGAL in patients with type 2 diabetes. We

compared serum and urine NGAL levels with urine albumin excretion and other parameters. We additionally studied NGAL levels regarding the presence of diabetic retinopathy.

Material and methods

In our cross-sectional study, there were 82 subjects who were diagnosed as type 2 diabetes mellitus according to the criteria of the American Diabetes Association [27]. Patients were followed in the outpatient clinic of the Internal Medicine Department of the Istanbul Fatih Sultan Mehmet Education and Research Hospital. They were classified into two main groups considering albuminuria and retinopathy. Based on the presence of albuminuria, two subgroups were created: a normoalbuminuric group ($n=66$) and microalbuminuric group ($n=16$). Based on the presence of retinopathy, two additional subgroups were designated: a group which had no diabetic retinopathy ($n=66$) and a group with nonproliferative diabetic retinopathy ($n=16$). Diabetic nephropathy was measured in urine samples over 24 hours at two different times within three months. The albumin/creatinine ratio (ACR) of the normoalbuminuric group was less than 30 mg/g creatinine and the ACR of the microalbuminuric group was 30–300 mg/g creatinine. All subjects had a funduscopic examination performed by the same ophthalmologist after pupillary dilatation.

The demographic features and the medical history of the subjects were recorded and a complete physical examination was performed. BMI was calculated as weight in kilograms divided by the square of height in meters. Patients with a medical history of hypertension, pregnancy, menstruation, or active inflammatory disease (infection, malignancy, and rheumatologic diseases), and who had a creatinine level higher than at least 1.5 mg/dl and macroalbuminuria ($\text{ACR} \geq 300 \text{ mg/g creatinine}$) were excluded. Subjects with antihypertensive treatment or exposed to nephrotoxic drugs were also excluded. Blood pressure was measured three times and those whose average readings were less than 140/90 mmHg were included to the study. The study was approved by the local ethical committee and a signed informed consent was obtained from the volunteers.

Laboratory methodology

The venous samples were collected from the subjects after 12 hours of overnight fasting. Serum creatinine, creatinine in 24 hours of urine samples, plasma glucose, uric acid, and serum cholesterol profile [total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglyceride] were measured with commercial kits by enzymatic colorimetry in an auto analyzer (Beckman Coulter UniCel 800). Blood hemoglobin A1c percentage (HbA1c%) was measured qualitatively by HPLC (Tosoh G7 variant). High-sensitive C-reactive protein was measured with

commercial kits by the nephelometric method (Dade Behring BN II). Albumin levels in urine samples over 24 hours were measured with commercial kits by the immunonephelometric method (Dade Behring BN II). The samples for measuring NGAL in urine and serum were centrifuged at 4000 rpm for 10 minutes. The serum and supernatant samples were conserved at -35°C in dry tubes. The measurement was performed within two months after the samples were obtained. The Human NGAL ELISA kit (BIOPORTO, DANMARK) which was used was available commercially. The tests were done with the enzyme-linked immunosorbent method, and the commercial kits were covered with polyclonal antihuman Lipocalin-2 and read spectrophotometrically at 450 nm.

Estimated glomerular filtration rate (eGFR) was calculated measuring both creatinine clearance (Jaffe method, based on creatinine levels in 24 hours of urine and serum) and the Cockcroft-Gault formula $[(140 - \text{age}) \times \text{kilograms}/(\text{serum creatinine} [\text{mg/dl}] \times 72)]$ (in females $\times 0.85$) [28].

Statistical analyses

For statistical analysis, the SPSS 15.0 Windows program was used. Definitive statistics were given as number and percent for categorical variations, mean, and SD for numeric variables. In numeric variables, for the independent two group comparison, the *t* test was used for comparing variable means which are convenient for the normal distribution range, and the Mann-Whitney U test was used for comparing variables which did not maintain the normal distribution range. Spearman correlation analysis was made to determine the relationship between variabilities. Statistical alpha meaning level was accepted as $P < 0.05$.

Results

The mean age of the 82 subjects with type 2 diabetes mellitus was 52.3 ± 6.5 , and the mean duration of the disease was 5.8 ± 5.6 years. The range of the duration of diabetes mellitus varied from 1 to 20 years. Thirty-nine patients (47.6%) were male. The demographic, anthropometric, and laboratory data of the patients with type 2 diabetes mellitus are presented in Table 1.

The mean serum and urine NGAL levels were slightly lower in the microalbuminuric group than in the normoalbuminuric group, which presented a nonsignificant statistical result. The comparison of the two groups is illustrated in Table 2. In our study, only three patients had both microalbuminuria and diabetic retinopathy.

The mean serum and urine NGAL levels were lower in patients who had nonproliferative diabetic retinopathy in comparison to patients who did not have diabetic retinopathy, as presented in Table 3.

We did not find any significant correlation between serum and urine NGAL levels and urinary albumin excretion

Table 1 Descriptive statistics for the patients included in the study

Parameter	Mean value
Demographic data	
Age (years)	52.3 ± 6.5
Duration of disease (years)	5.8 ± 5.6
Anthropometric data	
BMI (kg/m^2)	29.2 ± 5.8
Laboratory data	
Urinary albumin excretion (mg/day)	21.7 ± 29.5
Creatinine	$0.76 \pm 0.16 \text{ mg/dl}$ ($67.18 \pm 14.14 \mu\text{mol/l}$)
Estimated GFR ($\text{ml}/\text{min}/\text{m}^2$)	134.7 ± 35.5
Hemoglobin A1c	$9 \pm 2.4\%$ [$74.9 \pm 29.9 \text{ mmol/mol}$]
Serum HDL-C	$41 \pm 9 \text{ mg/dl}$ [$1.06 \pm 0.23 \text{ mmol/l}$]
Serum LDL-C	$120 \pm 35 \text{ mg/dl}$ [$3.1 \pm 0.9 \text{ mmol/l}$]
Serum total cholesterol	$191 \pm 43 \text{ mg/dl}$ [$4.93 \pm 1.11 \text{ mmol/l}$]
Serum triglyceride	$148 \pm 79 \text{ mg/dl}$ [$1.67 \pm 0.89 \text{ mmol/l}$]
High-sensitivity CRP	$2.8 \pm 2.7 \text{ mg/l}$ [$26.66 \pm 25.71 \text{ nmol/l}$]
Serum uric acid	$4.3 \pm 1.2 \text{ mg/dl}$ [$255.76 \pm 71.38 \mu\text{mol/l}$]
Serum NGAL (ng/ml)	379 ± 559^a
Urine NGAL (ng/ml)	60 ± 100^b

CRP, C-reactive protein; GFR, glomerular filtration rate; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; NGAL, neutrophil gelatinase-associated lipocalin.

^aMedian of serum NGAL was 74.5 ng/ml .

^bMedian of urine NGAL was 26.5 ng/ml .

Table 2 Comparisons of urine and serum NGAL levels in normoalbuminuric and microalbuminuric patients

Parameter	Normoalbuminuric patients ($n=66$)	Microalbuminuric patients ($n=16$)	<i>P</i> value
sNGAL, ng/ml	379 ± 545	378 ± 630	0.991
uNGAL, ng/ml	62 ± 106	50 ± 71	0.833

sNGAL, serum neutrophil gelatinase-associated lipocalin; uNGAL, urine neutrophil gelatinase-associated lipocalin.

Table 3 Comparisons of urine and serum NGAL levels in patients with nonproliferative diabetic retinopathy and without diabetic retinopathy

Parameter	Patients without diabetic retinopathy ($n=66$)	Patients with nonproliferative diabetic retinopathy ($n=16$)	<i>P</i> value
sNGAL, ng/ml	397 ± 580	298 ± 463	0.955
uNGAL, ng/ml	68 ± 108	23 ± 20	0.317

sNGAL, serum neutrophil gelatinase-associated lipocalin; uNGAL, urine neutrophil gelatinase-associated lipocalin.

Table 4 Correlation between sNGAL, uNGAL and urinary albumin excretion

Parameter	All patients ($n=82$)		Normoalbuminuric patients ($n=66$)		Microalbuminuric patients ($n=16$)	
	ρ	<i>P</i> value	ρ	<i>P</i> value	ρ	<i>P</i> value
sNGAL, ng/ml	-0.043	0.707	-0.051	0.691	0.018	0.948
uNGAL, ng/ml	0.004	0.975	0.032	0.803	-0.206	0.443

(Table 4). There was no significant correlation between serum NGAL (sNGAL) levels, urine NGAL (uNGAL) levels, and other parameters (Table 5).

Discussion

As NGAL is a smaller protein than albumin, it is presumed that it is rapidly filtered and reabsorbed by

Table 5 Correlation between sNGAL, uNGAL and other parameters

Parameter	sNGAL (ng/ml), Patients (n=82)		uNGAL (ng/ml), Patients (n=82)	
	rho	P value	rho	P value
Age	-0.073	0.512	0.053	0.636
Duration of disease	-0.160	0.180	-0.063	0.598
BMI	-0.027	0.811	0.132	0.245
Glomerular filtration rate (ml/min)	0.054	0.640	0.150	0.192
Glomerular filtration rate, (mg/dl) (formula)	0.041	0.718	0.138	0.218
Hemoglobin A1c	-0.091	0.423	-0.025	0.826
Serum high-density lipoprotein cholesterol	-0.007	0.950	-0.057	0.612
Serum low-density lipoprotein cholesterol	-0.054	0.629	0.032	0.774
Serum total cholesterol	-0.056	0.620	0.012	0.912
Serum triglyceride	-0.065	0.565	0.063	0.574
High-sensitivity C-reactive protein	0.063	0.577	0.080	0.479
Serum uric acid	0.063	0.573	-0.182	0.102

sNGAL, serum neutrophil gelatinase-associated lipocalin; uNGAL, urine neutrophil gelatinase-associated lipocalin.

proximal tubules in normal circumstances. Only 0.1–0.2% of filtrated NGAL is expected in urine [24]. In comparison with glomerular signs, the increasing levels in NGAL occur earlier and this condition might be secondary to subclinical tubular impairment [4,29]. Fioretto *et al.* [30] reported that only 29% had typical histological glomerular changings of diabetic nephropathy in patients with diabetes with microalbuminuria; on the other hand, 42% had severe tubulointerstitial lesions. Based on these findings, studies are performed for investigating the role of tubular impairment through biomarkers in patients with diabetic nephropathy.

Nielsen *et al.* [31,32] found that the uNGAL level was higher in patients with type 1 diabetes, when either microalbuminuria or macroalbuminuria was present. Zachwieja *et al.* [4] stated that because serum and urine NGAL levels were elevated in children with diabetes without albuminuria, normoalbuminuria would not exclude diabetic nephropathy regarding NGAL measurements. Bolignano *et al.* [10,33] had similar results in patients with type 2 diabetes, suggesting uNGAL and sNGAL were positively correlated with albuminuria without early signs of glomerular damages; but they did not find any significant difference between microalbuminuric and normoalbuminuric groups. Fu *et al.* [34] supported similar findings, with uNGAL significantly increased from the normoalbuminuric patients to the macroalbuminuric subjects. Fu *et al.* [34] also showed a correlation between uNGAL and eGFR. Jiao *et al.* [35] demonstrated an increase in NGAL levels in urine and in serum considering the severity of diabetic nephropathy in patients with type 2 diabetes mellitus. Thrailkill *et al.* [36] reported a positive correlation between uNGAL levels and the duration of diabetes mellitus. Garg *et al.* [37] performed a study both in patients with diabetes and prediabetes, and they observed that uNGAL levels were significantly higher in the microalbuminuria group compared to normoalbuminuria groups.

Hafez *et al.* [38] found uNGAL levels higher in microalbuminuric patients; however, they did not observe a significant difference compared with normoalbuminuric patients. Yürük Yıldırım *et al.* [39] also did not find any statistically significant difference in their study between microalbuminuric patients and subjects without microalbuminuria, but they did present a positive correlation between urine albumin and uNGAL levels. In a study of adolescents, Demir *et al.* [40] found similar sNGAL levels in patients with diabetes and healthy controls, stating that sNGAL levels do not always elevate in diabetic nephropathy. Nielsen *et al.* demonstrated that uNGAL in the microalbuminuric group was not significantly different in either the normoalbuminuric and macroalbuminuric groups. They also studied sNGAL levels and found no difference between microalbuminuric, normoalbuminuric, and control subjects. In their study, Nielsen *et al.* [32] observed the effect of Lisinopril treatment over uNGAL levels and found approximately a 15% reduction in uNGAL levels after two months of treatment and which did not reach statistical significance.

Yang *et al.* [41] performed a one-year follow-up study on uNGAL levels in diabetic nephropathy. They did not find any difference in uNGAL levels between the normoalbuminuric and microalbuminuric groups at the baseline or over the first year. In addition, the observed sNGAL levels were more useful in detecting the early stage of diabetic nephropathy, because the sNGAL changes were more significant than the uNGAL [41].

Our study found that in normoalbuminuric and microalbuminuric groups, there was no significant difference either in serum or uNGAL levels in patients with type 2 diabetes mellitus. We also did not find any correlation between NGAL levels and urinary albumin excretion.

Lacquaniti *et al.* [42] showed that NGAL increases in subjects with type 1 diabetes in normoalbuminuric phase, but they did not find any correlation between serum and urine NGAL levels. They presented a direct correlation between sNGAL and HbA1c [42]. Elevated HbA1c has been associated with the microangiopathic process. HbA1c has an affinity for oxygen that causes anoxia in tissues [43].

In a recent study, Chung *et al.* observed a significant positive association between sNGAL levels and diabetic retinopathy in patients with type 2 diabetes [44]. Because NGAL has been reported to play a role in iron delivery to cytoplasm, it has been suggested that an intracellular iron overload might result in oxidative stress, retinal neurodegeneration and be a contributor to inflammatory response [45]. NGAL also could have a possible relation to the formation of advanced glycation end-products [46]. We did not find any correlation between plasma and urine NGAL levels and the presence of diabetic retinopathy.

Our study had some limitations. It was a single-center study, the cohort of the patients was small, and the sample size of the two groups was not equal. The small number of patients with retinopathy or microalbuminuria increased the probability of a type 2 statistical error (i.e., false negative association). Second, we did not have a control group. On the other hand, cutoff values for normal NGAL levels in plasma and urine have not been clearly defined. We observed different values in different studies when the same or different NGAL kits were used. Mean NGAL levels varied largely from 46.4 to 655.29 ng/ml in serum, and 6.5 to 156.53 ng/ml in urine [4,42]. Further studies are needed to define the standard values of normality [37].

In conclusion, in our study, serum and uNGAL levels did not show any difference between normoalbuminuric and microalbuminuric patients with type 2 diabetes. The marker also did not present any difference between patients with or without diabetic retinopathy. On the other hand, the mean NGAL levels (especially in serum) that we found are compatible with other studies. The reason may be that diabetes is assumed to be a hyperglycemic and a proinflammatory state which induces increased NGAL production in extra-renal tissues [47]. Larger studies are still needed.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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