Urinary Neutrophil Gelatinase-Associated Lipocalin Levels in Diabetics; Correlation with Albuminuria

Diyabetiklerde İdrar Nötrofil Jelatinazla İlişkili Lipokalin Düzeyleri ve Albuminüri ile Korelasyonu

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ABSTRACT Objective: In this study, we aimed to investigate urinary neutrophil gelatinase-associated lipocalin (NGAL) levels of type II diabetic patients with various degrees of albuminuria and compare it with healthy controls. **Material and Methods:** The study included a total of 228 individuals; 153 type II diabetic patients and 75 healthy controls. Patients were divided into 3 groups as normoalbuminuric group (group 1) with microalbumin/creatinine (mau/cr) ratio<20 mg/g cr, microalbuminuric group (group 2) with mau/cr ratio 20-300 mg/g cr and macroalbuminuric group (group 3) with mau/cr ratio >300 mg/g cr. Urinary NGAL levels were measured by chemiluminescent microparticule immunoassay at Architect i400SR analyzer (Abbott, USA). **Results:** NGAL/cr levels of patient groups differed significantly from each other (p=0.0005). No significant difference was observed between NGAL levels of normo and microalbuminuric patients while both groups differed significantly from macroalbuminuric group (p<0.05). NGAL/cr levels were found to correlate better with protein/cr (r=0.4960, p<0.0001) than microalbumin/creatinine (mau/cr) (r=0.2606, p=0.0011) in macroalbuminuric group. After adjusting for GFR, NGAL/cr showed no association with mau/cr while it was even highly correlated with Protein/cr (r=0.6230,p<0.0001). A significant negative correlation was found between NGAL/cr and GFR in macroalbuminuric patients (r=-0.4045, p=0.0048). In macroalbuminuric group; serum creatinine, Protein/cr and BUN values were independent predictors for NGAL/cr (R^2=0.6690, p<0.001). **Conclusion:** Increase in urine NGAL levels seems to be the result of tubular insufficiency added on glomerular damage in macroalbuminuric diabetics. It doesn't seem to be early marker in diabetic nephropathy.

Key Words: Diabetic nephropathies; diabetes mellitus, type 2; LCN2 protein, human

ÖZET Amaç: Biz bu çalışmada, farklı düzeylerde albuminüri ve proteinürisi olan tip II diyabetli hastalarda idrar nötrofil jelatinazla ilişkili lipokalin (NGAL) düzeylerini çalışıp sağlıklı kontrollerle karşılaştırmayı amaçladık. **Gereç ve Yöntemler:** Çalışma; 153 tip II diyabetli hasta ve 75 sağlıklı kontrol olmak üzere toplam 228 olgudan oluşturuldu. Hastalar mikroalbuminüri düzeylerine göre 3 gruba ayrıldı. Mikroalbumin/kreatinin (mau/cr) oranı <20 mg/g cr olanlar normoalbuminürik grup (grup 1), mau/cr oranı 20-300 mg/g cr olanlar mikroalbuminürik grup (grup 2) ve mau/cr oranı >300 mg/g cr olanlar makroalbuminürik grup (grup 3) olarak adlandırıldı. İdrar NGAL düzeyleri kemilüminesan mikropartikül immunassay yöntemle Architect i400SR (Abbott, USA) cihazında çalışıldı. **Bulgular:** Üç grup hastanın NGAL/cr değerleri birbirinden farklı bulundu (p=0,0005). İkili karşılaştırmalarda normo ve mikroalbuminürik hastalar arasında anlamlı farklılık saptanmazken her iki grup da makroalbuminürik gruptan farklı bulundu (p<0,05). Makroalbuminürik grupta NGAL/cr'nin protein/cr ile olan korelasyonu (r=0,4960, p<0,0001) mau/cr'den (r=0,2606, p=0,0011) daha iyi düzeyde bulundu. GFR düzeltmesi yapıldıktan sonra NGAL/cr ve mau/cr ilişkisi kaybolurken NGAL/cr ve Protein/cr uyum katsayısı arttı (r=0,6230, p<0,0001). Makroalbuminürik hastalarda NGAL/cr ile GFR arasında anlamlı negatif korelasyon saptandı (r=-0,4045, p=0,0048). Makroalbuminürik hastalarda serum kreatinin, Protein/cr ve BUN düzeylerinin NGAL/cr düzeylerini belirleyici bağımsız değişkenler olduğu saptandı (R^2=0,6690, p<0,.001). **Sonuç:** Makroalbuminürik hastalarda idrarda NGAL/cr artışı glomerular hasara eklenen tubular yetersizlik sonucu oluşur ancak NGAL/cr testi diyabetik nefropatinin erken belirteci gibi görünmemektedir.

Anahtar Kelimeler: Diyabetik nefropati; diabetes mellitus, tip 2; LCN2 protein, insan

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Diabetic nephropaty (DN), is one of the most common microvascular complications of diabetes mellitus (DM) and the leading cause of end stage renal disease.[1,2] It occurs in 20-40% of patients with diabetes.[3] As prevalence of type 2 DM is steadily increasing, the number of patients with DN is expanding day by day. Adverse outcomes of renal failure can be prevented or delayed through early detection and treatment.[2]

Urinary albumin excretion (AER), is the most frequently used marker for early detection of renal damage. Clinically, diabetic nephropathy is characterized by the onset of hyperfiltration and microalbuminuria that are followed by sustained proteinuria with declining renal function.[4] As an indicator of renal damage, however, urinary albumin excretion has some limitations. First, some patients do not progress to macroalbuminuria but remain at microalbuminuria or even regress to normoalbuminuria.[5] Second, some patients follow a non-albuminuric pathway to renal impairment, where the increases in AER and decreases in glomerular filtration rate (GFR) are not closely related.[6] Thus, more sensitive and specific renal biomarkers than AER will be valuable in predicting early kidney injury and the progression or regression of renal damage in type 2 DM patients.

Recent studies have shown that not only glomerular but also tubulointerstitial damage is an important factor in the progression of diabetic nephropathy.[7] The microalbuminuria and proteinuria, in general, are reflectors of glomerular damage. Interestingly, the pathophysiology of proteinuria and tubulointerstitial damage has been found to be interwined. By reabsorption of increased amounts of protein from the tubular lumen, proinflammatory and profibrotic responses are induced in tubular cells leading to inflammation and fibrosis in the tubulointerstitial compartment.[4] Therefore, markers of tubular damage could potentially be useful in the evaluation of prognosis and for monitoring the effectiveness of treatment in DN.[8]

Recently, several different markers of tubular damage have been proposed. NGAL also known as lipocalin 2 (LCN2), is a member of the lipocalin family which consists of more than 20 low molecular weight proteins. It is stored mainly in the specific granules of neutrophils, but also expressed at very low-levels in several human tissues, including kidney, trachea, lungs, stomach, and colon.[9] NGAL is found to possess diverse functions such as transporting and activating matrix metalloproteinase 9, inducing apoptosis, regulating immune response and so on. NGAL can be filtered and reabsorbed by kidneys and because of this characteristic it is thought to serve as a marker for both glomerular filtration rate (GFR) and proximal tubular function. NGAL is hyperproduced in the kidney tubules within a few hours after deleterious stimuli.[10] Several studies on acute kidney injury (AKI) have shown that both serum and urine NGAL levels increase within a few hours after the onset of AKI, before the increase in serum creatinine. This makes NGAL a sensitive and specific biomarker with significant potential for early diagnosis of AKI.[10] On the other hand, several studies have also defined the role of NGAL in chronic kidney disease (CKD) and showed that serum and urinary NGAL levels are markers of kidney disease and severity of CKD.[11-14] It is now widely accepted that in some CKD-associated diseases such as DN, the rate of deterioration in renal function and the overall outcome are more accurately associated with tubulointerstitial damage than glomerular lesions whether the primary pathology is glomerular or not.[15]

In this study, we aimed to investigate urinary NGAL levels of diabetic patients with various degrees of albuminuria/proteinuria and compare it with healthy controls.

MATERIAL AND METHODS

The study included a total of 228 subjects; 153 type II DM patients followed by the diabetes clinic of our hospital and 75 healthy control subjects. Fasting serum creatinine and BUN levels were measured. Morning first urine samples were collected on the same day. Routine urine examinations were done and microalbumin, protein and creatinine levels were measured from the same specimen. Portions of urine samples were stored at -80° C until the day of analysis for NGAL. The healthy population consisted of people who visited the hospital for peri-

odic medical checkups and proved to have no specific clinical or laboratory problems. Diabetics with accompanying chronic diseases, infections or inflammatory conditions and renal disease other than diabetic nephropathy were excluded. The mean of three microalbumin values measured during last six months with 2 months intervals were calculated and these mean values were used instead of a single microalbumin value. Patients were divided into 3 groups as normoalbuminuric group (group 1) with microalbumin/creatinine (mau/cr) ratio <20 mg/g cr, microalbuminuric group (group 2) with mau/cr ratio 20-300 mg/g cr, macroalbuminuric group (group 3) with mau/cr ratio >300 mg/g cr. GFR of the patients were calculated with Modification of Diet in Renal Disease (MDRD) formula: $MDRD=186 \times [\text{serum creatinine (mg/dL)}^{-1.154}] \times age^{-0.203}$. A correction factor of 0.742 was used for women.[16]

NGAL levels were measured by chemiluminescent microparticule immunoassay with urine NGAL kit (ref: B1P37T) at Architect i400SR (Abbott, USA). Urine albumin levels were measured by immunoturbidimetric method with urine albumin kit (ref: OSR6167) and urine creatinines with urine creatinine kit (ref: OSR6578) at AU 2700 (Beckman Coulter, USA) Serum creatinine and BUN levels were measured at AU 2700 (Beckman Coulter, USA) autoanalyzer with routine methods.

Statistical analysis were carried out using MedCalc (Medical Calculation Version 12.4.0; Belgium). Mann Whitney U, Kruskal-Wallis, Spearman correlation and multiple regression analysis were used. Concentrations were expressed as median (2.5-97.5 percentile). Study was approved by our institute's scientific commitee and all patients signed an informed consent.

RESULTS

The inter-assay CV's of our method for NGAL were; 2.86% for 250 ng/mL and 5.8% for 12.3 ng/mL.

NGAL/cr values of healthy controls were found significantly different from total diabetics (p=0.0014) (Table 1).

153 patients were classified as; 54 normoalbuminuric (35%), 52 microalbuminuric (34%) and 47(31%) macroalbuminuric. Mau/cr values of Group 1 was 4.9 (1.857-17.848); Group 2: 90.2 (20.5-246); Group 3: 670 (334-1677) mg/g cr. NGAL/cr levels of patient groups differed significantly from each other in multiple comparisons (p=0.0005). Group 1 and 2 both differed significantly from group 3 in post hoc comparisons (p<0.05) But there were no significant difference between group 1 and group 2 (Table 2).

There was no significant correlation between NGAL/cr and GFR in total diabetic group while a significant negative correlation was found in macroalbuminuric patients (r=-0.4045, p=0.0048). There was no significant correlation between patient ages and NGAL/cr or patient ages and GFR in total diabetic group. Urine NGAL/cr levels significantly correlated with urine protein and urine microalbumin levels in both total diabetics and

TABLE 1: Study group characteristics.

Characteristics	Healthy Control	Diabetic Group	P Value
Number of patients	75	153	-
Age (years)	40(21.7-61.6)	62(46-74.6)	<0.0001
Gender (women/men)	46/29	78/75	0.0879
Diabetes duration (years)	-	14(5.3-29.6)	-
GFR (ml/min)	114(74.5-202)	85(23.6-186)	<0.0001
Serum creatinine (mg/dL)	0.64(0.39-1.05)	0.82(0.44-2.434)	<0.0001
Protein/cr (mg/g cr)	25(2.3-163)	137.3(14.45-4070)	<0.0001
BUN (mg/dL)	11.4(6-19.9)	15.100(7.165 - 48.11)	<0.0001
NGAL/cr (ng/g cr)	10.8(1.20-100)	12.785(1.030-275.884)	0.0014

TABLE 2: Characteristics of diabetic group.

Characteristics	Group 1	Group 2	Group 3	P value
Number of patients	54	52	47	-
Age (years)	58 (41.7-71.4)	62 (49.8-74.2)	65 (44.7-79.6)	0.0203
Gender (women/men)	33/21	27/25	18/29	0.07
Weight (kg)	80 (51.7-100)	82.5 (57.6-116)	82 (59.6-103)	0.6892
Diabetes duration (years)	12 (3.8-23.4)	15.5 (4.8-29.6)	14(6-33.9)	0.0071
GFR (ml/min)	96 (35.8-152.7)	95 (25.4-197.8)	64 (9.67-145.8)	< 0.0001
Serum creatinine (mg/dL)	0.78 (0.47-1.64)	0.76 (0.41-2.70)	1.04 (0.54-5.89)	< 0.0001
Protein/cr (mg/g cr)	52 (10-296)	133 (39.5-1359)	899 (160-6428)	< 0.0001
BUN (mg/dL)	13.5 (8.8-29.4)	14.95 (7-50.8)	17.6 (2.17-64.6)	0.0004
NGAL/cr (ng/g cr)	10.6 (0.46-125)	8.83 (1.11-340.5)	30.9 (1.123-446)	0.0005

macroalbuminuric patients. In both cases (total diabetics and Group 3 patients) correlation was better with protein levels than with microalbumin levels; besides it was better in group 3 than in total group (Table 3). In Group 3 no association with mau/cr was found after adjusting for GFR.

We investigated the association of age, weight, diabetes duration, GFR, serum creatinine, mau/cr, protein/cr and BUN levels with NGAL/cr values in macroalbuminuric patients. Serum creatinine, Protein/cr and BUN values were independent predictors for NGAL/cr (R^2=0.6690, p<0.001).

DISCUSSION

In this study, we investigated whether a tubular damage marker urine NGAL could contribute to early diagnose of DN compared with recently used markers urine microalbumin and protein. Urine NGAL/cr levels of diabetic patients with different stages of albuminuria were evaluated. NGAL/cr values of healthy controls were found significantly different from total diabetics. NGAL/cr levels were found to correlate better with protein/cr than mau/cr in total diabetics and macroalbuminuric patients. After adjusting for GFR the association with NGAL/cr with Protein/cr still remained significant while no association with mau/cr was present. We thought that urinary NGAL/cr levels seemed to be a result of tubular injury and increased as DN progresses.

Several studies investigated renal injury in pathogenesis of DM. mRNA expression of NGAL was found significantly higher in diabetic/obese mice or obese human beings, and associated closely with insulin resistance and hyperglycemia.[17] It is claimed that NGAL is produced principally by the injured tubule cells to prevent kidney from early injury. It was found to be able to induce cell apoptosis through an autocrine/paracrine pathway. NGAL may protect the diabetic kidney through restraining inflammation reaction and inducing apoptosis of neutrophil granulocytes in the renal tubules and interstitium.[18] The injured tubules likely resulted in both increased production and re-

TABLE 3: Correlation of NGAL/cr with GFR, Mau/cr and protein/cr in total diabetics and in Group 3.

Variables	Diabetic group (n=75)		Group 3 (n=47)		Group 3 (GFR adjusted)	
	r	p	r	p	r	p
GFR (ml/min)	-0.08576	0.2919	-0.4045	0.0048	-	-
Mau/cr (mg/g cr)	0.2606	0.0011	0.2930	<0.0001	0.1883	0.2102
Protein/cr (mg/g cr)	0.4960	<0.0001	0.6230	<0.0001	0.5838	<0.0001

duced reabsorption of NGAL. Consistent with these reports, results of our study showed that urinary NGAL levels were significantly elevated in diabetic patients compared to the normal control group. In our study, patients with macroalbuminuria showed higher NGAL levels than all the other groups demonstrating presentation of tubular damage with worsening glomerular functions. Similarly in Yang's study, urine NGAL levels of macroalbuminuric group were found higher than normo and microalbuminuric groups, although no differences were found between normo and microalbuminuric groups.[18] A few studies found high levels of NGAL in normo and microalbuminuric patients groups and introduced urine NGAL as an early marker of tubular damage in diabetics.[19,20] We were unable to show an early increase in NGAL/cr levels in normo and microalbuminuric patients thus we can not regard NGAL as an early predictor; but it seemed to closely relate with tubular injury as indicated by high correlation with proteinuria in later stages of DN. Proteinuria is a clinical situation with both glomerular and tubular aspects. Sustained passage of plasma proteins into tubular lumen is a source of injury to epithelial cells. This leads to tubular inflammation, tubular function loss and then to tubulointerstitial fibrosis which ultimately signals the appearence of an irreversible renal impairment, leading to chronic renal failure.[21] In diabetic kidney disease, progression to chronic kidney disease is attributed not only to glomerular lesions but also on tubulointerstitial damage.[22,23] In Bolignano study, urine NGAL/cr concentrations were found directly correlated with the extend of protein loss in nephritic patients.[21] Patients with higher NGAL/cr levels were shown to have a greater risk of developing a severe decrease in renal function compared to others. The augmented urine NGAL excretion in our patients may be the consequence of an increased loss of circulating NGAL through the damaged glomerular membrane, as happens for other blood proteins. This suggestion would be partially confirmed by the finding that urine NGAL levels correlate well with the extent of proteinuria.

In macroalbuminuric patients with a decreased GFR, we found a correlation between NGAL and urinary excretion of protein and albumin. Albuminuria, resulting from glomerular damage didn't significantly correlate with NGAL, when adjusted for GFR. But proteinuria levels still correlated with levels of NGAL. Increase in urine NGAL levels was because of the same pathophysiologic mechanism that caused proteinuria; that is the tubular function loss following primary glomerular injury.This direct correlation between proteinuria and urinary NGAL levels are shown in previous reports.[11,13,21] Whether tubular injury presenting after glomerular lesions may be a result of the tubulotoxic effect of albumin and other proteins that are leaked into the tubular lumen is still debated.[12,24-26]

CONCLUSION

Urinary NGAL/cr levels seemed to be a result of tubular injury and increased as DN progresses. But it doesn't seem to add more information as an early marker of DN in current clinical practice.

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