Urinary N-Acetyl-Beta-D Glucosaminidase Activity is Associated with Inflammation and Proteinuria in Diabetic and Non-Diabetic Patients with Different Stages of Chronic Kidney Disease

İdrar N-Asetil-Beta-D Glukosaminidaz Aktivitesinin Değişik Evrelerdeki Diyabetik ve Diyabetik Olmayan Kronik Böbrek Hastalarında İnflamasyon ve Proteinüriyle İlişkisi

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ABSTRACT

OBJECTIVE: Clinical studies have demonstrated that tubulointerstitial rather than glomerular pathology correlates with the degree and progression of renal impairment. Urinary n-acetyl-beta-D-glucosaminidase (NAG) is a biomarker of tubular damage, shown to be elevated in patients with glomerulonephritis and acute kidney injury. However, it has not been assessed longitudinally in chronic kidney disease (CKD). The aim of the present study was to determine urinary NAG activity and its possible associations with metabolic and inflammatory parameters in CKD.

MATERIAL and METHODS: A total of 72 patients (mean age: 64.5±15.7) with stage 1-5 CKD were included. Of the 72 patients 23 (32%) had diabetic nephropathy and 49 (68%) had different types of primary glomerular diseases. Fasting blood samples were collected to analyse complete blood count, urea, creatinine, albumin, lipid parameters, C-reactive protein, uric acid and parathyroid hormone. 24-hour urine was collected to determine protein excretion. Urinary NAG and creatinine levels were analysed from the first morning urine samples. The NAG index (urinary NAG/ creatinine) was used to exclude dilutional errors.

RESULTS: Mean eGFR was 38.3±21.7 ml/min. The urinary NAG index was significantly higher in stage 3 compared to stage 2 (32.1±23.5 vs. 7.5±3.3 U/gr-creatinine; p=0.002) and lower in stage 5 compared to stage 3 CKD (8.2±7.6 vs. 32.1±23.5; p=0.017). The urinary NAG index was positively correlated with 24-hour urine protein excretion (r=0.43; p=0.0001) and serum CRP (r=0.549; p=0.04) and negatively correlated with hemoglobin levels (r-0.394; p=0.004).

CONCLUSION: The present study demonstrated that urinary NAG correlates with systemic inflammation and proteinuria and may be associated with progression of CKD.

KEY WORDS: Chronic kidney disease, Proteinuria, Inflammation, N-acetyl-beta-D-glucosaminidase

ÖZ

AMAÇ: Klinik çalışmalar glomerüler patolojiden ziyade tubulointerstisyel hasarın böbrek yetmezliği derecesi ve ilerlemesi ile ilişkili olduğunu göstermiştir. İdrarda saptanan n-asetil-beta-D-glukosaminidaz (NAG) enzimi glomerülonefrit ve akut böbrek hasarında yükselen bir tübüler hasar biyomarkeridir ancak NAG düzeyi, kronik böbrek hastalığının (KBH) değişen aşamalarında değerlendirilmemiştir. Çalışmada, farklı evrelerdeki kronik böbrek hastalarında idrar NAG aktivitesi, NAG aktivitesi ile metabolik ve inflamatuvar parametreler arasında olası ilişki araştırılmıştır.

GEREÇ ve YÖNTEMLER: Evre 1-5 KBH tanılı toplam 72 hasta (ortalama yaş:64,5 ± 15,7) çalışmaya alındı. Hastaların 23'ünde (% 32) diyabetik nefropati ve 49'una (% 68) farklı türde primer glomerüler hastalıklar mevcuttu. Açlık kan örneklerinde C-reaktif protein, ürik asit ve paratiroid hormon, hemogram, üre, kreatinin, albümin, lipid parametreleri çalışıldı. Kantitatif protein analizi için 24 saatlik idrar örnekleri kullanıldı. İdrar NAG ve kreatinin seviyeleri sabah ilk idrar örneklerinden analiz edildi. Dilüsyonel hataları dışlamak için NAG indeksi (idrar NAG / kreatinin:mg/gr) hesaplanarak değerlendirilmelerde kullanıldı.

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BULGULAR: İdrar NAG indeksi(iNAG) evre 3 hastalarda evre 2'ye göre daha yüksek (32,1±23,5 vs 7,5±3,3 U/gr-kreatinin; p=0,002), evre 5 hastalarda ise evre 3'e göre daha düşük (8,2±7,6 vs 32,1±23,5 ;p=0,017) idi. İNAG, 24 saatlik idrar protein atılımı ve (r=0,43; p=0,0001), CRP (r=0,549; p=0,04) ile pozitif, hemoglobin düzeyleri ile negatif korele idi (r=-0,394; p=0,004).

SONUÇ: Çalışma idrar NAG indeksinin KBH'da sistemik inflamasyon ve proteinüri ile ilişkili olduğunu ve progresyon riskini belirlemede yararlı olabileceğini düşündürmektedir.

ANAHTAR SÖZCÜKLER: Kronik böbrek hastalığı, Proteinüri, İnflamasyon, n-asetil-beta-D-glukosaminidaz

INTRODUCTION

Chronic kidney disease (CKD) is a major health problem worldwide. Early identification of signals that reveal progression is important for monitoring response to interventions. Existing measures such as estimated glomerular filtration rate (eGFR) and proteinuria assist the stratification and detection of patients that are likely to show progression (1,2). However proteinuria has limitations as a biomarker of CKD progression (3).

N-acetyl-beta-D-glucosaminidase (NAG) is predominantly a biomarker of proximal tubular damage, but may also be a biomarker of injury to other parts of the nephron (4-7). NAG is an enzyme of hydrolase class that is abundant in the kidney, predominantly in the lysosymes of proximal tubular cells. It is physiologically excreted in low amounts in urine as a consequence of the normal exocytosis process (8). The increased excretion of NAG is thought to be a specific marker of functional tubular impairment in many renal pathologies (9). An experimental study has demonstrated that urinary NAG activity is a measure of altered function of renal tubules due to increased protein excretion that is presented to tubular cells rather than a simple indicator of damage. A nested case-control study from the Diabetes Control and Complications Trial found that baseline urinary NAG predicted both micro- and macro-albuminuria in type 1 diabetics (10). NAG is also elevated in the urine of the patients with glomerulonephritis compared to healthy controls (11). A substantial number of studies demonstrated that NAG has been a useful marker of acute kidney injury (AKI) (12,13) AKI is increasingly recognised as a prelude to CKD (14). However NAG activity and its relationship with stages of CKD, amount of proteinuria or markers of inflammation has not been assessed longitudinally in patients with CKD. The present study aimed to investigate the association between urinary NAG activity and glomerular filtration rate, level of proteinuria, inflammation and possible tubulotoxic metabolic parameters (uric acid, triglyceride, cholesterol) in diabetic and non-diabetic patients with different stages of CKD.

MATERIALS and METHODS

At the begining of the study we had a group of 230 patients with chronic kidney disease (CKD) who were at different stages of CKD with different etiologies and were attending regular clinical visits. We aimed to investigate a possible relationship between urinary N-acetyl B-D- Glucosaminidase (uNAG; as a biomarker of renal injury) and degree of inflammation, proteinuria, and estimated glomerular filtration rate (eGFR). The expected r coefficient between these quantitative continuous variables was between 0.25 and 0.5 with 80% power and 5% tolerance (two-sided). The suitable sample size for our cross-sectional study was therefore between n=23 and n=90. After we applied the exclusion criteria, 72 patients were eligible for the study (Figure 1).

A total of 72 patients (38 males, 34 females; mean age: 64.5±15.7 years) were involved in the study. All of the patients were attending regular monthly or bi-monthly clinical visits to the Nephrology Clinic with a diagnosis of CKD. Of the 72 patients, 23 (32%) had diabetic nephropathy and 49 (68%) had biopsy-proven diagnoses of different types of primary glomerular diseases (membranous nephropathy in 16, focal segmental glomerulosclerosis in 14, membranoproliferative glomerulonephritis in 7, IgA nephropathy in 7, minimal change disease in 3 and amyloidosis in 2 patients). The study protocol was approved by the Baskent University Faculty of Medicine Local Ethics Committee.

The inclusion criteria were established as follows: age 18-70 years, chronic diabetic or non-diabetic nephropathy, blood pressure above 125/75 mmHg and below 160/95 mmHg, no steroids or immunosuppressive treatment for a minimum of six months before the study, eGFR>10 ml/minute and dialysis treatment has not been started yet. All the patients were taking at least one angiotensin converting enzyme inhibitor (ACEi) or angiotensin receptor blocker (ARB). However, none of the patients were on treatment with both ACEi and ARB at the same time. Diabetic patients involved in the study were under treatment with insulin, oral hypoglycemic agents or both and the hemoglobin A1C levels were below 7% as studied in the last month. All of the patients were taking oral iron preparations (ferroglycine sulphate), oral phosphate binders (calcium carbonate, calcium acetate or sevelamer), oral sodium bicarbonate, vitamin B complex and essential amino acid preparations as supportive treatment of CKD. However, none of the 72 patients was under treatment with Erythropoietin, intravenous iron or active vitamin D preparations at the time of the study.

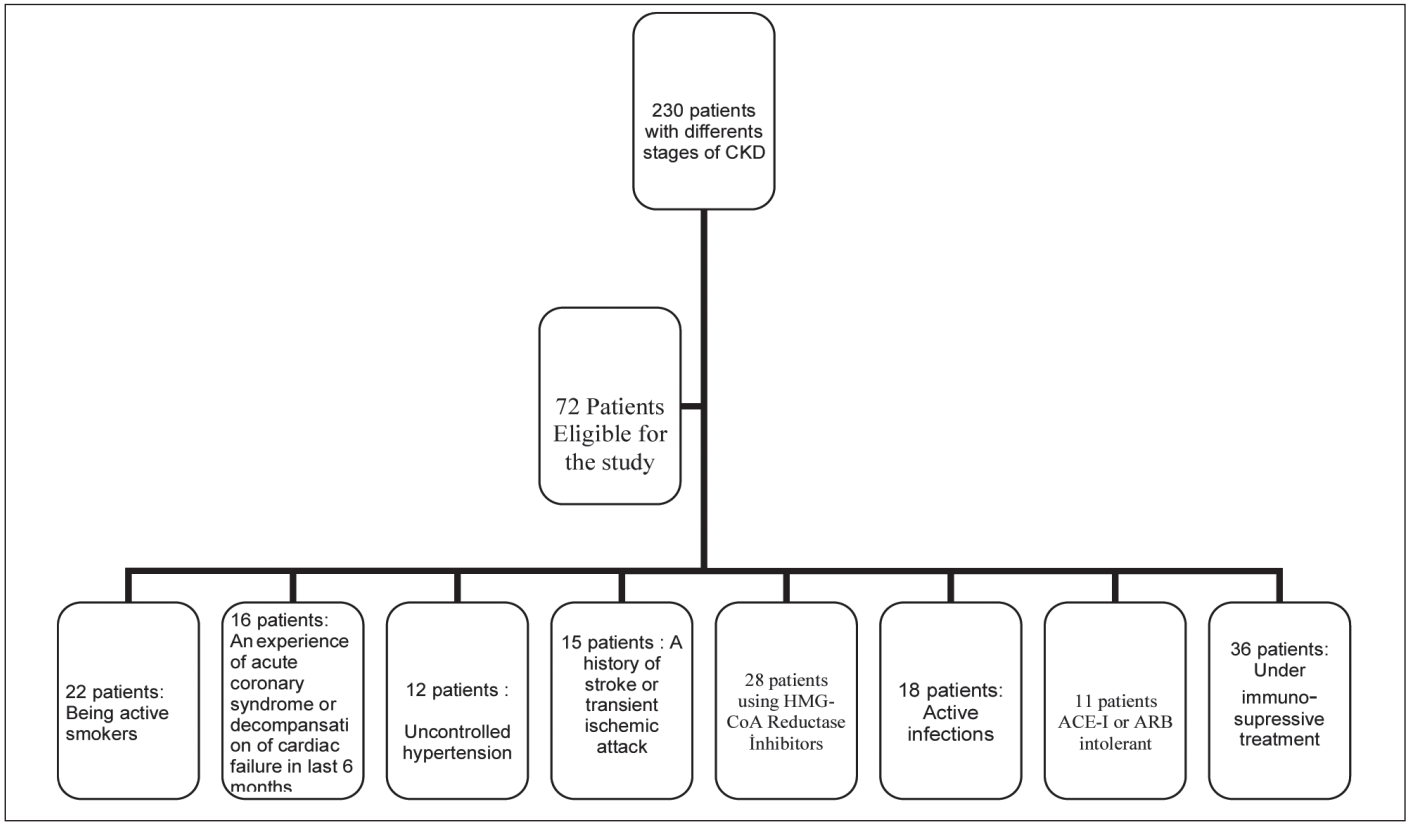

Figure 1: *Number of patients included in the study and number of patients excluded from the study due to different causes.*

Exclusion Criteria

Active smokers, patients with unstable coronary heart disease or decompensated congestive heart failure in the previous 6 months, and subjects with an episode of malignant hypertension or stroke or uncontrolled diabetes, active malignancy or infectious disease were excluded. Patients under treatment with an HMG-CoA reductase inhibitor were also excluded.

All of the patients involved in the study were determined according to these criteria during clinical visits. Oral and written informed consent was obtained from all patients. The study was performed in accordance to Declaration of Helsinki.

Estimated glomerular filtration rate (eGFR) was calculated using the MDRD formula and patients were grouped according to the classification of KDOQI guidelines (15). Patients with stage 5 CKD that were still not on dialysis treatment were also included in the study.

Laboratory Analyses

Blood samples were collected at 08:00 after an overnight fast for the hemogram and biochemical analyses (urea, creatinine, calcium, phosphorus, albumin, total protein, albumin, C-reactive protein, total cholesterol, triglyceride, LDL-cholesterol, glucose and hemoglobin A1c). All biochemical analyses were performed using the Architect C8000 (Abbott Laboratories, Illinois, U.S.A) biochemical analyser. Blood counts were performed using the Cell Dyn 3700 (Abbott Laboratories. Abbott Park, Illinois, U.S.A) analyser that works with laser measurement and impedance transducer technology. Twenty-four hour urine samples were collected from all patients starting from the day before the blood samples were drawn and those samples were used to study urinary protein and creatinine excretion (mg/day). Urinary creatinine was studied by Jaffe colorimetric method and protein was studied after denaturation with benzethonium chloride using the Architect C8000 (Abbott Laboratories., Illinois, U.S.A) biochemical analyser.

Determination of Urinary NAG Index:

Processing and Storage of Urinary Samples:

Fresh first morning urinary samples were obtained at the time of enrollment and were centrifuged immediately to remove insoluble elements after routine test-strip urinalysis. The urine sediment was examined by light microscopy and evaluated for the presence or absence of granular casts. The supernatant was treated with a protease inhibitor cocktail tablet (Complete, Mini; Roche Diagnostics, Mannheim, Germany) and stored at –80°C until assayed. This cocktail tablet inhibits a broad spectrum of serine, cysteine, and metalloproteases as well as calpains that are present in mammalian tissues (23).

Measurement of Urinary NAG Activity:

NAG activity was measured in the urine by a colorimetric assay (Roche Diagnostics GmbH.Mannheim/ Germany). In brief, this method uses the substrate 3-cresolsulfonphthaleinyl-N-acetyl-β-D-glucosaminidinase and borax, which is hydrolyzed by NAG when present in the urinary sample. This reaction releases 3-cresolsulfonphthalein-sodium, which is measured by spectrophotometry. According to the manufacturer's instructions, 1 ml of the substrate solution was incubated for 5 min at 37°C. A 50-µl aliquot of the urinary sample then was added to the substrate solution, mixed, and incubated for 15 min at 37°C. After incubation, 2 ml of the stop reagent solution that contained sodium carbonate was added to the sample mixture and allowed to stand for 10 min at room temperature. The absorbance was then measured by a spectrophotometer (Beckman Coulter, Fullerton, CA) set at 580 nm. A single measurement was performed per sample. The inter- and intra-assay coefficients of variation were 4.3 and 6.0%, respectively. Results were normalized to urinary creatinine values and expressed in U/gr creatinine (23).

Statistical Analyses

Statistical analyses were performed by using the SPSS 11.0.1 software (April 2002; IBM Corp.; NY; USA). Assumption of normal (Gaussian) distribution was tested by the One-Sample Kolmogorov-Smirnov test. Simple correlations were performed using Pearson's or Spearman's correlation analyses as appropriate. Comparisons of variables between groups were performed with Student's t test or the Mann-Whitney U tests in accordance with the distribution pattern of the variable.

Comparison of parameters between stages of CKD was performed with the ''One-way ANOVA'' variance analysis with post-hoc tests Tukey's and Tamhane's T2 in accordance with the distribution pattern of the variable.

According to the mean values and standard deviations of the NAG index in each stage of CKD with a power of 80% and tolerance of 5%, 8 patients in each group was adequate to detect a significant difference between the groups.

RESULTS

Baseline demographic and laboratory characteristics of the study group are revealed in Table I. The mean value of the urinary NAG index (uNAG) was 21.6±19.9 in all groups.

The mean BP of all study groups was 132.4±11.3 mm-Hg and the mean number of antihypertensive medications was 2.5±11.3.

The urinary NAG index was positively correlated with 24-hour protein excretion (r=0.590; p=0.0001) and C-reactive protein (r=0.258; p=0.04) levels (Figure 2). The NAG index

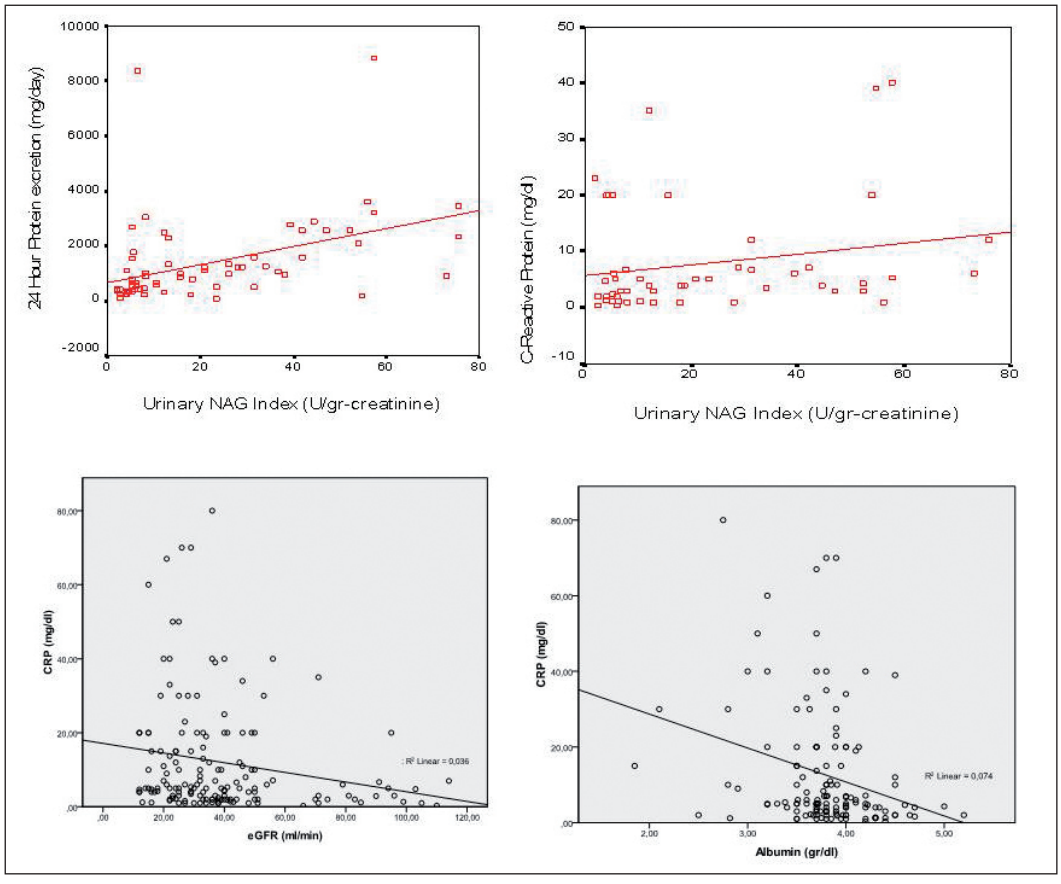

Figure 2: *Correlations between NAG index vs. 24 hour urine protein excretion and CRP. Correlations between CRP vs. eGFR and albumin levels.*

Table I: Baseline characteristics of the study group

	Minimum	Maximum	Mean	Std. Deviation
Age	22	70	63.7	15.9
Mean Office Systolic Blood Pressure (mm-Hg)	130	155	132.4	11.3
Mean Office Diastolic Blood Pressure (mm-Hg)	80	95	82.2	13.5
Mean number of antihypertensive medications	1	4	2.5	11.3
uNAG Index (U/gr-creatinine)	2.00	75.8	21.57	19.91
24 hour urine protein excretion (mg/day)	766	20246.0	1646.7	2715.3
Albumin (gr/dl)	2.85	5.20	3.8	0.49
ALT (IU/L)	4.0	44.0	19.81	13.51
CRP (mg/L)	0.27	48.00	12.05	15.37
LDL-Cholesterol (mg/dl)	32.00	175.00	112.19	35.10
Triglyceride (mg/dl)	46.00	389.00	154.50	78.28
Uric acid (mg/dl)	2.70	9.8	6.67	1.68
PTH (pg/ml)	3.00	689.00	182.97	151.80
eGFR (ml/min)	11.00	124.00	39.68	23.01
Creatinine (mg/dl)	0.89	5.49	1.97	0.87
Ferritin (ng/ml)	4.60	1174.00	172.80	189.49
Hemoglobin (gr/dl)	9.43	17.70	11.94	1.89

uNAG: Urinary NAG index, **eGFR:** Estimated glomerular filtration rate, **PTH:** Parathyroid hormone, **LDL:** Low density lipoprotein, **CRP:** C-reactive protein (normal range:0.0-5.0 mg/L), **L:** Liter, **ml:** Mililiter, **ng:** Nanograms, **gr:** Grams.

showed no correlation with age, serum albumin, uric acid, calcium, phosphorus, hemoglobin, ferritin, lipid parameters, eGFR and parathyroid hormone levels ($p>0.05$). The NAG index was similar between men and women (25.6±22.1 vs. 18.3±17.5 u/gr; $p=0.11$) and diabetic and non-diabetic patients (20.3±16.5 vs. 22 ± 21.1u/gr; $p=0.75$). In addition to the NAG index, C-reactive protein had negative associations with eGFR ($r=-0.224$; $p=0.005$) and serum albumin ($r=-0.286$; $p=0.001$) levels, but showed no correlation with proteinuria (Figure 2).

The urinary NAG index was significantly higher in stage 3 compared to stage 2 (32.1±23.5 vs. 7.5±3.3 U/gr-creatinine; $p=0.002$) and lower in stage 5 compared to stage 3 CKD (8.2±7.6 vs. 32.1±23.5; $p=0.017$). There was no positive linear correlation between stage of CKD and NAG index and fluctuating values were observed. The NAG index values in all stages of CKD are presented in Figure 3. Age, gender and proportion of the diabetic patients were similar between all stages of CKD. Hemoglobin levels were significantly decreased while C-reactive protein, PTH and uric acid levels were significantly increased with increasing stage of CKD. 24-hour urine protein excretion was similar between all stages of CKD (Table II).

DISCUSSION

The key feature of our study is that it is the first time that urinary N-acetyl-β-D-glucosaminidase activity (uNAG) is evaluated in diabetic and non-diabetic patients with different stages of CKD who are under treatment with a renin angiotensin aldosterone axis blocker (ACEi or ARB). Urinary NAG index was similar between diabetic and non-diabetic patients and also men and women. However, the NAG index was positively correlated with the proteinuria and there was no difference in protein excretion among all stages. Patients with stage 3 CKD had higher NAG index compared to stage 2 and the lowest levels were detected in patients with stage 5. Therefore we suggest that increased value of NAG index in stage 3 compared to stage 2 could be caused by the increase in tubulointerstitial injury. Decreased value of NAG index at stage 5 CKD might be a result of enormous loss of nephron mass. Increased activity of urinary NAG is revealed to be sensitive to active renal disease or a toxic insult but falls to normal levels on recovery or removal of the toxin (16). However there is no follow-up study for its level in the course of CKD. A study on patients with congenital and acquired solitary kidney revealed that urinary NAG levels

increased in 52.6% and 60.6% of the patients respectively (17). They showed an inverse correlation between urinary NAG and estimated glomerular filtration rate (eGFR). We could not detect a direct correlation between eGFR and the NAG index, and found fluctuating values of the NAG index in different stages. Increased activity may be related with increasing activity of underlying disease. Decreased activity may represent both recovery of tubulointersititial injury or a transformation of tubulointersititial injury to fibrosis and so may still be associated with a decline in eGFR.

Our study was a cross-sectional analysis of patients who are on different stages of CKD and it is therefore not possible to comment on the relationship between the NAG index and progression risk of CKD. However, a significant positive correlation between proteinuria and the NAG index suggests that an increased NAG index might be a risk factor for progression of CKD.

C-reactive protein had positive correlation with the NAG index and negative correlations with eGFR and serum albumin levels in the present study. Previously Fox et al demonstrated that Afro-Americans with CKD had higher CRP levels compared to those without CKD (18). Tonelli M. et.al demonstrated that a higher baseline CRP level was associated with faster loss of kidney functions (19). Elevated CRP is associated with endothelial injury and impaired vasodilatation, both of which may lead to glomerular damage and progressive loss of kidney function (20). Although we do not have baseline CRP levels, an inverse correlation between CRP and eGFR in the present study may represent a vicious cycle that results in tubulointersititial injury. On the other hand, a positive association between the NAG index and CRP supports the hypothesis that uNAG may be related to a risk of tubulointersititial damage.

In patients with active lupus nephritis, urinary NAG activity has been shown to be increased in association with proteinuria compared to controls (21). After 30 days of prednisone treatment, the NAG level was shown to decrease but still nearly ten-fold increased compared to the controls. There was no follow-up after the first 30 days of the treatment and it was suggested that the reduction in urinary NAG set in later than the decline in proteinuria and the improvement in GFR.

Given that progression of renal disease is linked to chronic inflammatory state and proteinuria (10, 22, 23), the relationship between the NAG index and proteinuria and CRP levels in the present study reveal that it may be a valuable parameter in the estimation of the risk of progression in CKD. On the other hand, the NAG index could be determined by the balance between tubulointersititial injury and fibrosis in advanced stages.

Limitations of the present study include the following: the patient number in each stage of CKD was low, we could not perform a kidney biopsy at the time of the study to prove a relationship between tubulointerstitial injury and the NAG index, the study

Table II: Comparison of demographic, clinical and laboratory parameters between stages of CKD.

Variable	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	p
Number of Patients	10	12	24	18	8	
Sex (M/F)	4 / 6	8 / 4	14 / 10	8 / 10	4 / 4	0.67
Percent of Diabetics (%)	30	34	33	27.7	37.5	0.59
uNAG index (U/gr-creatinine)	27.7±20.2	7.5±3.3	32.1±23.5	20.4±16.2	8.2±7.6	0.001
24 hour urine protein excretion (mg/day)	1428±1172	1555±2174	2103±3580	1421±2386	864±830	0.73
Albumin (gr/dl)	3.9±0.4	3.8±0.7	3.9±0.5	3.7±0.5	4.1±0.3	0.26
LDL-Cholesterol (mg/dl)	136.2±28.5	138±49	112.8±32	101.5±32.7	85.3±31	0.001
Triglyceride (mg/dl)	182.6±123	174±68.3	148.2±65.6	155.2±88.1	134.3±53	0.6
Uric acid (mg/dl)	4.8±1.4	5.9±0.9	6.9±1.6	6.8±1.7	6.6±1.3	0.0001
PTH (pg/ml)	102.1±72	105.8±44	122.9±75	237±175	312±222	0.0001
eGFR (ml/min)	102.1±9.1	77.6±15.4	40.7±6.9	21.9±4.7	12.6±0.7	0.0001
Ferritin (ng/ml)	109.1±146	137.4±127.3	131.9±123	246.8±248	109.1±108	0.009
Hemoglobin (gr/dl)	13.3±1.7	13.8±1.8	12.1±1.9	11.3±1.7	10.6±1.6	0.0001
CRP (mg/L)	5.2±5.7	5.5±10.5	10.9±13.5	16.3±18.8	19.1±8.5	0.043

uNAG: Urinary NAG index, **eGFR:** Estimated glomerular filtration rate, **PTH:** Parathyroid hormone, **LDL:** Low density lipoprotein, **CRP:** C-reactive protein (normal range:0.0-5.0 mg/L), **L:** Liter, **ml:** Mililiter, **ng:** Nanograms, **gr:** Grams.

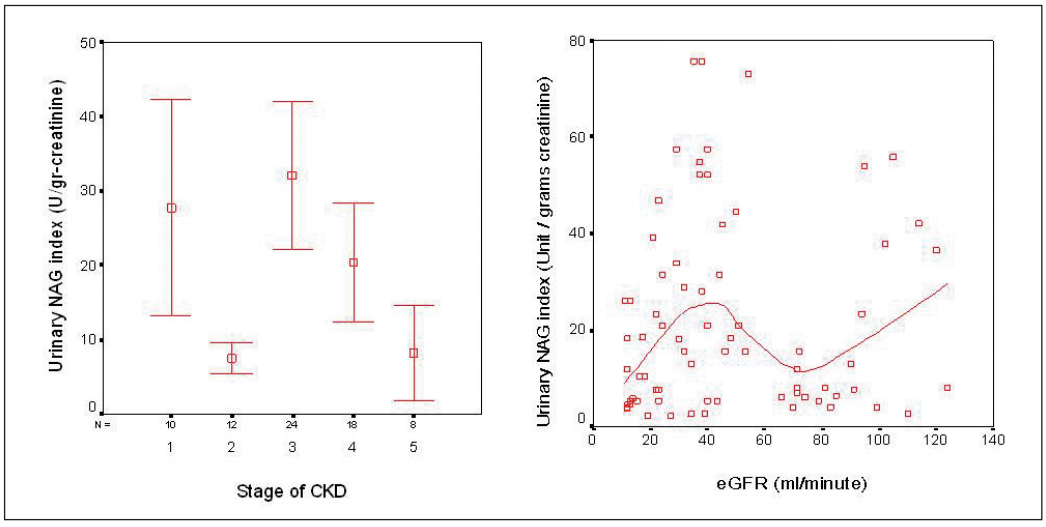

Figure 3: *Urinary NAG index in stage 1 to 5 of CKD, variation in NAG index compared to eGFR.*

design was cross-sectional and we had no serial measurements of the NAG index at different stages of CKD in the same patient group. Although larger prospective cohort studies are needed, our preliminary results demonstrate that the NAG index as a marker of tubulo-interstitial injury could stay high even in the advanced stages of CKD. Therefore we suggest that anti-inflammatory and anti-proteinuric approaches continue even in advanced CKD.

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