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Original Article

Biochemistry Section

Urinary N-acetyl Glucosaminidase as an Early Marker of Diabetic Nephropathy: A Cross-sectional Study

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ABSTRACT

Introduction: Diabetic nephropathy is one of the microvascular complications of diabetes mellitus. Even though microalbuminuria is the most widely used test for the development of diabetic nephropathy, it does not manifest clinically until stage 3 of the five stages in the development of diabetic nephropathy. N-acetylglucosaminidase (NAG) activity has been shown to be elevated in urine samples of patients developing diabetic nephropathy earlier than the appearance of microalbuminuria.

Aim: To study the role of Urinary N-Acetyl Glucosaminidase (NAG) as an early marker of diabetic nephropathy.

Materials and Methods: The present cross-sectional study was conducted from August 2022-October 2022 at KMCH Institute of Health Sciences and Research, Coimbatore, Tamil Nadu, India. A total of 50 diabetic patients (Group 1) and 50 age and gender matched controls (Group 2) were included in the study following the inclusion and exclusion criteria. Data collected includes age, gender, height, weight, Body Mass Index (BMI), duration of diabetes mellitus, drug history, history of diabetic retinopathy, ischaemic heart disease and the analytes measured were serum creatinine, eGFR, Glycated Haemoglobin (HbA1c) and urine NAG levels. Urine NAG levels between cases and controls were compared using independent t-test. Urine NAG levels between different subgroups of cases, based on the urine albumin levels were compared using Analysis of Variance (ANOVA).

Results: The mean age of the study population was 55.92 in group 1 and 46.04 years in group 2. Out of 50 subjects, 30 were males and 20 were females. Independent t-test showed that there was a significant difference in means of urine NAG levels in cases and controls with t=2.024 and p=0.02 (<0.05). One-way ANOVA done to compare the urine NAG levels in three subgroups of patients divided according to the urine albumin levels as normal (3 mg/dL), microalbuminuria (3-30 mg/dL), macroalbuminuria (>30 mg/dL) showed significant differences in urine NAG levels between the three groups.

Conclusion: There is a statistically significant difference between the cases and controls in urine NAG levels. There was a statistically significant difference in urine NAG levels between patients with different grades of albuminuria.

Keywords: Creatinine, Diabetes mellitus, Glycated haemoglobin, Microalbuminuria

INTRODUCTION

Diabetes Mellitus is a surging health concern now-a-days which needs medical attention and awareness. As per statistics, India ranks second after China in the global diabetes epidemic with 77 million people with diabetes. Of these, 12.1 million are aged >65 years, which is estimated to increase to 27.5 million in the year 2045 [1]. Diabetic nephropathy is one of the microvascular complications of diabetes mellitus. It is characterised by long-lasting albuminuria, increasing arterial blood pressure and a decline in glomerular filtration rate. This places significant financial, social, and medical constraints on the patient and the healthcare system. Kidney replacement therapy such as dialysis or renal transplantation are two options for managing End-Stage Renal Disease (ESRD), both of which lower patients' quality of life [2]. The cost of renal replacement treatment is an economical burden for the stakeholders.

Microalbuminuria, a most widely used test for detection of diabetic nephropathy, it does not manifest clinically until stage 3 of the five stages in diabetic nephropathy. A protein called NAG (EC 3.2.1.50) is found in the liver, spleen, and other organs [3]. Owing to its high molecular weight, it is not filtered by the glomerulus. It is also present in the lysosomes of the Proximal Convoluted Tubule (PCT) cells [4]. N-acetyl-glucosaminidase activity has been shown to be elevated in urine samples of patients developing diabetic nephropathy earlier than the appearance of microalbuminuria [5]. Hence, present study was conducted to measure urinary NAG levels using spectrophotometer in diabetic patients, so that it could be used as an early marker of diabetic nephropathy. This could be an easier and cost-effective diagnostic method for preventing end-stage renal disease in diabetic patients. This study is based on the hypothesis that NAG, will be excreted in the urine in the earlier stages of renal failure and hence it can be used as an early marker of diabetic nephropathy.

Kim SR et al., have measured urinary NAG levels in diabetic patients using an automated chemistry analyser [4]. Zhang D et al., have measured urine NAG levels in diabetic patients using immunonephelometry technique whereas Sheira G et al., have measured the same using ELISA [6,7]. The present study was the first to measure urinary NAG levels in the current study population using a manual method.

Thus, present study was conducted to study the role of urinary NAG as an early marker of diabetic nephropathy.

MATERIALS AND METHODS

The present cross-sectional observational study was conducted at KMCH Institute of Health Sciences and Research, a tertiary care hospital in Coimbatore, Tamilnadu, India for a period of three months from August 2022-October 2022. The Institutional Human Ethics Committee approval was obtained (IEC no. 41/IHEC/2022). Study participants were recruited based on the inclusion and exclusion criteria after obtaining the written informed consent.

Inclusion criteria

Cases: All type 2 Diabetes mellitus patients attending endocrinology Outpatient Department (OPD) during the study period irrespective of duration of the disease and medication history.

Controls: Age and gender-matched patients without any disease.

Exclusion criteria

Cases: Diabetes mellitus patients with any acute illness or surgery in the last three months, urinary tract infection in the last three months.

Controls: Patients with any known diseases, any other renal disease, any acute illness or surgery in the last three months, urinary tract infection in the last three months.

Sample size calculation: The sample size was calculated using the formula: $n= 2S_p^2 (Z_{1-}\alpha_{/2} +Z_{1-}\beta)^2/\mu d^2$; $S_p^2= S_1^2 + S_2^2/2$. Based on the findings of Omozee EB et al., [5], S1(Standard deviation in group I)= 4.42, S2(Standard deviation in group II)= 1.5, Mean difference-3.04, Effect size- 1.027027, Alpha error (%)-5, Power (1- beta) %-99.5, 2 sided. Substituting these values in the above formula, The sample size for cases was calculated to be 50. Taking controls in the ratio of 1:1, sample size for controls is 50.

Group 1: all the cases were considered as group 1.

Group 2: all the controls were considered as group 2.

Study Procedure

After obtaining informed consent, relevant data including age, gender, height, weight, BMI, duration of diabetes mellitus, drug history, history of diabetic retinopathy, ischaemic heart disease were collected from the participants using a preset questionnaire. Participants' serum creatinine, estimated Glomerular Filtration Rate (eGFR) were also noted from the case sheets. Random urine samples were collected in sterile containers. They were stored by freezing and thawed before analysis.

Methodology for urine NAG measurement: The urine samples (10 mL) were subjected to centrifugation for 5 minutes at 4000 rpm. After centrifugation, 250 µL of sample was aliquoted into another labelled test tube. Then, 500µL of citric acid-disodium phosphate buffer of pH 4.5 was added. In the next step, 1 mL of 1.5 mmol/L of P-NP β-D glucosaminidine is added. This mixture is then incubated at 37°C for 30 minutes. The reaction is then stopped by the addition of sodium carbonate-bicarbonate buffer of pH 10. Then, the reading is taken in Hitachi UV 500 spectrophotometer at a wavelength of 405 nm.

After taking the OD values, NAG activity is calculated by the formula:

$$\frac{\text{A (U-B) X dilution factor}}{\text{A (S-RB) X incubation time}}$$

This methodology is based on the experiments done by Horak E et al., [8]. Urine Albumin was estimated by using immunoturbidimetric assay. The reference range for all the measured parameters is shown in [Table/Fig-1].

Analyte	Reference range
Urine NAG (U/L)	Males <19.4 Females <15.7
Spot urine albumin (mg/dL)	Normal urine albumin- <30 Microalbuminuria-30-300 Macroalbuminuria >300
Serum creatinine (mg/dL)	Males 0.7-1.4 Females 0.6-1.2
eGFR (mL/min)	90-110

[Table/Fig-1]: Reference range of various parameters.

STATISTICAL ANALYSIS

Statistical analysis was done using Statistical Package for Social Sciences (SPSS) software version 28.0. Continuous variables were represented using descriptive statistics like mean and standard deviation. Urine NAG levels between cases and controls were compared using independent t-test. Urine NAG levels between different subgroups of cases, based on the urine albumin levels were compared using ANOVA. The p-value less than 0.05 was considered as significant. Pearson correlation coefficient was used to find out the correlation between different parameters.

RESULTS

The mean age of the study population was 55.92±10.62 in group 1 and 46.04±12.47 years in group 2. Out of 50 group 1 subjects, 30 (60%) were males and 20 (40%) were females. The mean BMI of the study population was 23.57±6.17 Kg/m^2. Majority of the patients in both group 1 and 2 were having class I obesity [Table/Fig-2]. In present study, 17 (34%) patients had diabetes mellitus for more than 10 years. A total of 29 (58%) patients were on metformin and 21 (42%) patients were on dipeptidyl peptidase-4 inhibitors. Among the patients with diabetes mellitus 5 (10%) had history of diabetic retinopathy, 19 (38%) had history of ischaemic heart disease and 18 (36%) patients have systemic hypertension. The drug history and the duration of diabetes mellitus in patients are shown in [Table/Fig-3].

Parameters		Group 1	Group 2	p-value
Mean age±SD		55.92±10.62	46.04±12.47	0.08
Gender	Males	30	30	0.16
	Females	20	20	
BMI category:				
Underweight (<18.5)		3	2	0.02
Normal (18.5-22.9)		15	17	0.23
Overweight (23-24.9)		8	10	0.02
Obesity class I (25-29.9)		19	18	0.26
Obesity class II (>30)		5	3	0.10
BMI (mean±SD)		23.1±7.3	24.2±3.25	0.24
HbA1c level (mean ±std)		8.7±2.86	5.56±0.52	0.01

[Table/Fig-2]: Age, gender and BMI distribution of cases and controls.
Statistical test applied: Mean, standard deviation, t-test

Drug class	Frequency
Metformin	29
Sulphonyl urea	15
DPP-4 inhibitors	21
SGLT-2 inhibitors	5
Alpha glucosidase inhibitors	3
Insulin	18
Antihypertensives	18
Aspirin	11
Statins	13
Duration of diabetes (in years)	**Frequency**
<1	4
1-2	6
3-5	13
5-10	10
>10	17

[Table/Fig-3]: Drug history and duration of diabetes among cases (N=50).

The mean HbA1c of the group 1 was 8.7±2.8% and of the group 2 was 5.56±0.52%. The mean serum creatinine level in group 1 was 0.78±0.25 mg/dL and in group 2 it was 0.74±0.15 mg/dL. The mean eGFR was 99±18 mL/min and 110±14 mL/min in group 1 and group 2, respectively. The mean spot urine albumin level was 21±5 mg/dL and 7.9±4 mg/dL in group 1 and 2. The mean urine NAG level was 425.3±10 U/L and 219± 8 U/L in group 1 and 2, respectively [Table/Fig-4].

Analyte	Group 1	Group 2	p-value
Urine NAG (U/L)	425.3±10	219± 8	0.003
Spot urine albumin (mg/dL)	21±5	7.9±4	0.02
Serum Creatinine (mg/dL)	0.78±0.25 mg/dL	0.74±0.15	0.17
eGFR (mL/min)	99±18	110±14	0.0005

[Table/Fig-4]: Mean levels of various parameters in cases and controls.
Statistical test applied: Mean; Standard deviation; t-test

Independent t-test showed that there was a significant difference in means of urine NAG levels in group 1 and 2 with t=2.024 and p=0.02 (<0.05). One-way ANOVA done to compare the urine NAG levels in three subgroups of patients divided according to the urine albumin levels as normal (3 mg/dL), microalbuminuria (3-30 mg/dL),macroalbuminuria (>30 mg/dL) showed significant differences in urine NAG levels between the three groups. The f-ratio value was 7.31197. The p-value was 0.001719. The result was significant at p <0.05 as shown in [Table/Fig-5].

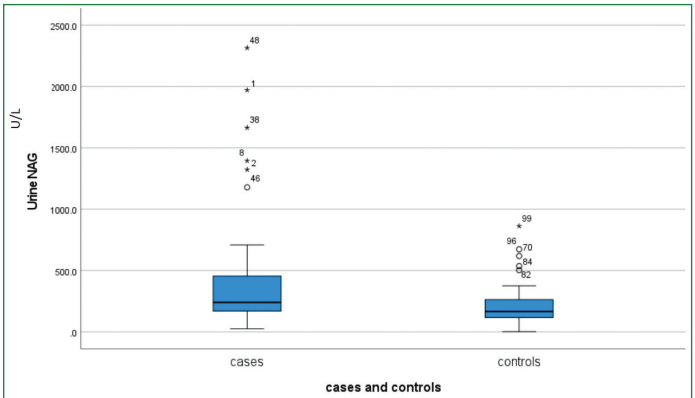
Parameters	n (%)	Urine NAG (U/L) (mean+/-std dev)	Urine NAG	
			F-ratio	p-value
Normal urine albumin	9 (18%)	227.6±93.2	7.31197	0.001719
Microalbuminuria	34 (68%)	357.1±413.3		
Macroalbuminuria	7 (14%)	1010.71±746		

[Table/Fig-5]: Comparison of urine NAG levels in cases with different grades of albuminuria using ANOVA.
Statistical test applied: Mean; standard deviation; ANOVA; t-test

Pearson correlation test was done to find the correlation between urine NAG levels and other variables like age, gender, BMI, HbA1c, duration of diabetes mellitus, serum creatinine and urine albumin. There was statistically significant correlation between urine NAG levels and serum creatinine, eGFR and urine albumin levels. There was no significant correlation between urine NAG levels and drug intake by Pearson correlation test [Table/Fig-6]. The mean urine NAG levels in Group 1 and 2 are shown in [Table/Fig-7].

Parameters	Urine NAG	
	Pearson correlation	p-value (2-tailed)
Serum creatinine	0.469	<0.001
eGFR	0.446	0.001
Urine albumin	0.518	<0.001
HbA1c	-0.037	0.797
Age	0.02	0.887
Gender	0.217	0.131
BMI	-0.190	0.187
Duration of diabetes mellitus	-0.009	0.950
Intake of anti-diabetic medications	-0.050	0.729

[Table/Fig-6]: Correlation between urine NAG levels and other variables.
Statistical test applied: Pearson correlation coefficient; t-test



[Table/Fig-7]: Urine NAG levels in Group 1 and 2.

DISCUSSION

One of the chronic complications of diabetes mellitus is diabetic nephropathy which eventually leads to renal failure necessitating dialysis or transplantation. This, apart from increasing the morbidity and mortality in diabetic patients, also causes an economic burden to the country. The situation becomes grim in the rural setup with limited access to the healthcare.

In this study, urine NAG was significantly elevated in diabetic patients when compared to age and gender matched controls. This is similar to the findings of Kim SR et al., [4], Zhang D et al., Al-Hazmi SF et al., [4,6,9]. NAG is a lysosomal enzyme present in the renal tubular epithelial cells [10]. Owing to its high molecular weight, NAG is not filtered through the glomerulus and whatever NAG present in urine is from the renal tubules. Elevated urinary NAG in diabetic patients is due to the renal tubular injury seen in diabetic patients [11]. Renal tubular injury is said to precede the glomerular damage in renal diseases.

Spot urine albumin levels of <30 mg/dL are said to be normal, between 30-300 mg/dL is said to be microalbuminuria and > 300mg/dL is said to be macroalbuminuria [3]. One-way ANOVA done to compare the levels of urine NAG between these three groups of samples was found to be statistically significant. This is similar to the findings of Ghobrial EE et al., and Tanaka A et al., [12,13]. This could be because of the tubular damage associated with increasing grades of albuminuria seen in diabetes mellitus [14].

There was positive correlation between serum creatinine and urine NAG levels. This is an expected finding which depicts the worsening renal damage. As the kidney disease progresses both serum creatinine levels and urinary NAG levels increases. Our findings are similar to that of Zhang D et al., [6].

There was a negative correlation between eGFR estimated by CKD-EPI equation and urine NAG levels. This negative correlation shows the decreasing renal function as predicted by decreasing eGFR and increasing urinary NAG levels. This is in line with the findings of Kim SR et al., Nauta FL et al., have demonstrated in their study that this inverse relationship between eGFR and urine NAG does not exist if adjusted for age, gender and albuminuria [4,14].

There was no significant correlation between urine NAG and intake of any oral hypoglycemic agents. This is in contrast with the findings of the study done by Sato S et al., where they have suggested that SGLT-2 inhibitors had a protective effect against renal dysfunction. This may be because of intake of oral hypoglycemic drugs in various combinations in our study population unlike the study population of Sato et al., where the patients were only on SGLT-2 inhibitors [15].

In this study, there was no significant correlation between urine NAG and BMI, age, gender. This is in contrast with the findings of Kim SR et al., where there was significant correlation between urine NAG and these parameters [4]. This may be because of the variability in the population under study.

Limitation(s)

This study is done in a single hospital-based population and spot urine NAG levels were not normalised with urine creatinine values.

CONCLUSION(S)

Urine NAG level was estimated using a manual method in this case-control study. There is a statistically significant difference between the cases and controls in urine NAG levels. There was a statistically significant difference in urine NAG levels between patients with different grades of albuminuria. There was a positive correlation between urine NAG levels and serum creatinine levels, urine albumin levels. There was a negative correlation between urine NAG levels and eGFR. This is a single center study done in a small population. Further, multicentric large studies are required to confirm the findings of the study. Reference interval studies could be done to establish the reference levels for urine NAG levels in our Indian population.

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