Clin. Lab. 2020;66:XXX-XXX

ORIGINAL ARTICLE

Urinary Neutrophil Gelatinase-Associated Lipocalin (NGAL) as a Marker of Diabetic Nephropathy in Type 1 Diabetic Patients

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SUMMARY

Background: **Glomerular and tubulointerstitial damage plays a role in renal function failure in diabetic patients. While both serum and urine levels of neutrophil gelatinase-associated lipocalin (NGAL) show significantly increased levels in acute renal pathologies, the NGAL increase in active phase indicates a reversible condition in chronic cases.**

Materials and methods: **52 type 1 diabetic patients and 30 healthy volunteers participated in the study. The diabetic participants were separated into two groups as follows: a normoalbuminuria group consisting of those with an albumin/creatinine ratio less than 30 mg/g and an albuminuria group consisting of those with an albumin/creatinine ratio equal or greater than 30 mg/g. Albumin, creatinine and NGAL were measured in all participants.**

Results: **Urinary NGAL median level was 21.1 ng/mL for diabetic patients and 11.9 ng/mL for healthy controls, and the difference between the two groups was statistically significant. When diabetic patients were compared as those with and without albuminuria, the median urinary NGAL levels of normoalbuminuria and albuminuria were 24.7 and 16.1 ng/mL, respectively, but the difference was not statistically significant. Statistically similar results were obtained through evaluation of the ratio of urinary NGAL excretion to creatinine excretion. The NGAL/Cr ratio was significantly higher in diabetic patients than in healthy controls, but no statistically significant difference was found between the diabetic patients with and without albuminuria.**

Conclusions: **Urinary NGAL excretion in type 1 diabetic patients is found to be increased over a wide range, but it does not correlate with urinary albumin excretion. In this regard, urinary NGAL excretion should not be used as an alternative to microalbuminuria in detecting diabetic nephropathy. The greater amount of NGAL excretion among diabetic patients may be due to diabetic nephropathy with possible tubulointerstitial damage pathologies.**
(Clin. Lab. 2020;66:xx-xx. DOI: 10.7754/Clin.Lab.2019.190326)

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Manuscript accepted June 25, 2019

KEY WORDS

neutrophil gelatinase-associated lipocalin (NGAL), type 1 diabetes, diabetic nephropathy

INTRODUCTION

Nephropathy, one of the most significant causes of mortality and morbidity among diabetic patients, develops in approximately 30 - 50% of diabetic patients [1,2]. Prevention and delay of the onset of end-stage renal failure via early detection and intensive treatment of patients at high risk for nephropathy are among the most crucial aspects of diabetic patient follow-up and treat-

ment. Diabetic nephropathy develops mainly following glomerular damage. The patients initially have selective increase in urinary albumin secretion and later experience significant amounts of protein excretion. Detection of increased urinary albumin excretion, which is called microalbuminuria, is a laboratory tool used for the early diagnosis of diabetic nephropathy [3,4].

Studies have reported that tubulointerstitial damage with glomerular damage plays a substantial role in renal function loss in diabetic patients [5,6]. To demonstrate tubulointerstitial damage, many studies with numerous markers have been conducted. Kidney injury molecule 1 (KIM1), N-acetyl beta D glucosaminidase (NAG) and neutrophil gelatinase-associated lipocalin (NGAL) are markers that have been studied frequently in recent years [7-9]. NGAL is a member of the lipocalin group, which is a group of small transport proteins that function between extracellular and intracellular structures [10,11]. Both the serum level and the urinary excretion of NGAL, which can be intensively synthesized in tubular epithelial cells, are significantly increased in acute renal injuries [9,12]. Some studies have shown that serum NGAL may also be elevated in chronic renal diseases [13,14]. Some investigators have suggested that the increase in serum NGAL levels in patients with chronic kidney disease occurs passively due to loss of renal function and that the increase in the urinary excretion of NGAL in the active phase of the event is indicative of a reversible condition [9,11,15].

In this study, we determined if urinary excretion of NGAL can be used as an early indicator of diabetic nephropathy, which causes chronic renal damage. We compared urinary NGAL excretions both between type 1 diabetic patients and the healthy control group and between the patients with and without albuminuria within the type 1 diabetic group. We also investigated the correlation between urinary albumin and NGAL excretion. The goal of this study was to investigate if NGAL excretion is sensitive enough for clinical use as an early indicator of nephropathy in diabetic patients.

MATERIALS AND METHODS

Fifty-two type 1 diabetic patients with no history of malignancy, chronic liver failure, refractory hypertension, rapidly increasing proteinuria, chronic renal failure, or use of nephrotoxic agent were included in the study. The symptoms and findings of other diseases that could cause proteinuria in diabetic patients included to our study were not present in physical examination. Urine analysis, estimated glomerular filtration rate (eGFR), and serum creatinine levels were performed in all the patients. Patients with elevated fever, acute infections, or leukocyturia and/or bacteriuria upon urine analyses were excluded. Also, patients with hematuria, cylinduria, and eGFR < 60 mL/min were excluded from the study. Patients with normal serum creatinine levels were included in the study. Thirty healthy controls from the outpatient clinic for control or consultation were also included in the study. The local ethics committee approved the study protocol. A written consent was obtained from each participant prior to enrollment. Spot urine and fasting blood samples were taken from the patients during clinical examinations. Serum samples were immediately studied, and samples obtained for urinary albumin and NGAL measurements were stored at -80°C until the day of the analysis.

Albumin, creatinine, and NGAL were measured in the morning spot urine specimens of the patients and control group. The creatinine and NGAL concentrations were measured in two urine samples of the participants, and the average values were recorded.

Fasting blood samples were centrifuged at 1,500 x g, and serum urea, creatinine, total protein, albumin, total cholesterol (T-Chol), triglycerides (TG), high-density lipoprotein-cholesterol (HDL-Chol), and glucose levels were measured the same day. In the patient group, the hemoglobin A1c (HbA1c) level was measured in the blood sample collected in an ethylenediaminetetraacetic acid tube. In addition, the patients with diabetes were questioned regarding the presence of retinopathy and antihypertensive drugs. Concentrations of serum glucose, urea, total protein, albumin, T-Chol, HDL-Chol, TG, creatinine, and urine creatinine were measured via photometric methods using the Abbott brand kit on an Architect C16000 instrument. Urine albumin concentrations were measured immunoturbidimetrically using the Abbott analyzer. HbA1c level was measured by boronate affinity chromatography using the Premier Hb9210 (Trinity Biotech) device.

Urinary NGAL measurements were performed on an Architect i2000 using the Abbott brand kit, which utilizes the chemiluminescent microparticle immunoassay method. There was no sample over the established upper limit of measurement (1,500 ng/mL) specified by the kit manufacturer. The ratio of NGAL/Creatinine (µg/mg) was used as the ratio of measured urine NGAL concentration (ng/mL) to urine creatinine (mg/dL). For the urine NGAL level, the samples stored at -80°C were thawed on the day of the analysis and were all analyzed on the same day. Coefficients of variation (CV%) were found to be 4.07% and 4.08% in the reproducibility study of the sample pool containing NGAL at normal (mean 5.25 ng/mL) and high (154.9 ng/mL) levels, respectively.

Statistical Package for Social Sciences (SPSS) version 20.0 software was used for statistical analyses. Parameters with normal distribution are presented as the mean ± standard deviation, and nonparametric variables are provided as median (minimum-maximum). Tukey (parametric values) or Mann-Whitney *U* tests (nonparametric values) were performed following ANOVA or Kruskal-Wallis tests to analyze between group differences. Spearman's correlation analysis was applied for correlation analyses. A p-value < 0.05 was considered statistically significant.

Table 1. Demographic and biochemical parameters of healthy control and diabetic patient groups.

	Control	Type 1 DM	p	Normoalb.	Alb.
n (female/male)	30 (19/11)	52 (33/19)	-	39 (27/12)	13 (6/7)
Age (year)	37.3 ± 9.4	32.1 ± 10.4	0.01	31.2 ± 9.7 [a *]	34.5 ± 12.4
Diabetes age (year)	-	12.5 (2.0 - 30.0)	-	12.0 (3.0 - 30.0)	16.0 (2.0 - 25.0)
Antihypertensive (%)	-	21.1	-	9.3	57.1
Retinopathy (%)	-	19.2	-	13.9	35.7
Glucose (mg/dL)	89.5 ± 10.8	211.3 ± 89.0	0.001	206.9 ± 90.4 [a ***]	224.3 ± 87.0 [a ***]
HbA1c (%)	-	7.8 (5.7 - 15.7)	-	7.8 (5.7 - 15.7)	7.9 (6.6 - 11.9)
Urea (mg/dL)	23 (16 - 47)	27 (6 - 79)	0.062	25 (6 - 55)	35 (14 - 79) [a **, b **]
Creatinine (mg/dL)	0.71 (0.52 - 1.01)	0.77 (0.6 - 3.0)	0.058	0.75 (0.60 - 1.58)	0.90 (0.6 - 3.0) [a **, b **]
Total Protein (g/dL)	7.2 (6.5 - 8.2)	7.0 (4.9 - 8.0)	0.02	7.2 (4.9 - 7.9)	6.7 (5.5 - 8.0) [a **]
Albumin (g/dL)	4.2 (2.7 - 4.8)	4.0 (2.6 - 4.6)	0.01	4.1 (2.7 - 4.6)	3.7 (2.6 - 4.2) [a **, b **]
TK (mg/dL)	187 ± 46	186 ± 36	0.86	186 ± 40	184 ± 22
HDL-K (mg/dL)	45.4 ± 11.7	49.2 ± 13.2	0.15	51.8 ± 12.5 [a *]	41.6 ± 12.8 [b *]
TG (mg/dL)	119 (30 - 476)	91 (41 - 491)	0.18	88 (41 - 491) [a *]	151 (62 - 377) [b *]

[a] - Comparison with control group, [b] - Comparison with normoalbuminuric group; * p < 0.05, ** p < 0.01, *** p < 0.001.
HT - hypertension, HbA1c - hemoglobin A1c, TK - total cholesterol, HDL-K - high density lipoprotein-cholesterol, TG - triglycerides.

Table 2. Urinary albumin and NGAL excretion results of healthy control and diabetic patient groups.

	Control (n = 30)	Type 1 DM (n = 52)	Normoalb. T1DM (n = 39)	Alb. T1DM (n = 13)
Alb/Cr (µg/mg)	8 (0 - 30)	14 (4 - 3,220) [a ***]	12 (4 - 28)	105 (39 - 3,220) [b ***]
NGAL (ng/mL)	11.9 (1.0 - 176.4)	21.1 (1.8 - 328) [a *]	24.7 (1.8 - 328)	16.1 (5.7 - 181.5)
NGAL/Cr (µg /mg)	10.5 (0.92 - 215.7)	41.9 (1.3 - 753.7) [a **]	34.5 (1.3 - 753.7)	51.5 (8.6 - 525)

[a] - Comparison of control group, [b] - Comparison of normoalbuminuric group; * p < 0.05, ** p < 0.01, *** p < 0.001.

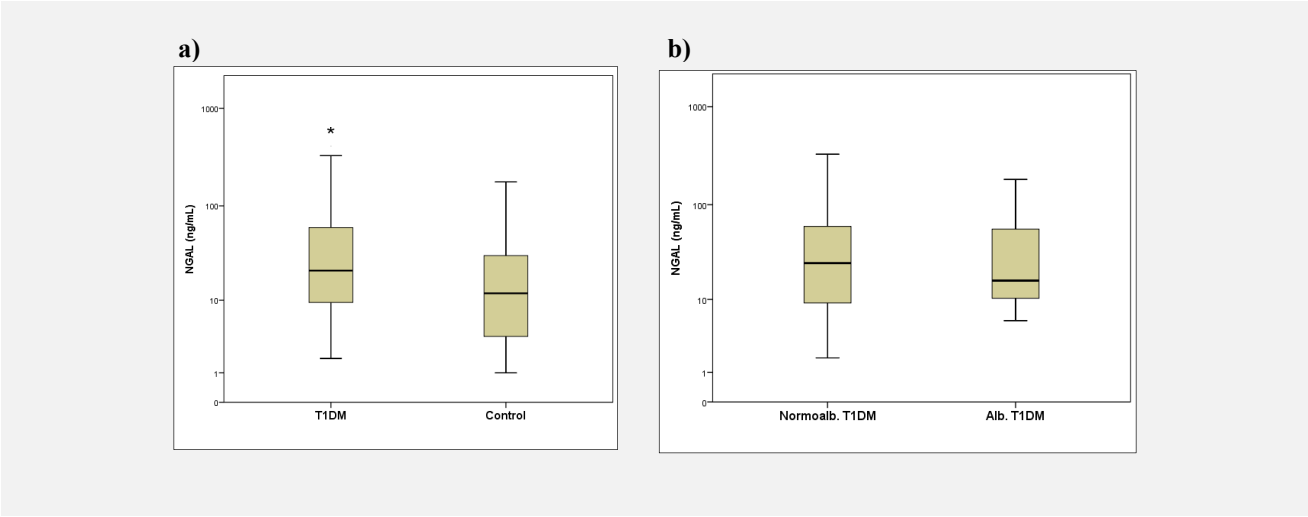

Figure 1. Logarithmic graph of urinary NGAL excretion.

a) Control group vs. type 1 diabetic patients (T1DM). b) Type 1 diabetic patients with and without albuminuria (* p < 0,05).

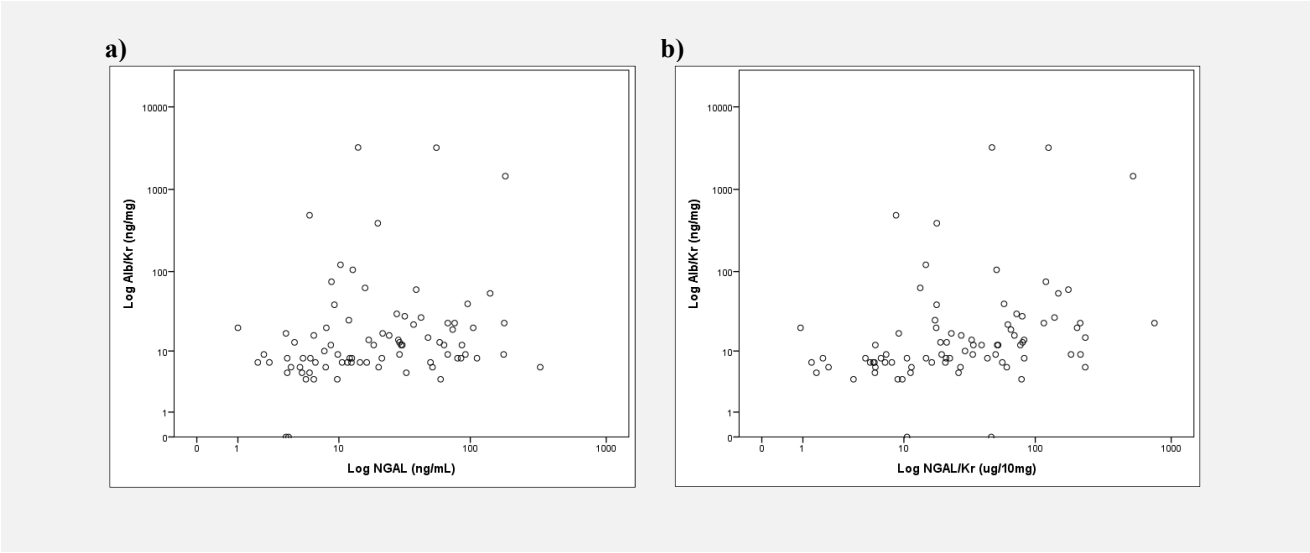

Figure 2. Correlation between urine Alb/Cr ratios of all participants in the study.

a) urine NGAL measurements (r = 0.072). b) Logarithmic values of urine NGAL/Cr ratios (r = 0.165) (p > 0.05).

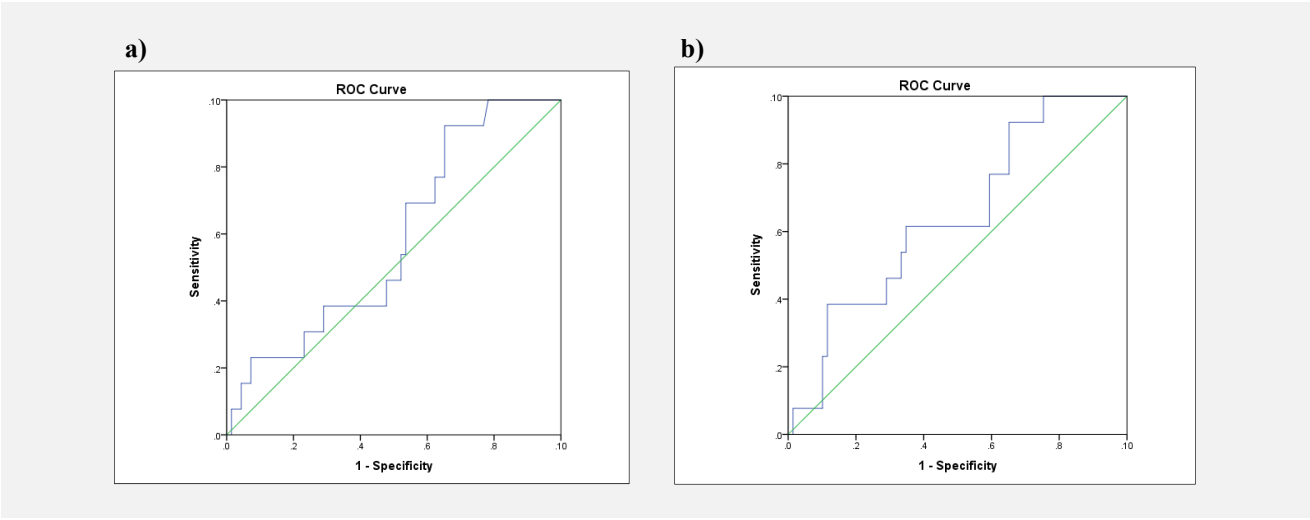

Figure 3. Type 1 diabetic patients with urinary albumin excretion greater than 30 µg/mg are considered to have diabetic nephropathy as demonstrated by ROC curves of a) urine NGAL level and b) urine NGAL/Cr ratio (p > 0.05).

RESULTS

In the study, 52 type 1 diabetic patients were separated into 2 subgroups based on their spot urine albumin/ creatinine (Alb/Cr) levels as follows: normoalbuminuria group (those with a ratio less than 30 mg/g) and albuminuria group (those with a ratio equal or greater than 30 mg/g). The control group was formed by patients within a similar age group, but the patient group was significantly younger than the control group due to the cases excluded owing to suspected urinary infection (Table 1). When the subgroups were examined, the ages of the normoalbuminuria and the albuminuria groups were similar. Serum glucose, HbA1c, and diabetes mellitus age did not differ between normoalbuminuria and albuminuria subgroups. Albuminuria patients had higher creatinine and TG values but lower albumin and HDL-Chol levels compared to normoalbuminuric type 1

diabetic patients.

Urinary NGAL levels, Alb/Cr ratios, and NGAL/Cr ratios of the healthy control and type 1 diabetic patient groups were compared. Diabetic patients were divided into those with and without albuminuria and compared with respect to the same parameters (Table 2). Urinary NGAL median level was 21.1 ng/mL for diabetic patients and 11.9 ng/mL for healthy controls, and the difference between the two groups was statistically significant (Figure 1a).

When diabetic patients were compared to those with and without albuminuria, the median urinary NGAL levels of normoalbuminuria and albuminuria were 24.7 and 16.1 ng/mL, respectively, but the difference was not statistically significant (Figure 1b). Statistically similar results were obtained through evaluation of the ratio of urinary NGAL excretion to creatinine excretion. The NGAL/Cr ratio was significantly higher in diabetic patients than in healthy controls ($p < 0.01$), but no statistically significant difference was found between the diabetic patients with and without albuminuria.

A correlation analysis was performed for the urine Alb/Cr ratio, the earliest clinical indicator of diabetic nephropathy, to urinary NGAL excretion and urinary NGAL/Cr ratios. There was no significant correlation between urinary Alb/Cr ratio and these parameters (Figure 2). A correlation analysis was also performed for urinary NGAL excretion-urinary NGAL/Cr ratios with duration of diabetes, HbA1c, and serum creatinine levels. There was no significant correlation between urinary NGAL excretion, urinary NGAL/Cr ratios, and these parameters. In addition, there was no statistically significant correlation between albuminuria and duration of diabetes, HbA1C, and serum creatinine levels. ROC curve analysis was performed to assess the utility of NGAL excretion as a marker of diabetic nephropathy in the urine. When the diabetic patient group was examined as two subgroups (with and without albuminuria), the area under the curve of urinary NGAL excretion in the ROC curve was found to be 0.582, and the area under the curve of urine NGAL/Cr ratio was 0.641 (Figure 3). Sensitivity and specificity of both parameters were too low to detect patients with microalbuminuria ($p > 0.05$).

DISCUSSION

The early detection of patients with a high risk of diabetic nephropathy is crucial in delaying the progression of nephropathy and preventing entry into renal failure [1,2]. Biochemical parameters, such as serum urea and creatinine, which are used to detect kidney function impairment, generally increase in stages where renal function is markedly decreased [3,5]. In everyday practice, demonstrating increased urinary albumin excretion is used as the earliest signal of diabetic nephropathy [16]. Although microalbuminuria is an important signal for diabetic nephropathy, more precise techniques are needed due to the possibility of false positive or negative results. Microalbuminuria patients do not demonstrate progression of renal parenchymal damage, and renal parenchymal damage can occur without preceding microalbuminuria in some diabetic patients [17]. These findings suggest that physiopathological changes other than glomerular injury play a role in the development of chronic renal failure due to diabetic nephropathy. Increased urinary albumin excretion is primarily due to glomerular pathologies. Tubulointerstitial damage has been investigated in patients with diabetic nephropathy and has been shown to contribute to the development of renal failure [5,6,18,19].

Recently, liver-type fatty acid binding protein (L-FABP), KIM1, NAG, and NGAL are markers that have been studied, frequently detecting tubulointerstitial damage. It has been found that urinary L-FABP showed the strongest association with tubular dysfunction. Urinary NGAL and then urinary KIM-1 followed L-FABL [20,21].

The main source of NGAL in the serum are neutrophil leukocytes. Serum NGAL concentration increases significantly, especially after bacterial infections. Under normal conditions, NGAL, which is a small protein (25 kDa), passes freely through the glomerulus to the glomerular filtrate and is reabsorbed almost entirely through the proximal tubules. Investigations have shown that NGAL can be produced in many tissues other than neutrophils [11]. Kidney tubules are one of the tissues where NGAL can be produced. In experimentally formed ischemic renal injury cases, the level of serum NGAL significantly increases. In tubular injuries, NGAL production in tubule cells is increased, and both serum NGAL levels and urinary excretion significantly increase [10,20,21]. Serum and urine NGAL measurements have been reported in various studies to be appropriate to be used as the earliest indicator of acute renal injury [11,12,22,23]. Bunz et al. found a significant relationship between the level of urinary NGAL and the severity of acute kidney injury [24]. In this study, it was observed that the length of hospitalization in patients with high urinary NGAL levels was long [24]. As a cue of chronic renal injury, the use of NGAL is not as clear as in acute injury. Studies of patients with chronic renal disease have led to suggest that NGAL is not an indicator of chronic renal dysfunction but rather an indicator of the acute tubular effects of the disease [13,14].

Studies on the use of NGAL in early detection of diabetic nephropathy are few and controversial [7,8,10,25-29]. Fu et al. showed that the level of serum NGAL is reduced in diabetic patients with glomerular hyperfiltration [30]. Glomerular hyperfiltration in early stages of diabetic nephropathy has shown a negative correlation, though not statistically significant, with serum NGAL levels ($r = -0.159$). Another study conducted by Fu et al. emphasized that urine NGAL excretion is increased in type 2 diabetic patients and that this is a promising laboratory method in terms of showing early tubular injury [31]. Bolignano et al. studied 56 type 2 diabetic patients

and 18 healthy subjects and showed that NGAL excretion is significantly higher among diabetics compared to the control group, but they showed no significant difference between normoalbuminuric and microalbuminuric groups [32]. This group also demonstrated that serum NGAL levels are also elevated with increased urinary NGAL excretion among diabetics. Based on these findings, Bolignano et al. suggested that the increase in NGAL may be due to an adaptation to diabetic changes. Kim et al. investigated tubular damage markers in urine from 118 patients with type 2 diabetes and found that the urinary NGAL levels are significantly higher in the microalbuminuria group than in the normoalbuminuric group [33], and they did not report any correlation between albuminuria and urinary excretion of NGAL in their study [34].

In our study, we included only type 1 diabetic patients. Patients were divided into 2 groups, namely, patients with and without albuminuria and were compared with respect to urinary NGAL excretion (Table 2). Urinary NGAL excretion was significantly higher in type 1 diabetic patients than in healthy controls (Figure 1a). When we compared the ratio of urinary NGAL excretion to creatinine excretion, we found that this ratio was also significantly higher among diabetic patients compared to the healthy controls. However, when we separated the diabetic patients into 2 groups as those with and without albuminuria, there was no difference in NGAL excretion and NGAL/creatinine excretion ratio between subgroups although the albuminuria group had very high urinary albumin excretion (Table 2). Furthermore, no correlation was found between albuminuria level and urinary NGAL excretion or NGAL/creatinine ratio (Figure 2). When we considered patients with microalbuminuria as having diabetic nephropathy, we concluded that the amount of NGAL excretion had low sensitivity and specificity for detecting diabetic nephropathy (Figure 3). When microalbuminuria is accepted as a reliable indicator of diabetic nephropathy in type 1 diabetic patients, our findings indicate that urinary NGAL measurement is unlikely to be used as a reliable laboratory method to detect nephropathy.

Studies of patients with proteinuria have shown that proteinuria may lead to renal tubular cell damage and to chronic renal failure in the long-term. Proteinuria has also been shown to cause inflammation and damage to tubular epithelial cells [35,36]. Bolignano et al. showed significantly high levels of urinary NGAL excretion in patients with massive proteinuria due to non-diabetic causes [37], and they hypothesized that the cause of increased NGAL excretion in the case of proteinuria at nephrotic levels may be due to damaged glomeruli or to the response of distal tubules to proteinuria. In our study, we could not detect urinary NGAL excretion levels in a parallel manner to albuminuria. The fact that the patients in our study had low levels of proteinuria or that protein excretion other than albumin did not increase may explain why our results differed from those of other studies.

Under normal conditions, NGAL, a small protein (25 kD), passes fully through the glomeruli to the filtrate and is completely reabsorbed in the proximal tubules. Approximately 5 ng/mL NGAL is excreted via urine. In our study, urinary NGAL excretion was significantly higher in diabetic patients than in healthy controls. This increase may indicate the development of diabetic nephropathy, but we did not observe urinary NGAL excretion correlating with urinary albumin excretion. It is known that some diabetic patients may develop end-stage renal failure without microalbuminuria. Our results showing increased urinary NGAL excretion in type 1 diabetic patients suggests that physiopathological mechanisms other than glomerulopathy may play a role in the development of diabetic nephropathy. In normoalbuminuric diabetic patients, the increase in urinary NGAL excretion may be due to tubulointerstitial injury, and detection of increased levels of NGAL among diabetic patients may be due to tubulointerstitial damage. The renal-angiotensin system plays an important role in the pathogenesis of diabetic nephropathy. Increased glomerular pressure due to increased angiotensin levels before microalbuminuria development may lead to basal membrane pathology, and angiotensin may increase renal tubular apoptosis in addition to intra-glomerular injury [38]. Increased urinary NGAL excretion without urinary albumin excretion in our study suggests that angiotensin may have tubulointerstitial effects prior to glomerular effects. These patients may develop diabetic nephropathy as a result of tubulointerstitial damage in the long-term. Lacquaniti et al. suggested that NGAL excretion may be increased in non-microalbuminuric individuals and may be indicative of earlier renal injury in type 1 diabetic patients [39]. To better interpret the role of tubulointerstitial pathologies in the development of diabetic nephropathy, type 1 diabetic patients with high and low excretion of NGAL should be investigated with respect to their renal function loss rate and end-stage renal failure development pace.

The most important limitation of our study is that it is not a long-term study following patients in terms of progression of diabetic nephropathy. Second, although there are many tubular dysfunction markers, in this cross-sectional study microalbuminuria was considered as a definite indicator of diabetic nephropathy because of its wide use and comparison was performed with microalbuminuria. By interpreting our results according to the presence of microalbuminuria, it can be concluded that NGAL excretion is not a reliable parameter in detecting diabetic nephropathy. However, many diabetics develop end-stage renal failure without microalbuminuria. By interpreting our results according to the presence of microalbuminuria, it can be concluded that NGAL excretion is not a reliable parameter in detecting diabetic nephropathy. However, many diabetics develop end-stage renal failure without microalbuminuria.

CONCLUSION

Urinary NGAL excretion in type 1 diabetic patients is found to be increased over a wide range, but it does not correlate with urinary albumin excretion. In this regard, urinary NGAL excretion should not be used as an alternative to microalbuminuria in detecting diabetic nephropathy. The higher detection rate of NGAL excretion among type 1 diabetic patients compared to healthy controls may be due to the presence of diabetic nephropathy among non-microalbuminuric cases. These patients may be more likely to develop end-stage renal failure. There is a need for further studies examining the long-term changes in NGAL excretion levels and renal functions.

Source of Funding:
No funding was received for this study.

Declaration of Interest:
The authors state that they have no conflict of interest.

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