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ORIGINAL RESEARCH

Urinary N-Acetyl-β-d-Glucosaminidase (NAG) Levels and Risk of Cardiovascular Events in Diabetic Patients

Weiwei Lou[1]
Qun Cheng[1]
Yanqiu Liang[1]
Ding Xia[2]

[1]Department of Rehabilitation, Tongji Hospital, Huazhong University of Science and Technology, Wuhan, Hubei, 430030, People's Republic of China; [2]Urology Surgery, Tongji Hospital, Tongji Medical College of Huazhong University of Science and Technology, Wuhan, Hubei, 430030, People's Republic of China

Background: Cardiovascular diseases (CVDs) have a high incidence rate in population with diabetic patients. Studies on the association between urinary N-acetyl-β-d-glucosaminidase (NAG) levels, the biomarker of renal tubular damage, with cardiovascular (CV) events diabetic patients was still few.

Methods: The relationship between urinary NAG levels and CV events was analyzed in a prospective cohort including 357 patients with type 2 diabetes mellitus at a follow-up of 5 years.

Results: Twenty-six (7.3%) patients have CV events. Kaplan–Meier analysis suggested that diabetic patients with urine NAG levels ≥37.5 IU/L had a higher rate of CV events than those with urine NAG levels <37.5 IU/L (Log rank test, P = 0.021). Cox analysis revealed that elevated urine NAG levels significantly contributed to increased risk of CV events (HR = 1.43, 95% CI 1.23–1.93, $P < 0.001$) after adjusting for clinical confounding factors. Interestingly, we also found that "abnormal renal function" has an effect modification on the association between urine NAG levels and CV events. ROC-AUC analysis suggested that the urine NAG (AUC = 0.81, P < 0.001) had a better predictive value than eGFR (AUC = 0.74, P = 0.012).

Conclusion: Elevated urine NAG levels are associated with higher risk of CV events in patients with type 2 diabetes. These results might further suggested that urinary NAG is a value urinary biomarker for early detecting CV events among diabetic patients.

Keywords: N-acetyl-β-d-glucosaminidase, type 2 diabetes mellitus, cardiovascular event, diabetic nephropathy

Introduction

Cardiovascular (CV) events, such as coronary artery disease, heart failure, stroke, cardiac arrhythmia and peripheral vascular diseases, are the main causes of morbidity and mortality among diabetic patients.[1–3] Compared with patients without diabetes, the incidence rate of CV events increased by 2–4 times in diabetic patients.[4] Hence, developing methods with more accuracy to detecting the risk of CV events is necessary to reduce CV mortality and improve prognosis for diabetic patients.

Some surrogate measures, like pulse wave velocity (PWV) and albuminuria, predicting CV events have been investigated. Existing studies showed that PWV has been considered as the gold standard for detecting arterial stiffness and is significantly associated with increased risk of atherosclerosis-related pathological

Correspondence: Ding Xia
Department of Urology, Tongji Hospital, 1095 Jiefang Ave, Wuhan, Hubei, 430030, People's Republic of China
Tel +86 18177494319
Email dingxia123123@126.com

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deterioration such as increased intima-media thickness of carotid artery and the presence of carotid plaques,[5,6] and CV outcomes such as coronary artery disease and stroke.[7,8] As an indicator of early renal injury, albuminuria has been regarded as a biomarker for determining diabetic kidney disease, suggesting positive independent associations with risk of cardiovascular disease (CVD) and mortality.[9] Interestingly, some recent studies reported significant associations between albuminuria and macrovascular complications including coronary artery calcification and PWV in patients with essential hypertension or diabetes mellitus.[9,10] However, the association between urinary N-acetyl-β-d-glucosaminidase (NAG), a marker of renal tubulopathy, and CV events and in patients with type 2 diabetes mellitus is still controversial. The controversy maybe originated from different characteristics of study populations including age, gender, ethnic diversity and duration of diabetes, as well as different methods used to measure CV events.[10]

In recent years, several biomarkers of renal tubular damage have attracted more and more attention due to their predictive value on predicting progression of early-stage diabetic kidney disease.[11–13] Injury of proximal renal tubular cells mainly contributes to increased level of urine. Furthermore, increases in urinary NAG has been found in mildly increased albuminuric patients with type 2 diabetes mellitus.[11] Some studies have also suggested that urinary NAG levels were correlated with macrovascular disease,[14] neuropathy[15] and retinopathy.[16] However, few or controversial study has focused on the association between urinary NAG levels and CV outcomes in patients with type 2 diabetes mellitus. We would like to investigate the relationship between urinary NAG and CV events in patients with type 2 diabetes mellitus.

Methods

We continuously included 377 hospitalized patients with type 2 diabetes mellitus who are from clinical research center from April 2012 to December 2014 by using a prospective design. These patients have also been detected for blood glucose parameters, urinary NAG, and have undergone cardiovascular examination (ECG, echocardiography and others) after admission. All included patients with type 2 diabetes mellitus did not have any CVD history and other serious chronic diseases (cancers, severe liver disease, active infectious disease and connective tissue disease) before admission. The definition for type 2 diabetes mellitus was based on the basis:[17] 1) when fasting plasma glucose >7.0mmol/L, diabetes can be diagnosed if the typical symptoms of diabetes; 2) when 2-hour postprandial blood glucose >11.1mmol/L, if accompanied by typical symptoms of diabetes and 3) clinically, in the case of reliable test methods, glycosylated hemoglobin (HbA1C) can also be used for the diagnosis of type 2 diabetes mellitus when HbA1C >6.5%. Furthermore, in order to achieve the purpose of the study, 1) the taking hypoglycemic therapy liking using insulin and oral hypoglycemic agents was defined type 2 diabetes mellitus. Additionally, patients were excluded from this cohort study due to an CVD history including coronary artery disease (N = 13) and stroke (N = 7) and other disease including cancers (N = 5), severe liver disease (N = 3), active infectious disease (N = 1) and connective tissue disease (N = 1) before admission. Clinical variables including age, gender, body mass index (BMI), smoking and drinking habits, systolic and diastolic blood pressure, diabetes duration, medical history and others were recorded. BMI was defined as weight divided by height squared (kg/m^2). The institutional review board of Tongji Hospital approved this study and all included patients gave the written informed consent, consistent with Declaration of Helsinki guidelines.

Finally, all included patients type 2 diabetes mellitus (N = 357) were followed up by telephone until the occurrence of CV events. If the status of these patients was not clear completely during the follow-up, referring cardiologists were invited to identify the patient's condition. During a mean follow-up of 5 years, CV events requiring hospitalization were defined by acute myocardial infarction (N = 12), ischemic or hemorrhagic stroke (N = 7), acute HF (N = 5) and death (N = 2) caused by these diseases. Twelve diabetic patients lost contact during a mean follow-up of 5 years. CV events occurred in 26 diabetic patients.

Measurement of Blood and Urine Markers

Urine samples of fasting morning were obtained from each included patient with type 2 diabetes mellitus and were measured for urinary NAG levels using a JCA-BM 6010/c automated chemistry analyzer (JEOL Ltd., Tokyo, Japan). Fasting blood samples from each included patient after admission blood were also tested for creatinine, uric acid, glucose, HbA1C, hemoglobin and lipid profiles. Measurement of HbA1c was performed by an Integra

800 CTS (Roche, Hercules, CA, USA). Serum lipid levels of profiles were measured by an enzymatic method (Asahi Kasei Pharma Co, Tokyo, Japan). Serum creatinine, uric acid, hemoglobin and glucose were tested by the Hitachi 7600 analyzer (Hitachi Ltd.). The estimated glomerular filtration rate (eGFR) was derived from the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI).[18] eGFR <60 mL/min per 1.73 m^2 was defined as renal function injury.

Statistical Analyses

All of the data were analyzed by using SPSS 25.0. $P \leq 0.05$ was considered to be statistically significant. A K–S test was performed for normality of all continuous variables. Baseline characteristics upon entry into the cohort were compared between tertiles of eGFR (<60 mL/min per 1.73 m^2 and ≥60 mL/min per 1.73 m^2). In multivariate analysis, no adjustment was made for confounding factors in Crude model. Adjustments of confounding factors including age, gender, smoking history, drinking history and BMI were made in Model 1. In Model 2, adjustments of age, gender, smoking history, current drinking history, BMI, systolic blood pressure, diastolic blood pressure and moderate physical activity were made.

Cox regression analysis was performed to identify the independent predictive value of urine NAG levels for CV events in patients with type 2 diabetes mellitus. To further evaluate the independent association, we further excluded the effect of "duration of diabetes" by sensitivity analysis. We also analyzed the association between urine NAG levels at baseline and CV events during the follow-up by stratified analysis by adding "eGFR ≥ 60 mL/min per 1.73 m^2" as the covariate. Finally, the performance of urine NAG for predicting CV events was analyzed by receiver operating characteristic (ROC) curves and area under the curve (AUC).

Table 1 Clinical Characteristics in Patients with Type 2 Diabetes Mellitus (N = 357)

Characteristic	eGFR ≥ 60 mL/Min per 1.73 m^2 (N = 178)	eGFR < 60 mL/Min per 1.73 m^2 (N = 179)	P value
Age (Y)	68.3±9.6	70.5±10.6	0.009
Male, n (%)	105 (58.9)	102 (57.0)	0.158
CV events, n (%)	8 (4.5)	18 (10.1)	<0.001
Duration of diabetes (years)	6.9 (4.1–12.4)	11.9 (7.3–13.3)	<0.001
Smoking history, n (%)	64 (36.0)	71 (39.7)	0.045
Drinker history, n (%)	102 (57.3)	111 (62.0)	0.052
BMI (kg/m^2)	27.3±6.3	29.5 ±7.1	<0.001
Systolic blood pressure (mmHg)	146.4±11.8	155.5±16.4	<0.001
Diastolic blood pressure (mmHg)	83.1±9.3	87.5±9.8	<0.001
Moderate physical activity, n (%)	66 (37.1)	72 (40.2)	0.096
Urinary NAG (IU/L)	32.6±4.2	41±4.8	<0.001
Urinary NAG (U/g creatinine)	4.3 (2.1–7.2)	12.6 (8.4–17.2)	<0.001
Estimated GFR (mL/min per 1.73 m2)	70.6±5.7	48.4±6.9	<0.001
Serum albumin (g/L)	43.4±3.1	38.3±3.6	<0.001
Serum hemoglobin (g/L)	115±19	108±19	0.079
Serum C-reactive protein (mg/L)	1.9 (0.6–7.6)	7.3 (1.1–12.5)	<0.001
Serum total cholesterol (mmol/L)	5.1±0.2	5.4.±0.3	0.134
Serum triglycerides (mmol/L)	3.4±0.2	3.5±0.4	0.115
Serum HDL cholesterol (mmol/L)	1.4±0.1	1.6±0.2	<0.097
Serum LDL cholesterol (mmol/L)	1.6±0.4	1.7±0.5	0.231
Serum creatinine (mmol/L)	68 ±5	135 ±11	<0.001
Serum uric acid (mmol/L)	288 ±10	305 ±13	0.042
Hemoglobin A1c (%)	6.8±0.3	6.9±0.2	0.344
Fasting plasma glucose (mmol/L)	8.5±0.2	8.6±0.4	0.403

Notes: Data are presented as mean ± SD for normally distributed data, as median (interquartile range) for nonnormally distributed data, and as n (%) for categoric variables.
Abbreviations: NAG, N-acetyl-β-d-glucosaminidase; CV, cardiovascular; BMI, body mass index; eGFR, estimated glomerular filtration rate; HDL, high density lipoprotein; LDL, low density lipoprotein.

Table 2 Multivariate Cox Regression Analysis of Predicting CV Events in Patients with Type 2 Diabetes Mellitus (N = 357)

	Crude	Model 1	Model 2
Estimated GFR (mL/min per 1.73 m^2)	1.42 (1.22–1.76)	1.36 (1.18–1.76)	1.32 (1.14–1.68)
P value	0.017	0.019	0.021
Urinary NAG (IU/L)	1.59 (1.31–1.98)	1.47 (1.26–1.89)	1.43 (1.23–1.93)
P value	0.009	0.010	0.015
Urinary NAG (U/g creatinine)	1.64 (1.39–2.62)	1.57 (1.34–2.41)	1.51 (1.31–2.27)
P value	0.002	0.007	0.010

Notes: Model 1: Adjusted for no. Model 2: Adjusted for age, gender, smoking history, drinking history and BMI. Model 3: Adjusted for age, gender, smoking history, drinking history, BMI, systolic blood pressure, diastolic blood pressure and moderate physical activity.
Abbreviations: NAG, N-acetyl-β-d-glucosaminidase; CV, cardiovascular; BMI, body mass index; eGFR, estimated glomerular filtration rate.

Results

Clinical Characteristics of Patients with Type 2 Diabetes Mellitus

The clinical features of patients with type 2 diabetes mellitus are described in Table 1. These included patients were divided into two groups according to renal function injury (eGFR < 60 mL/min per 1.73 m^2). Compared with diabetic patients with normal renal function (eGFR ≥ 60 mL/min per 1.73 m), patients with renal function injury had a higher rate of CV events and higher urine NAG levels, which initially suggested that elevated urine NAG levels were potentially associated with an increased risk of CV events. Furthermore, these patients with renal function injury showed a higher age, BMI, duration of diabetes, systolic blood pressure and diastolic blood pressure and tended to have higher rate of smoking history. Information about laboratory indexes and medications was also described in detail (Table 1).

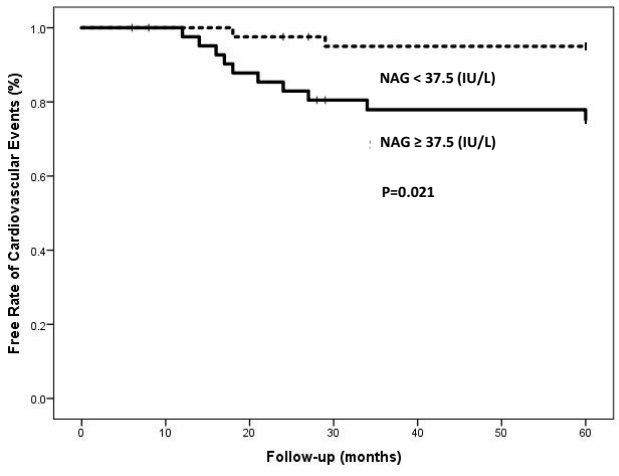

Figure 1 Kaplan–Meier analysis for cardiovascular events free stratified by urine NAG.

Elevated Urine NAG Levels Were Independently Related to CV Events in Patients with Type 2 Diabetes Mellitus

To investigate the association of urine NAG levels with CV events in patients with type 2 diabetes, Cox regression analysis was performed. Cox analysis revealed that elevated urine NAG levels significantly contributed to increased risk of CV events (HR = 1.43, 95% CI 1.23–1.93, $P < 0.001$, Table 2) after adjustments for age, gender, smoking history, drinking history, BMI, systolic blood pressure, diastolic blood pressure and moderate physical activity were made in Model 3. Kaplan–Meier analysis suggested that diabetic patients with urine NAG levels ≥ 37.5 IU/L had a higher rate of CV events than those with urine NAG levels <37.5 IU/L (Log rank test, P = 0.021, Figure 1).

To further clarify the association between urine NAG levels and CV events in patients with type 2 diabetes, an additional sensitivity analysis was performed by adding "duration of diabetes" as a covariate. Our results still showed that elevated urine NAG levels were still significantly associated with a higher risk of CV events (HR = 1.27, 95% CI 1.18–1.72, $P = 0.023$, Table 2) after adjusting age, gender, smoking history, drinking history, BMI, systolic blood pressure, diastolic blood pressure, moderate physical activity and duration of diabetes in Model 3 (Table 3).

Elevated Urine NAG Levels Were Independently Related to CV Events by Stratified Analysis in Patients with Type 2 Diabetes Mellitus

To confirm whether "eGFR ≥ 60 mL/min per 1.73 m^2" have an effect modification on the independent association

Table 3 Sensitivity Analysis of Predicting CV Events in Patients with Type 2 Diabetes Mellitus By Adding "Duration of Diabetes" as the covariate (N = 357)

	Crude	Model 1	Model 2
Estimated GFR (mL/min per 1.73 m^2)	1.32 (1.21–1.73)	1.25 (1.18–1.69)	1.23 (1.13–1.64)
P value	0.035	0.40	0.41
Urinary NAG (IU/L)	1.38 (1.25–1.84)	1.33 (1.21–1.76)	1.27 (1.18–1.72)
P value	0.011	0.018	0.023
Urinary NAG (U/g creatinine)	1.43 (1.27–2.35)	1.38 (1.25–2.14)	1.35 (1.23–2.09)
P value	0.007	0.009	0.012

Notes: Model 1: Adjusted for duration of diabetes. Model 2: Adjusted for age, gender, smoking history, drinking history, BMI, duration of diabetes and duration of diabetes. Model 3: Adjusted for age, gender, smoking history, drinking history, BMI, systolic blood pressure, diastolic blood pressure, moderate physical activity and duration of diabetes.
Abbreviations: NAG, N-acetyl-β-d-glucosaminidase; CV, cardiovascular; BMI, body mass index; eGFR, estimated glomerular filtration rate.

between urine NAG levels and CV events. These included patients were divided into two groups according to renal function injury (eGFR ≥ 60 mL/min per 1.73 m^2). In diabetic patients with eGFR ≥ 60 mL/min per 1.73 m^2, Our found that urine NAG levels did not associate with CV events (HR = 1.04, 95% CI 0.89–1.47, P = 0.157) in diabetic patients (Table 4). However, in diabetic patients with eGFR < 60 mL/min per 1.73 m^2, we still found that elevated urine NAG levels significantly contributed to increased risk of CV events (HR = 1.50, 95% CI 1.27–2.96, P = 0.016).

ROC Analysis of Predicting CV Events in Patients with Type 2 Diabetes Mellitus

To determine performance of urine NAG levels for predicting CV events, ROC-AUC analysis was used (Table 5). The urine NAG (AUC = 0.81, P < 0.001) had a better predictive value than eGFR (AUC = 0.74, P = 0.012). The result suggested that urine NAG might be a valuable biomarker for predicting CV event in patients with type 2 diabetes mellitus.

Discussion

The prevalence of diabetes mellitus has been increasing remarkably around the world and cardiovascular, neurological and renal complications are the main causes of death in diabetic patients.[1–3] For example, diabetes can lead to kidney damage and even kidney failure even if blood glucose levels are not controlled.[19] In recent years, several biomarkers of renal damage have attracted more and more attention due to their predictive value on predicting progression of early-stage diabetic kidney disease. Markers of

Table 4 Stratified Analysis of Predicting CV Events in Patients with Type 2 Diabetes Mellitus By Adding "eGFR ≥ 60 mL/min per 1.73 m^2" as the covariate (N = 357)

	Crude	Model 1	Model 2
eGFR ≥ 60 mL/min per 1.73 m^2			
Urinary NAG (IU/L)	1.15 (1.03–1.73)	1.08 (0.93–1.58)	1.04 (0.89–1.47)
P value	0.037	0.053	0.157
Urinary NAG (U/g creatinine)	1.06 (0.94–1.87)	1.04 (0.92–1.84)	1.02 (0.88–1.73)
P value	0.146	0.205	0.226
eGFR < 60 mL/min per 1.73 m^2			
Urinary NAG (IU/L)	1.53 (1.29–2.93)	1.53 (1.29–2.81)	1.50 (1.27–2.96)
P value	0.010	0.011	0.016
Urinary NAG (U/g creatinine)	1.56 (1.19–2.69)	1.49 (1.17–2.52)	1.44 (1.15–2.49)
P value	0.006	0.008	0.010

Notes: Model 1: Adjusted for duration of diabetes. Model 2: Adjusted for age, gender, smoking history, drinking history, BMI, duration of diabetes and duration of diabetes. Model 3: Adjusted for age, gender, smoking history, drinking history, BMI, systolic blood pressure, diastolic blood pressure, moderate physical activity and duration of diabetes.
Abbreviations: NAG, N-acetyl-β-d-glucosaminidase; CV, cardiovascular; BMI, body mass index; eGFR, estimated glomerular filtration rate.

Table 5 ROC Analysis of Predicting CV Events in Patients with Type 2 Diabetes Mellitus (N=357)

Variables	AUC	Standard Error	95% CI	P value
Estimated GFR (mL/min per 1.73 m^2)	0.74	0.03	0.65–0.93	0.012
Urinary NAG (IU/L)	0.78	0.04	0.68–0.96	0.009
Urinary NAG (U/g creatinine)	0.81	0.05	0.72–0.97	<0.001

Abbreviations: NAG, N-acetyl-β-d-glucosaminidase; CV, cardiovascular; eGFR, estimated glomerular filtration rate; AUC, area under the curve.

renal injury on predicting risk of CV events have also been investigated.[11–13] As a lysosomal enzyme, NAG is highly expressed in renal tubules and its urine levels is increased in nephropathy. Increased urine level of NAG is a sensitive biomarker for renal tubular damage because the molecular mass (130,000) of the protein precludes glomerular filtration.[20,21] Our study demonstrated that elevated urine NAG levels were associated with a higher risk of CV events in diabetic patients. The significant association in regression models suggested that urine NAG may have an ideal utility to predict the risk of CV events in diabetic patients. Additionally, we also found that "abnormal renal function" has an effect modification on the association between urine NAG levels and the risk of CV events.

In previous findings, NAG values are elevated with worsening renal function and increased diabetes duration. They have concluded that urine NAG level is helpful in the diagnosis of diabetic nephropathy.[22,23] Recently, the attention to markers of renal tubular damage as potential indicators for CVD is increasing rapidly. Evidence demonstrated that urinary levels of NAG are related to different CVD in various subjects. For instance, the GISSI-prevenzione trial suggested that increased urinary NAG was strongly associated with poor outcomes in population with chronic HF.[24] Weitgasser et al reported a significant relationship between urinary NAG and macrovascular disease in old patients with type 2 diabetes mellitus.[14] Ouchi et al suggested that elevated levels of urinary NAG were associated with increased arterial stiffness in subjects without diabetes.[25] In a study with a general population, urinary NAG was independently associated with high risks of ischemic stroke, myocardial infarction and all-cause mortality. Importantly, they did not found that traditional CV risk factors such as eGFR and albumin were associated with CV events when urinary NAG compared.[26] Consistently, increased urinary NAG levels were significantly associated with CV events in diabetic patients in our study. Differently, our study showed that elevated urinary NAG levels were associated with higher CV risk in diabetic patients with abnormal renal function but not in those subjects with normal renal function. "Abnormal renal function" has an effect modification on the association between urine NAG levels and the risk of CV events. The most likely explanation is that renal injury leads to abnormal renal function, aggravating cardiovascular events and increasing urine levels.

This study has some notable strengths. Firstly, our findings in this prospective cohort study can further provide clinical evidence that urinary levels of NAG contributes to elevated risk of CV events in diabetic patients. Secondly, we reported that urinary NAG, a renal tubulopathic marker, is more closely associated with CV events only in diabetic patients with abnormal renal function, suggesting that diabetic nephropathy can mediate the correlation between urinary NGA levels and CV events. Thirdly, although this is a single center clinical study, we collected a relatively large number of study samples (N = 357), which enhances the reliability of the results. Finally, sufficient clinical confounding factors were also adjusted to further confirm the stability of our results. However, our study also has several limitations. For instance, although some confounders, including renal function, were adjusted, which may be a very important determinant of urinary NAG levels, other confounders cannot be fully excluded because many determinants of urinary NAG are currently unknown. Similar to some other biomarkers of renal injury, urinary NAG can change substantially over time. We could not perform a repeated measures analysis in this cohort due to the measurement of urinary NAG only at a single time point, which may introduce some bias into our results. The follow-up durations of variables may have affected the observed results. Furthermore, the application of a Cox regression model using a relatively large number of covariates might cause overfitting of the model.

Conclusions

We provided the evidence that elevated urinary NAG levels were related to risk of CV outcomes in diabetic patients. The "abnormal renal function" has an effect

modification on the association between urine NAG levels and the risk of CV events. These results might further suggested that urinary NAG is a value urinary biomarker for early detecting CV events.

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Disclosure

The authors report no conflicts of interest in this work.

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