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Study of Urinary N-Acetyl-Beta-D-Glucosaminidase as a biomarker of Diabetic Nephropathy

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ABSTRACT

Background: Diabetic nephropathy (DN) is a major cause of morbidity and mortality in diabetic patients worldwide. Plenty of tubular damage biomarkers have been discovered. Urinary N-acetyl-β-D-glucosaminidase (NAG) is a hydrolytic enzyme that acts on glycosyl compounds. NAG is excreted in abnormally high amounts in many renal diseases.

Objective: The aim of this work was to study the importance of Urinary N acetyl-β-D-glucosaminidase level as an early biomarker for detection of DN and to assess the degree of kidney affection in various stages of DN.

Patients and methods: 100 subjects divided into five groups: Group 1: 20 healthy volunteers (control group), group 2: 20 pre diabetic persons, group 3: 20 normo-albuminuria diabetic patients, group 4: 20 micro-albuminuria diabetic patients (ACR 3 -300 mg/mmol) and group 5: 20 macro-albuminuria diabetic patients (ACR ≥ 300 mg/mmol).

All individuals were subjected to full history taking, ECG & echocardiography, abdominal ultrasound, laboratory investigations (HbA1c, fasting and post prandial blood glucose, lipid profile, oral glucose tolerance test (OGTT), blood urea, serum creatinine, serum uric acid, e-GFR and urinary NAG.

Results: There was high significant difference between the five groups regarding duration of the disease, fasting blood sugar, postprandial blood glucose, HbA1c, Serum cholesterol, albumin/creatinine ratio, serum urea and creatinine, e-GFR and urinary NAG. In addition, there was significant positive correlation between urinary NAG and albumin/creatinine ratio, blood urea, creatinine, and e-GFR.

Conclusion: The urinary NAG can be used as an early urinary biomarker for early detection and progression of diabetic nephropathy in type 2 diabetic patients.

Keywords: Diabetes, Nephropathy, Urinary N-Acetyl-Beta-D-glucosaminidase.

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder in which hyperglycemia is predominant due to an imbalance in insulin secretion, its effect, or both. Chronic hyperglycemia in diabetes is usually associated with dysfunction, long-term damage, and failure of many organs, especially the kidneys, eyes, heart, blood vessels, and nerves [(1)]. Diabetic nephropathy (DN) is defined as persistent albuminuria [albumin excretion rate (AER) > 300 mg/24 hours] in diabetic patient for more than 5 years with concomitant retinopathy, in the absence of other kidney disease, urinary tract infection (UTI) and heart failure. This process is often accompanied by high blood pressure [(2)]. The K/DOQI diagnosis of diabetic kidney disease is based on elevated urinary albumin, low glomerular filtration rate, or persistence of GFR (less than sixty mL/min/1.73 m2) of 3 months or more [(3)].

DN is diagnosed clinically in most individuals, as kidney biopsy will not alter the management of these patients. However, a kidney biopsy may be required in some diabetics with CKD to determine the underlying cause and thus referral to a nephrologist should be considered when there is uncertainty about the etiology of the kidney disease (patients with hematuria, heavy proteinuria, active urine deposits, rapid decrease in glomerular filtration rate, absence of retinopathy, or resistant hypertension) [(1)]. DN occurs in ~30% of people with type 1 DM and 25-40% of people with type 2 DM [(4)].

Several modifiable and non-modifiable risk factors, such as poor blood sugar control, smoking, high blood pressure, hyperlipidemia, urinary albumin excretion, and genetic factors may predict the development of diabetic nephropathy in albuminuric patients with type 2 diabetes mellitus [(5)]. Glucose amplifies the effects of intra-glomerular blood pressure by inducing impaired self-regulation in glomerular microcirculation. On the other hand, glomerular capillary hypertension enhances glucose transporter-1 (GLUT-1) expression with concomitant intracellular accumulation of glucose, amplifying the harmful effects of glucose and its metabolites within the kidney [(6)].

Biomarkers play an essential role in early detection of DN. Microalbuminuria is the most common. At the same time, microalbuminuria is a sign of the generalized endothelial dysfunction that occurs in diabetes, which explains the involvement of the kidneys with impaired brain and cardiovascular disease. Over time, it has been suggested that

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microalbuminuria reflects injury to the glomeruli as well as tubular lesions, as the filtered albumin will be reabsorbed at the tubular level [(7)]. Urinary biomarkers used for early detection of DN have proven useful in determining the prognosis of this disease, and thus preventive effects and treatment plans. Urinary biomarkers are easy and applicable, which allow screening of the population and can detect tubular lesions that occur early in DM [(7)].

N-acetyl-βeta-D-glucosaminidase (NAG) NAG is a hydrolytic enzyme of molecular weight 130000-140000 dalton that acts on glycosyl compounds, oligosaccharides derived from hyaluronic acid resulting in removal of the terminal non-reducing N-acetyl glucosamine residue and the next lower even number oligosaccharide. It is also involved in degradation of mucopolysaccharides and glycoproteins [(8)]. NAG is naturally excreted in the urine as a result of normal excretion of cells and scarce epithelial cells of the proximal renal tubules. This normal excretion is subjected to several factors such as diurnal contrast, with maximum excretion in the morning, so the second urine sample in the day proved to be the most reliable for NAG determination [(9)]. Also, the difference between the sexes is not of much significance, although NAG secretion is slightly higher in females than in males [(10)]. The aim of this work was to study the importance of urinary N acetyl-β-D-glucosaminidase level as an early biomarker for detection of DN and to assess the degree of kidney affection in various stages of DN.

PATIENTS AND METHODS

100 subjects with different age groups and of both sexes were enrolled in this cross sectional controlled study and divided into five groups: follow:

- Group 1: 20 non-diabetic non-hypertensive healthy volunteers of the same age and sex as control group.
- Group 2: 20 pre diabetic persons according to IGTT (ADA) criteria reported in 2014 (i.e. fasting blood glucose level 110-125 mg/dL (<7.0 mmol/L), or 2 h postprandial blood glucose level 140-199 mg/dL (<7.8 mmol/L), or HbA1c 5.7-6.4%) [(11)].
- Group 3: 20 diabetic patients have normoalbuminuria, albumin excretion rate (ACR albumin creatinine ratio ≤ 3 mg/mmol).
- Group 4: 20 diabetic patients have microalbuminuria ACR 3-300 mg/mmol
- Group 5: 20 diabetic patients have macroalbuminuria ACR ≥300 mg/mmol.

Exclusion criteria:

Subjects with acute or chronic inflammatory disorders, other endocrine diseases (except T2DM), pregnancy, hematological, rheumatological, neoplastic, autoimmune diseases, active hepatitis/ liver cirrhosis, congestive heart failure, chronic renal failure, hypertension, and other severe cardiovascular disease.

All participants included in the study were subjected to:

1-Full history taking and complete clinical examination (including blood pressure, fundus examination and BMI).

2- ECG & Echocardiography to rule out cardiovascular disease.

3- Laboratory investigations were done including: CBC, LFT, urea, creatinine, lipid profile,

HbA1c, fasting and post prandial blood glucose, oral glucose tolerance test (OGTT) and glomerular filtration rate using MDRD equation GFR (ml/min/1.73m^2)=175× (serum creatinine) 1.154×(age)−0.203× (0.742 if female) [(12)].

Urine analysis including albumin concentration, urine creatinine and urinary concentrations of NAG.

Collection and handling of blood and urine samples. **Blood sample,** four milliliters of venous blood were collected after 8 hours fasting under complete aseptic precautions, two ml on EDTA for determination of HbA1c & CBC. The rest of the sample was placed in a plain tube and left to coagulate for 30 min then centrifuged at 3000 r.p.m for 15 minutes. The separated serum was designated for the immediate assay of fasting glucose and serum creatinine. Two milliliters of venous blood were collected after 12 hours fasting under complete aseptic precautions. The sample was placed in a plain tube for the immediate assay of lipid profile.

Urine sample, the first urine of the day (mid-stream) about six milliliters aseptically collected, voided directly into a sterile container. Collected sample was divided into two aliquots: three ml for detection of microalbumin and three ml centrifuged to remove particulate matter, assay immediately or aliquot and store at ≤ -200 C. repeated freeze-thaw cycles were avoided. Detection of NAG in urine using a double-antibody sandwich enzyme-Linked immunosorbent assay (ELISA) for the second mid-stream morning urine sample.

Ethical approval:

The study was conducted in accordance with the ethical standards of the Helsinki Declaration of 1964, as revised in 2000, and was approved by Ethical Committee of Zagazig University. Written informed consents were obtained from all participants in the study.

Statistical analysis

Statistical analysis was performed by using the IBM SPSS software Statistics version 21.0 (IBM Corp., Armonk, NY, USA). Our research data were summarized in the form of mean, standard deviation, and percentage. For non-symmetrically distributed data and quantitative data, nonparametric (Mann–

Whitney U) test was used. The Chi-square test was used for analysis of qualitative data, while the Kruskal– Wallis H test was done for analysis of more than two variables [13].

RESULTS

Comparison between all the studied groups as regards age, sex and BMI showed non-statistically significant difference. Regarding duration of the disease, we found that there was significant increase in progression of diabetic nephropathy with long duration of the disease (increase in group 5 and group 4 than group 3). The mean duration of diabetes was significantly longer among the progressors to diabetic nephropathy compared to non-progressors to diabetic nephropathy (Table 1).

Table (1): The demographic data of studied groups as regard age

	G1	G2	G3	G4	G5	F. test	p. value
Age (year)							
Range	43 –50	45 – 52	45 – 52	39 – 52	47 – 59	1.247	0.296
Mean ± S.D	46.6 ± 2.46	48.45 ± 1.85	48.5 ± 2.65	46.4 ± 3.71	52.0 ± 3.40		
Sex							
Male	12	11	16	10	8	0.127	
Female	8	9	4	10	12		
Disease duration							
Mean ± S.D			8.3 ± 1.45	12.65 ± 0.93	12.40 ± 0.94	92.468	0.001*

Excretion of urinary-NAG was gradually increased with the duration of diabetes and appeared before microalbuminuria and increased serum creatinine. There was significant increase in urinary NAG in pre-diabetic patients than healthy control people. e-GFR was significantly lower in diabetic patients than e-GFR in the healthy control group. Serum cholesterol showed a statistically significant increase in group 5 than in group 4 and group 4 than group 3 and group 3 than group 2 and all were more than group 1 (Table 2).

Urinary NAG was significantly higher in pre diabetic persons and in type 2 diabetic patients with normo, micro and macroalbuminuria than in non-diabetic controls, and its value increased in parallel with the severity of renal involvement (Tables 2 & 3).

Significant positive correlation was observed between urinary NAG and ACR, HbA1c, age and serum creatinine (Table 4 and figs 1, 2).

Table (2): Laboratory results of all studied groups

	G1 Control	G2 Prediabetes	G3 Normo-alb.	G4 Micro- alb.	G5 Macro-alb.	F. test	p. value
Fasting blood sugar (mg /dl)							
Mean ± S.D	87.65 ± 10.20	116 ± 5.53	137.0 ± 20.55	208.15 ± 43.98	243.0 ± 41.56	50.121	0.001*
2hr Postprandial sugar(mg/dl)							
Mean ± S.D	119.5 ± 11.1	167.9 ± 13.1	181.0 ± 22.7	295.8 ± 51.3	330.5 ± 42.9	65.452	0.001*
HbA1c (%)							
Mean ± S.D	4.89 ± 0.37	5.91 ± 0.16	6.56 ± 0.58	8.21 ± 0.78	9.79 ± 1.06	65.452	0.001*
Albumin /creatinine ratio(mg/mm)							
Mean ± S.D	1.83 ± 0.44	1.83 ± 0.34	2.51 ± 0.24	21.08 ± 4.64	48.05 ± 7.26	89.006	0.001*
Serum Creatinine (mg/dl)							
Mean ± S.D	0.75 ± 0.18	0.75 ± 0.18	0.98 ± 0.15	1.74 ± 0.32	2.10 ± 0.35	65.980	0.001*
Blood Urea (mg/dl)							
Mean ± S.D	29.65 ± 3.89	29.65 ± 3.89	35.8 ± 2.24	54.8 ± 3.61	63.35 ± 5.02	96.520	0.001*
e-GFR(ml/min)							
Mean ± S.D	116.0 ± 12.25	97.0 ± 2.41	94.70 ± 6.19	72.35 ± 6.67	57.15 ± 5.30	67.238	0.001*
S.Cholesterol (mg/dl)							
Mean ± S.D	182.2 ± 10.7	186.6 ± 14.2	190.5 ± 20.8	203.1 ± 18.6	228.5 ± 21.0	17.238	0.001*

FBS (fasting blood sugar), 2 hr pp (2hours postprandial sugar), eGFR: estimated glomerular filtration rate.

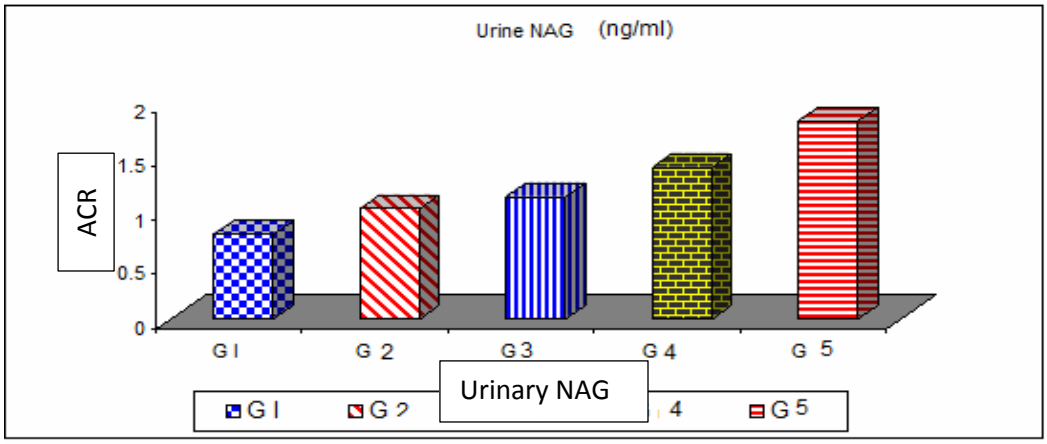
Table (3): Comparison of urinary NAG between studied groups

Urine NAG (ng/ml)	Range	Mean ± S. D	F. test	p. value
G I Control	0.5 – 1	0.78 ± 0.18	46.041	0.001*
G 2 prediabetes	0.9 – 1.15	1.02 ± 0.09		
G 3 normo- alb.	0.94 – 1.3	1.13 ± 0.11		
G 4 micro- alb.	1.1 – 1.63	1.39 ± 0.17		
G 5 macro-alb.	1.49 – 2.1	1.83 ± 0.17		

Table (4): Correlation between NAG and different data of the studied groups

	Urine NAG (ng/ml)	
	r	P
FBG(mg/dl)	0.824	0.001*
2 hr P.P(mg/dl)	0.843	0.001*
HbA1c(%)	0.887	0.001*
Alb / Creat ratio (mg/mol)	0.916	0.001*
Serum Creatinine(mg/dl)	0.788	0.001*
blood Urea(mg/dl)	0.855	0.001*
eGFR(ml/min)	- 0.877	0.001*
S. Cholesterol(mg/dl)	0.663	0.001*

Figure (1): Comparison of Urinary NAG between studied groups



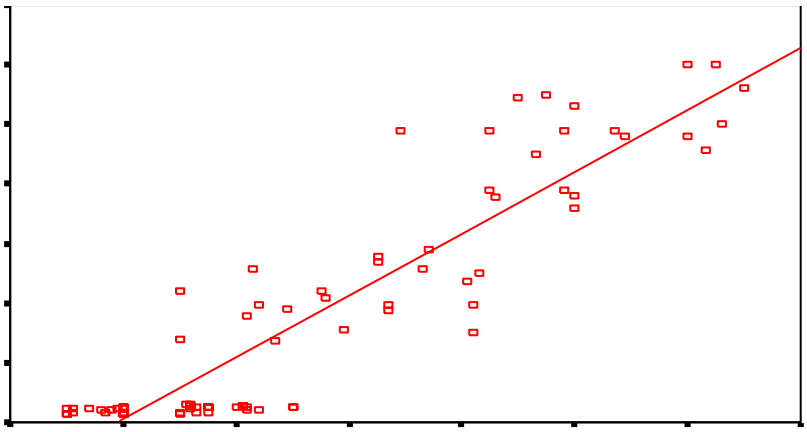


Figure (2): Correlation between NAG and Alb/creat ratio.

In our study, we found that increase in serum creatinine was associated with significant increase in urinary NAG denoting a positive correlation between serum creatinine and Urinary NAG level (Tables 4 & 5).

Table (5): Urinary NAG sensitivity specificity and predictive values in studied groups

Urine NAG	Sensitivity	Specificity	PPV	NPV	Accuracy
G 2	79	83	85	77	81
G 3	85	87	89	79	85
G 4	9o	89	88	84	87
G 5	93	9o	95	88	92

DISCUSSION

Diabetic nephropathy (DN) is one of the major complications of diabetes mellitus (DM) that became the main cause of end-stage renal disease all over the world [(1)].

In the current study, we investigated urinary level of N acetyl- β-Dglucosaminidase (NAG) as a marker of proximal tubular affection in prediabetic persons, diabetic patients and non-diabetic control subjects to evaluate the relationship of this marker to the diabetic kidney disease.

Our study reported that there was high significant correlation between NAG and diabetic nephropathy. This coincides with **Sheira *et al.*** [(14)] who stated that the urinary-NAG can predict progression of renal involvement in diabetic nephropathy. But, **Agardh *et al.*** [(15)] and **Mungan *et al.*** [(16)] were against our results and reported that urinary NAG activity did not predict development of diabetic nephropathy. In the present study, comparison between the five studied groups as regards age and sex showed non-statistically significant values. This coincides with **Afkhami-Ardekani *el al.*** [(17)]**.**

Comparison between the five studied groups as regards duration of disease denoted that there was significant increase in progression of diabetic nephropathy with long duration of disease (increased in group 5 and group 4 than in group 3). This coincides with **Jamal *et al.*** [(18)] and **Kiran and Patel** [(19)] who stated that urinary-NAG excretion increases gradually with the duration of diabetes and can be detected much before detection of microalbuminuria and elevation of serum creatinine. In this study, our results showed that there was significant increase in urinary NAG in studied pre diabetic patients than healthy control people. This coincides with **Hiratsuka *et al.*** [(20)] and **Fujita *et al.*** [(21)] who found that urinary NAG level is slightly but significantly higher in individuals with IGT than in control subjects.

Comparing the levels of urinary NAG in different stages of albuminuria showed that levels of urinary NAG was higher when patients had more progression of albuminuria. Moreover, urinary NAG significantly increased in patients with normal to mildly albuminuric T2DM compared to patients without diabetes. This coincides with **Kim *et al.*** [(22)]**.**

A significant positive correlation between urinary NAG and ACR is in agreement with **Al-Futaisi *et al.*** [(23)] and **Udomah *et al.*** [(24)] who found elevation of urinary NAG levels in African diabetics with strongly correlation to ACR.

In our study, we found significantly positive correlation between urinary NAG level and HbA1c supposing that urinary NAG may be used as a biomarker for the degree of renal affection in DN this coincides with studies done by **Prashant *et al.*** [(25)]**.** Against our results **Beatriz *et al.*** [(26)] found no correlation between urinary NAG excretion and glycemic state in type 2 diabetic patients.

In our study, we found that higher levels of blood urea and creatinine parallel to failing of e-GFR. This is in agreement with the findings of **Mitsnefes *et al.*** [(27)]. But, **Devarajan** [(28)] stated that in chronic kidney disease the blood urea was not elevated, and it elevated when more than 60% of kidney tissues are no longer functioning. Our study revealed that e-GFR was significantly lower in diabetic patients compared to the healthy control group, which is in agreement with **Malyszko *et al.*** [(29)] **and Jamal *et al.*** [(18)]**.** But, **Nauta *et al.*** [(30)] showed no association between e-GFR and urinary NAG levels after adjusting age, sex, and albuminuria. In our study, Correlation between NAG and age among the studied showed a positive significant correlation between NAG and the age. This is in agreement with **Agirbasli *et al.*** [(31)] who stated that urinary NAG levels increase with age.

Comparison between all the five studied groups as regard serum cholesterol showed a statistically significant increase in group 5 than in group 4 and group 4 than group 3 and group 3 than group 2 and all were more than group 1. This coincides with **Muntner *et al.*** [(32)] who stated that dyslipidemia is common in diabetes. In addition, **Krolewski *et al.*** [(33)] showed that hypercholesterolemia is a risk factor for and associated with the onset and progression of nephropathy in type 2 diabetics.

Our result showed elevation of urinary NAG excretion in the absence of any albuminuria denoting that renal tubular dysfunction may exist before the occurrence of glomerular affection, which is in agreement with **Kuźniar *et al.*** [(34)].

In our study, we found that NAG was high among pre diabetic group and normoalbuminuric group. This is in agreement with **Navarro *et al.*** [(35)] who supposed that NAG changes may occur before microalbuminuria, as the tubular cells can reabsorb the increased albumin load resulted from glomerular damage, but the increased NAG will be lost from the

affected cells. Moreover, **Nauta *et al.*** [30] showed that urine NAG level increases 9-folds in normoalbuminuric diabetic patients compared to control persons and it further increases with development and progression of microalbuminuria.

In our study, we found a significant positive correlation between serum creatinine and urinary NAG level. This is in agreement with **Kuzniar *et al.*** [34].

CONCLUSION

Urinary NAG could be used as an early non-invasive biomarker to detect diabetic nephropathy in type 2 DM patients.

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