Prospective Evaluation of Urinary *N*-Acetyl-β-D-Glucosaminidase With Respect to Macrovascular Disease in Elderly Type 2 Diabetic Patients

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OBJECTIVE — To analyze prospectively the importance of urinary *N*-acetyl-β-D-glucosaminidase (NAG), a marker for renal tubular function, in comparison with urinary albumin excretion (UAE), a marker for glomerular renal function, with respect to macrovascular disease in elderly patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS — We followed 124 patients over a mean period of 7.0 ± 0.5 years. At baseline, urinary NAG, UAE, age, diabetes duration, sex, blood pressure, lipids, and serum creatinine were determined. Also, history of myocardial infarction (MI), stroke, severe peripheral vascular disease (PVD), antidiabetic and concomitant medication, and smoking habits were recorded. After 7 years, patients were reevaluated, and a multivariate logistic regression analysis was used to test risk factors for significance in order to predict macrovascular disease. Subgroups of patients were analyzed with respect to severe macrovascular disease, with a separate analysis for surviving patients.

RESULTS — Compared with known cardiovascular risk factors such as microalbuminuria and total cholesterol, urinary NAG was similarly associated with cardiovascular disease for the total cohort ($P < 0.05$). Analyzing the subgroup of 65 patients still alive after follow-up care, urinary NAG and UAE were significantly elevated at baseline and at the time of follow-up care in patients with MI and PVD, but not in those with stroke ($P < 0.01$). There was a positive predictive trend of NAG excretion for the development of MI and PVD in our patients ($P = 0.07$).

CONCLUSIONS — Urinary NAG proved comparable to UAE when analyzed with respect to preexistence and development of severe macrovascular disease. It needs to be determined by further studies if urinary NAG will be of value to serve as an adjunct marker to UAE in type 2 diabetic patients.

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Diabetic macroangiopathy is the main reason for reduced life expectancy in type 2 diabetic patients (1–5). Besides predicting diabetic nephropathy (10,11) in type 1 and type 2 diabetic patients, microalbuminuria has been found to be a marker for patients at high risk for cardiovascular disease (6–9). An increased excretion rate of other "urinary markers," such as β-2-microglobulin, α-1-microglobulin, and *N*-acetyl-β-D-glucosaminidase, has been found to be associated with renal changes caused by diabetes (12). Data on long-term follow-up care of patients with microalbuminuria, with albumin excretion serving as a marker for glomerular renal function, have been published. The aim of our study was to look into changes regarding a marker for tubular renal function, *N*-acetyl-β-D-glucosaminidase (NAG), in comparison to urinary albumin excretion (UAE). As in other patients with chronic renal disorders, it has been shown that NAG excretion is enhanced in diabetic patients compared with normal subjects (13–15). In a cross-sectional survey, we found that NAG, a lysosomal enzyme fraction of proximal tubular renal cells, was associated with signs for severe atherosclerosis (16). We therefore designed a prospective study to follow type 2 diabetic patients with respect to end points of cardiovascular disease.

RESEARCH DESIGN AND METHODS

Patients

In a cross-sectional design, we studied all patients with type 2 diabetes who consecutively attended our outpatient clinic over a period of 3 months. Of 182 patients, 58 were excluded because of urinary infection and preexisting renal diseases known to increase UAE and urinary NAG activity ($n = 44$) or lost to follow-up care because of moving to other countries or districts ($n = 14$). From baseline and after a mean of 7 years, data were collected for the remaining 124 patients. Age, sex, diabetes duration, BMI, smoking habits, antidiabetic, further treatment, and laboratory examinations (see below) were recorded as independent variables at baseline and after 7 years. Cardiovascular disease, the dependent variable, was defined as a positive history of myocardial infarction (MI), stroke, and severe peripheral vascular disease (PVD) (gangrene, amputation, reconstruction, or percutaneous transluminal angioplasty); all of what had to be documented by hospital reports. Patients were followed

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Abbreviations: BUN, blood urinary nitrogen; MI, myocardial infarction; NAG, *N*-acetyl-β-D-glucosaminidase; OEQAS, Austrian Quality Assurance Scheme; PVD, peripheral vascular disease; UAE, urinary albumin excretion.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

Table 1—*Characteristics of patients with respect to presence or absence of cardiovascular disease at baseline*

Characteristics	Cardiovascular disease	
	Yes	No
n	66	58
Age (years)	70 ± 1	65 ± 1.5*
Sex (M/F)	33/33	24/34*
Insulin-treated patients (*n*)	19 (28)	51 (88)†
Smokers (*n*)	5 (7)	12 (20)†
Diabetes duration (years)	14 ± 1	10 ± 1*
BMI (kg/m^2)	27 ± 0.5	28 ± 0.5
HbA_{1c} (%)	7.8 ± 0	7.6 ± 0.1
Systolic blood pressure (mmHg)	165 ± 2	156 ± 3*
Diastolic blood pressure (mmHg)	88 ± 1	86 ± 10
Total cholesterol (mmol/l)	6.44 ± 0.2	5.92 ± 0.18*
HDL cholesterol (mmol/l)	1.16 ± 0.05	1.18 ± 0.05
Triglycerides (mmol/l)	6.28 ± 0.67	5.5 ± 0.67*
Urinary NAG (U/g creatinine)	7.7 ± 1.4	5.4 ± 0.5*
Urinary albumin (μg/min)	100 ± 20	60 ± 20*

Data are means ± SD or *n* (%).*$P < 0.05$; †$P < 0.01$.

for a mean of 7 ± 0.5 years. In patients still living 7 years after baseline, the above-mentioned measurements were performed again. All patients were evaluated according to cardiovascular end points, which were defined as MI, stroke, severe PVD, or death from one of these causes. For statistical purposes, patients were divided into different groups. Those with either a cardiovascular event or death caused by cardiovascular disease and those without cardiovascular disease, irrespectively, whether they had died or were still alive at the time of follow-up care.

Further subgroup analysis was performed for patients with MI, stroke, or severe PVD to look for possible differences with respect to various cardiovascular risk factors. Causes of death were determined by death certificates or, when not available, by reports from the responsible family doctor.

Laboratory procedures

Diabetic control was determined by HbA_{1c} measurements by high-performance liquid chromatography (HPLC) (Diamat; Bio-Rad, Richmond, CA).

The reference range was 4.2–6% (2 SD range) evaluated by OEQAS (Austrian Quality Assurance Scheme, Central Laboratory, Lainz Hospital, Vienna, Austria). Serum total cholesterol, HDL cholesterol, triglycerides, creatinine, and blood urinary nitrogen (BUN) were measured on a Hitachi automated analyzer with external quality control by OEQAS. Systolic and diastolic blood pressure was taken according to the guidelines of the American Heart Association at two visits in our out-patient clinic: eight measurements (two left and right upper arm measurements by a technician as well as by a physician at each of these visits) were used for calculating mean blood pressure. Overnight urine samples were used for determination of albumin and NAG excretion. All samples were tested for nitrite and leucocytes using test strips (Combur-9-Test; Boehringer Mannheim, Mannheim, Germany); when results were positive, testing was followed by microscopic examination of urine samples. Those patients with urinary infection on at least two occasions of testing, with chronic renal disease other than that caused by diabetes, and with a serum creatinine >132.6 μmol/l were excluded from analysis. Further testing in samples applicable for follow-up care was performed after storage at 4°C for up to 1 week. UAE was determined using an immunoturbidimetric assay (TurbiTimeSystem, Behring, Germany). Turbiquant reagents consisting of human serum proteins were used as standards. Microalbuminuria was defined as an albumin excretion rate of 20–200 μg/min, which was calculated from two overnight urine samples. The same urine samples were used for determination of NAG excretion. The procedure uses a color test assay with CPR-NAG (3-cresolsulfonphthaleinyl-β-*N*-acetyl-β-D-glucosaminide) as a substrate (NAG color test, Boehringer Mannheim). Urine creatinine was determined by an enzymatic method on a Hitachi automated analyzer (CREA plus, Boehringer Mannheim). NAG is expressed as the NAG:urine creatinine ratio (U/g creatinine). NAG activity is considered abnormally when >4 U/g creatinine (17).

Statistical analysis

The characteristics of various subgroups of patients are expressed in means ± SD. Where applicable, two-sided *t* tests or χ^2 tests were used for the comparison of two subgroups of patients, with respect to possible risk factors taken singly. Multivariate logistic regression analysis was used to test individual risk factors on the various cardiovascular end points. NAG excretion, UAE, age, diabetes duration, sex, insulin treatment, smoking, total serum cholesterol, triglycerides, and systolic and diastolic blood pressure were used as independent variables, and this choice was based both on the results of univariate analysis and dependent variables. Two-sided Mann-Whitney tests were used to test the association of MI, stroke, and PVD with the levels of urinary NAG excretion and UAE. Special emphasis was laid to a subgroup analysis of patients surviving after follow-up care, for whom all comparable data with respect to MI, stroke, and PVD could be obtained.

RESULTS — A total of 66 of 124 patients who could be followed for a mean of 7 years had significant cardiovascular disease at baseline, and 35 died from cardiovascular disease (Table 1). NAG excretion, UAE, age, diabetes duration, treatment without insulin, systolic blood pressure, serum total cholesterol, and triglycerides were found to be significantly associated with the prevalence of cardiovascular disease. In a multivariate logistic regression model using the variables of age, diabetes duration, serum total cholesterol, and systolic blood pressure, only age and systolic blood pressure emerged as independent predictive factors ($P < 0.02$).

From the subgroup of 59 patients who had died during follow-up care, 35 (more than half of patients) had died from cardiovascular causes, 17 from acute MI, 9 from severe heart failure after previous MI, 7 from stroke, and 2 from severe PVD (gangrene, amputation) leading to death. For end points in this subgroup, diabetes duration and serum triglycerides were found to be the only significant predictors ($P < 0.01$ for both).

From the 65 survivors, 32 with cardiovascular disease were divided into three sub-

Table 2—*Baseline and follow-up data for surviving patients*

	MI	Stroke	PVD	No CVD
n	15	12	5	33
Age (years)	68 ± 1	69 ± 2	67 ± 1	66 ± 2
Sex (M/F)	6/9	6/6	3/2	11/22
Insulin-treated patients (*n*)	9 (11)	5 (8)	5 (3)	28 (31)
Smokers (*n*)	1 (1)	2 (1)	2 (2)	11‡ (6)‡
Diabetes duration (years)	22 ± 2.5†	17 ± 2.5	24 ± 6†	16 ± 1
BMI (kg/m^2)	27 ± 1 (26 ± 1)	28 ± 1 (26 ± 1)	25 ± 2 (24 ± 2)	28 ± 1 (27 ± 1)
HbA_{1c} (%)	8.6 ± 0.6* (8.6 ± 0.6*)	8.0 ± 0.4 (8.0 ± 0.7)	8.0 ± 0.7 (8.1 ± 0.7)	8.0 ± 0.2 (8.6 ± 0.3*)
Systolic blood pressure (mmHg)	160 ± 4 (143 ± 5)	162 ± 3 (140 ± 6)	161 ± 9 (146 ± 10)	158 ± 2 (147 ± 3)
Diastolic blood pressure (mmHg)	88 ± 2 (77 ± 3)	89 ± 2 (76 ± 3)	87 ± 4 (76 ± 2)	87 ± 1 (80 ± 1)
Total cholesterol (mmol/l)	6.98 ± 0.4 (7.03 ± 0.38)	6.56 ± 0.69 (6.33 ± 0.4)	6.9 ± 1 (6 ± 0.74)	6.18 ± 0.15 (6.23 ± 0.2)
HDL cholesterol (mmol/l)	1.16 ± 0.07 (1.16 ± 0.1)	1.29 ± 0.07 (1.1 ± 0.15)	1.29 ± 0.18 (0.95 ± 0.1)	1.2 ± 0.05 (1.2 ± 0.07)
Triglycerides (mmol/l)	5.89 ± 0.5* (4.9 ± 1.06)	7.42 ± 2.79 (4.57 ± 0.69)	6.77 ± 2.12 (4.03 ± 0.69)	5.87 ± 0.67* (4.7 ± 0.43)
Urinary NAG (U/g creatinine)	7.0 ± 0.4† (10 ± 3.5†)	3.5 ± 0.7 (4.9 ± 1.2)	9.1 ± 2.8† (7.3 ± 3†)	5.8 ± 0.7‡ (6.2 ± 1.2)
Urinary albumin (μg/min)	100 ± 40† (480 ± 180†)	45 ± 20 (300 ± 165)	100 ± 55† (420 ± 320†)	6 ± 1‡ (19 ± 9‡)

Data are means ± SD or *n* (%). Follow-up data for surviving patients are in parentheses. *$P < 0.05$ for MI and no CVD vs. stroke and PVD; †$P < 0.05$ for MI and PVD vs. stroke and no CVD; ‡$P < 0.05$ for no CVD vs. MI, stroke, and PVD.

groups of patients reflecting the end points MI, stroke, and severe PVD and compared with patients without cardiovascular disease during follow-up care (Table 2). An association of UAE with MI as well as PVD at baseline and at the time of follow-up care was found (190 ± 100 vs. 750 ± 250 μg/min; $P < 0.01$). The same association was true for NAG excretion in these patients (11.1 ± 4.6 and 13.4 ± 5.6 vs. 5.3 ± 0.6 and 6.2 ± 1.2 U/g creatinine; $P < 0.01$).

Comparison of data for the small group of patients without cardiovascular disease at baseline with respect to development of cardiovascular disease over the study period ($n = 11$) gave a positive predictive trend for NAG excretion (6.5 ± 1.3 vs. 8.3 ± 2.6 U/g creatinine; $P = 0.07$).

For microalbuminuria, this was statistically significant (200 ± 12 vs. 750 ± 25 μg/min; $P < 0.02$). When comparing patients with and without hypertension, we found no difference in UAE and NAG excretion rates.

Regarding known cardiovascular risk factors depicted in Table 2, typical for the era before prospective studies with lipid-lowering agents (statins) had been performed, most of our patients were not adequately treated for their dyslipidemia. Lipid profiles thus did not change significantly after follow-up care. However, most patients with arterial hypertension were treated adequately, predominantly with ACE inhibitors. Blood pressure values were significantly lower during follow-up care compared with baseline data in the majority of our patients.

CONCLUSIONS — Compared with previous studies on diabetes and atherosclerosis (1–3,11,18–23), we also found age, diabetes duration, systolic blood pressure, serum total cholesterol, serum triglycerides, and microalbuminuria significantly associated with end points of cardiovascular disease.

Microalbuminuria has been found to be a predictive marker for the development of cardiovascular disease in various studies (6–9,22,24,25). Because there is no information on clinical long-term data of other renal markers in diabetic patients, our interest focused on urinary NAG with respect to cardiovascular disease. In contrast to urinary albumin, which serves as a marker for glomerular renal function, urinary NAG represents tubular renal function. In our study, this high molecular weight lysosomal enzyme was associated with cardiovascular disease to a lesser extent but was parallel to microalbuminuria. This finding was most evident in the subgroup of patients with MI and PVD who survived over 7 years. Because poor metabolic control has been discussed to be present in patients with a higher rate of urinary NAG excretion (26–29), we also looked into possible correlations with HbA_{1c} levels. A recent study by Yamanouchi et al. (30) described urinary NAG excretion as being associated with high blood glucose. Additionally, to fasting blood glucose levels and HbA_{1c}, they found a positive correlation with serum 1,5-anhydroglucitol, a marker for glycemia that is reabsorbed by the renal tubule. In contrast in our study population, we were not able to prove a correlation between NAG and UAE excretion with metabolic control as measured by HbA_{1c}.

This is in keeping with data in type 1 diabetic subjects published by Jones et al. (27). For the time being, conflicting data on diabetic control and NAG excretion have thus been accumulated. To further clarify the role of NAG excretion, some studies were undertaken to look into associations with microvascular diabetic complications. In studies in type 1 diabetic patients, retinopathy and peripheral neuropathy were found to be correlated with NAG excretion rates and microalbuminuria (14,15,26,28,31). Because sufficient data for the association of NAG excretion with macrovascular disease are still lacking, we tried to find answers in our study.

In 1974, Belfiore et al. (32) published data on serum NAG activity in patients without diabetes, indicating that NAG participates in the degradation of mucopolysaccharides accumulating in atherosclerotic lesions. The increase of extracellular excretion of lysosomal enzymes such as NAG, and, consequently, a rise in urinary excretion rate could thus be one of the reasons for our finding that NAG seems to be associated with macrovascular disease in diabetic patients. In contrast to microalbuminuria, NAG excretion is not uniformally found to be correlated with high blood pressure (16,33–35). Interestingly, in our cohorts, NAG excretion as well as that of urinary albumin are correlated with MI and PVD, but only to a

lesser extent with the hypertension-associated end point for stroke. A reasonable explanation for the difference might be the shorter diabetes duration in the subgroup of patients with stroke. However, we are aware of the fact that the overall small number in the analyzed subgroups needs a cautious interpretation of data.

In summary, our study provides evidence for similar deterioration of glomerular and tubular renal function in diabetic patients with macrovascular disease. It needs to be determined by further studies whether urinary NAG excretion, which is easily detected by using a color test set (36) commercially available and simple to perform, will be of value to serve as an adjunct marker to UAE. Particularly because our data represent a group of elderly type 2 diabetic patients, at present, it is not known if our findings can be extrapolated to other age-groups or to type 1 diabetic patients.

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