Urinary Adiponectin Excretion

A Novel Marker for Vascular Damage in Type 2 Diabetes

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OBJECTIVE—Markers reliably identifying vascular damage and risk in diabetic patients are rare, and reports on associations of serum adiponectin with macrovascular disease have been inconsistent. In contrast to existing data on serum adiponectin, this study assesses whether urinary adiponectin excretion might represent a more consistent vascular damage marker in type 2 diabetes.

RESEARCH DESIGN AND METHODS—Adiponectin distribution in human kidney biopsies was assessed by immunohistochemistry, and urinary adiponectin isoforms were characterized by Western blot analysis. Total urinary adiponectin excretion rate was measured in 156 patients with type 2 diabetes who had a history of diabetic nephropathy and 40 healthy control subjects using enzyme-linked immunosorbent assay. Atherosclerotic burden was assessed by common carotid artery intima-media-thickness (IMT).

RESULTS—A homogenous staining of adiponectin was found on the endothelial surface of glomerular capillaries and intrarenal arterioles in nondiabetic kidneys, whereas staining was decreased in diabetic nephropathy. Low-molecular adiponectin isoforms (~30–70 kDa) were detected in urine by Western blot analysis. Urinary adiponectin was significantly increased in type 2 diabetes (7.68 ± 14.26 vs. control subjects: 2.91 ± 3.85 μg/g creatinine, $P = 0.008$). Among type 2 diabetic patients, adiponectinuria was associated with IMT ($r = 0.479$, $P < 0.001$) and proved to be a powerful independent predictor of IMT ($\beta = 0.360$, $P < 0.001$) in multivariable regression analyses. In a risk prediction model including variables of the UK Prospective Diabetes Study coronary heart disease risk engine urinary adiponectin, but not the albumin excretion rate, added significant value for the prediction of increased IMT ($P = 0.007$).

CONCLUSIONS—Quantification of urinary adiponectin excretion appears to be an independent indicator of vascular damage potentially identifying an increased risk for vascular events. ***Diabetes*** **58:2093–2099, 2009**

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Cardiovascular disease (CVD) is the leading cause of mortality in patients with type 2 diabetes, and the identification of individual risk patterns is fundamental for the prevention and treatment of CVD. However, risk stratification in patients with diabetes is still vague (1,2), and, consequently, numbers needed to treat for prevention of a single cardiovascular event in clinical trials are ~100–200 patients per year (3–5).

Most of the recently described risk markers are metabolic or inflammatory molecules that do not directly indicate vascular damage. Therefore, these indirect markers show variations in risk prediction depending on the metabolic status of the study group (6,7). This becomes evident reviewing data on serum adiponectin; whereas low-circulating adiponectin was significantly associated with increased primary CVD risk in apparently healthy men (8), subsequent studies in high-risk populations, as well as patients with prevalent coronary heart disease (CHD), failed to confirm this association (9,10). A reason for this discrepancy between different groups of risk patients could be a reverse causality, where silent or apparent CVD might lead to compensatory rises in serum adiponectin. Consistently, it was shown in type 2 diabetic patients that adiponectin is lowest in the presence of impaired glucose regulation and early diabetes, whereas long diabetes duration is associated with a significant increase in circulating adiponectin (11).

Adiponectin is a 30-kDa adipocyte-derived vasoactive peptide closely linked to components of the metabolic syndrome (rev. in 12). It has anti-inflammatory and anti-atherosclerotic properties on endothelial cells by decreasing vascular inflammation, foam cell formation, and cell adhesion, which all are involved in the initiation and progression of vascular lesions (12). Recently, it was reported that adiponectin has a distinct role for glomerular homeostasis in an experimental model (13). Hence, adiponectin could be present on human renal endothelium and glomerular capillary stress in diabetes may promote shedding of adiponectin from endothelial surfaces by proteolytic cleavage, causing degradation of high-order complexes of adiponectin and subsequent appearance of the adiponectin monomer (~28 kDa), dimer (~56 kDa), and trimer (~68 kDa) in urine.

We hypothesized that adiponectin appears in urine consequently reflecting early glomerular vascular damage in type 2 diabetes rather than the metabolic changes associated with serum adiponectin. To characterize a possible diagnostic value of urinary adiponectin excretion, patients with type 2 diabetes and early diabetic nephrop-

TABLE 1
Characteristics of control and type 2 diabetic subjects and Spearman's correlation coefficients between serum or urinary adiponectin levels and cardiovascular risk factors and measures of subclinical atherosclerosis in type 2 diabetic patients

	Control subjects	Type 2 diabetic patients	P*	Serum adiponectin		Urinary adiponectin	
				r	P†	r	P†
n	40	156					
Sex (male)	25 (61.0)	117 (75.0)	0.017	−0.290	<0.001	−0.009	0.915
Age (years)	51.9 ± 9.5	60.5 ± 7.8	<0.001	0.327	<0.001	0.181	0.025
BMI (kg/m²)	25.4 ± 4.6	33.1 ± 5.9	<0.001	−0.041	0.613	−0.098	0.231
WHR	0.87 ± 0.09	1.02 ± 0.08	<0.001	−0.286	<0.001	0.062	0.453
HDL cholesterol (mmol/l)	1.52 ± 0.42	1.17 ± 0.35	<0.001	0.413	<0.001	0.018	0.826
LDL cholesterol (mmol/l)	3.12 ± 0.72	2.66 ± 0.92	<0.001	0.137	0.106	−0.013	0.877
Triglycerides (mmol/l)‡	1.06 (0.80–1.71)	1.87 (1.19–2.86)	<0.001	−0.311	<0.001	−0.069	0.400
FPG (mmol/l)	5.06 ± 0.6	8.03 ± 2.74	<0.001	−0.167	0.041	0.073	0.375
A1C (%)	5.5 ± 0.5	7.2 ± 1.2	<0.001	−0.143	0.085	0.080	0.342
Creatinine clearance (ml/min)	119.3 ± 41.2	109.6 ± 40.7	0.146	−0.223	0.011	−0.156	0.074
Cystatin C (mg/l)	0.57 ± 0.13	0.79 ± 0.31	<0.001	0.141	0.084	−0.025	0.759
AER (mg/24h)‡	<10	43.9 (21.5–103.1)	<0.001	−0.009	0.911	−0.031	0.707
High-sensitive CRP (mg/l)‡	0.2 (0.1–0.6)	2.3 (1.0–4.9)	<0.001	−0.188	0.024	−0.152	0.069
Systolic blood pressure, 24 h (mmHG)	124 ± 14	140 ± 16	<0.001	−0.025	0.770	0.124	0.137
Diastolic blood pressure, 24 h (mmHG)	76 ± 9	80 ± 8	0.010	−0.093	0.265	0.031	0.715
Mean arterial pressure, 24 h (mmHG)	92 ± 9	99 ± 10	<0.001	−0.060	0.472	0.073	0.383
Duration of diabetes (years)‡		11.0 (6.0–16.0)		0.135	0.099	0.211	0.009
Current smoker (yes)	13 (31.7)	25 (16.0)	0.027	−0.054	0.509	−0.022	0.788
Patient with angiotensin converting enzyme-inhibitor or angiotensin receptor blocker (yes: n; %)		131 (84.0)		0.104	0.205	−0.191	0.019
Patient with statins (yes: n; %)		91 (58.3)		0.074	0.364	0.016	0.846
Carotid IMT (mm)		0.88 ± 0.15		−0.202	0.017	0.479	<0.001
Serum adiponectin (μg/ml)	10.3 ± 6.6	7.0 ± 4.9	0.033			0.184	0.025
Urinary adiponectin (μg/g creatinine)	2.91 ± 3.85	7.68 ± 14.26	0.008	0.184	0.025		

Data are means ± SD, n (%), or median (interquartile range). *P for type 2 diabetic versus control subjects (by independent t test for normally distributed data or Mann-Whitney U test for not normally distributed data). †P by bivariate Spearman correlation analysis.

athy (i.e., a history of microalbuminuria) were studied, and the atherosclerotic burden of these patients was assessed by quantification of common carotid artery intima-media-thickness (IMT). Both urinary adiponectin and urinary albumin excretion rate (AER) as an established marker of micro- and macrovascular dysfunction in type 2 diabetes were evaluated for the prediction of increased IMT in comparative analyses.

RESEARCH DESIGN AND METHODS

Study population and data collection. Patients (156) with type 2 diabetes, according to the American Diabetes Association (ADA) criteria (14), were recruited from family practices being referred to the diabetes outpatient clinic at the University Hospital Heidelberg for specialist treatment. For eligibility, patients had to have a documented history of microalbuminuria in at least two separate urine samples [albumin creatinine ratio (ACR) >30 mg/g creatinine or AER >30 mg/24h]. Healthy control subjects (40) were recruited at the outpatient clinic of the University Hospital Wuerzburg. In the latter, manifest CVD and/or diabetes, fasting plasma glucose (FPG) level ≥7 mmol/l, acute or chronic kidney disease, present microalbuminuria, history of hypertension and/or lipid disorder, and intake of any regular medication was an exclusion criterion. Detailed patient characteristics are shown in Table 1. In all individuals, 24-h urine samples were collected on 3 consecutive days and the mean of AER and of the creatinine clearance was taken for statistical evaluation. All blood values, as well as ambulatory 24-h blood pressure values (given as mean of 24 h), were taken on day 1. The study complied with the Declaration of Helsinki, and all subjects gave written informed consent. The study was approved by the ethics committees of the Universities of Heidelberg and Wuerzburg.

Adiponectin immunohistochemistry in human kidneys. Immunohistochemical analyses were performed in human kidney biopsies from type 2 diabetic patients (n = 6) and tumor-distant kidney tissues from nondiabetic patients after tumornephrectomy (n = 6). Early diabetic nephropathy (n = 3) was classified as presenting with micro- or macroalbuminuria and calculated glomerular filtration rate >60 ml/min. Indication for kidney biopsy was based on progressive increase in proteinuria >1 g/24 h in two subjects and progressive increase in serum creatinine in one. Late diabetic nephropathy (n = 3) was classified by micro- or macroalbuminuria and calculated glomerular filtration rate <30 ml/min. Indication for kidney biopsy in these patients was progressive nephrotic proteinuria. In nondiabetic control subjects, tumor-distant kidney tissue was embedded in paraffin blocks and according histological samples were qualified to show normal kidney morphology by two independent experts. Utilization of noncancerous regions of resected kidneys distant from the tumor as control tissue has previously been reported (15,16). None of these patients had an FPG level ≥7 mmol/l or present microalbuminuria. Immunohistochemical staining was performed on 3-μm sections of formaldehyde-fixed and paraffin-embedded tissue using the avidin-biotin complex method. Tissues were deparaffinized with xylene and rehydrated through graded concentrations of ethanol. After rehydration, the sections were pretreated by microwave heating in citrate buffer (pH 6.0) for 20 min. The primary antibody against adiponectin (Adiponectin/Acrp30 biotinylated affinity purified polyclonal antibody) was obtained from R&D Systems (Minneapolis, MN) and used at a dilution of 1:25 for 2 h at room temperature, followed by incubation with horseradish peroxidase–labeled streptavidin (Vector Laboratories, Burlingame, CA) diluted 1:200 for 1 h. NovaRED (Vector) was applied for visualization. Control subjects, omitting the first antibody for each paraffin block tested, were negative.

Detection and characterization of adiponectin multimers in urine by Western blot. SDS-PAGE was performed after modification of the protocol as previously described (17). Urinary samples (16 μl) were mixed with SDS-sample buffer and then incubated at room temperature for 1 h. Urinary proteins were separated by 10% SDS-PAGE, and transferred to a nitrocellulose membrane. Membranes were blocked with Tris-buffered saline Tween-5% skim milk and then incubated with a goat anti-human adiponectin polyclonal antibody (1:200; R&D Systems, Wiesbaden, Germany) for 1 h at room temperature. After washing (three times for 5 min), membranes were incubated with horseradish peroxidase–conjugated donkey anti-goat antibody (1:3,200; Santa Cruz Biotechnology, Heidelberg, Germany) for 1 h at room temperature and then washed thoroughly (three times for 5 min). Signals were visualized using lumi-light Western blotting substrate (Roche Diagnostics, Mannheim, Germany) and the image was acquired by Kodak IS440CF Imaging Station. Specificity of bands was shown in a competition experiment. In

competition experiments, membranes were preincubated overnight with 2 μg/ml recombinant human adiponectin (R&D Systems) as a competitor before Western blotting.

Clinical chemistry. Blood was drawn on day 1 in a fasting state under standardized conditions and stored at −80°C until analysis. Total urinary adiponectin concentrations were measured in duplicates by a high-sensitive enzyme-linked immunosorbent assay (ELISA; BioVendor, Brno, Czech Republic) according to the manufacturer's protocol. The intra- and interassay variations were 5.4 and 9.0%, respectively. Urinary adiponectin levels (nanogram per milliliter) were adjusted for urinary creatinine excretion and expressed as micrograms per gram of creatinine for statistical analysis. In type 2 diabetic patients, presence of adiponectinuria was defined as level >mean + 2 SD (95. percentile) of the adiponectin excretion rate in healthy control subjects (→urinary adiponectin >10.61 μg/g creatinine). FPG was measured by a glucose oxidase method. Triglyceride, total cholesterol, and HDL cholesterol levels were quantified by standard laboratory methods, and LDL cholesterol levels were calculated by using the Friedewald formula. A1C was measured by high-performance liquid chromatography on a Variant II device (Bio-Rad Laboratories, Munich, Germany). Cystatin C was measured by ELISA (BioVendor, Brno, Czech Republic), and high-sensitive C-reactive protein (hsCRP) levels were determined by nephelometry (Dade Behring, Cupertino, CA). AER was assessed and performed in three consecutive 24-h urinary collections. For collection of the urine sample, a 3-l plastic container was used, and the volume of urine was measured to the nearest 50 ml. Urine aliquots were stored deep frozen at −80°C until analyses. Albumin levels were determined by turbidimetry (Siemens Healthcare Diagnostics, Eschborn, Germany). AER was expressed as milligrams per 24 h. Patients with an AER of 30–300 mg/24 h were defined to have microalbuminuria and an AER >300 mg/24 h was defined as macroalbuminuria.

Assessment of carotid atherosclerosis. IMT was detected using high-resolution B-mode ultrasound (Voluson 730 Kretz, Tiefenbach, Austria) of the extracranial carotid arteries bilaterally under continuous detection of the heart cycle using a three-lead electrocardiogram. The whole imaging and quantification procedure was performed digitally (Voluson 730 Kretz, Tiefenbach, Austria) at the time of study entry by a single investigator blinded for clinical data. For the purpose of this study, IMTs of the far wall of the common carotid artery were detected in end-diastolic frames, ~10 mm proximal to the carotid bulb, according to a previously described scanning protocol (18). The measurements were performed in four points of both common carotid arteries avoiding areas of atherosclerotic plaque formation. The mean of the resulting eight single measurements was taken as mean IMT for statistical analyses in this study.

Statistical analyses. Statistical analyses were performed using SPSS software version 15.0 (SPSS, Chicago, IL). Spearman correlation coefficients were used to describe the association between urinary adiponectin and the variables of interest. Comparison between two sets of patients was performed by independent t test or Mann-Whitney U test. Multivariable linear regression analyses evaluated the association of urinary adiponectin with IMT. The models fitted for IMT as a dependent variable, included age, sex, waist-to-hip ratio (WHR), HDL, LDL, hsCRP, A1C, AER, smoking status, mean arterial pressure (MAP), diabetes duration, serum, and urinary adiponectin concentrations. The UK Prospective Diabetes Study (UKPDS) risk engine was previously introduced as a parametric model that provides the estimates of primary coronary heart disease (CHD) risk in type 2 diabetes (19). In contrast to previous models for CHD, such as the Framingham risk equations, the diabetes-specific approach of the UKPDS risk engine includes A1C as continuous variable. Age as a risk factor is replaced by two diabetes-specific variables: age at diagnosis of type 2 diabetes and time since diagnosis. Furthermore, as there is evidence that diabetic dyslipidemia is qualitatively different from dyslipidemia in the general population, total and HDL cholesterol are included instead of LDL cholesterol. The complete model incorporates age at diagnosis of diabetes, diabetes duration, sex, smoking status, A1C values, systolic blood pressure, and the total and/or HDL cholesterol ratio (19). We plotted receiver operating characteristic (ROC) curves and the area under the curve (AUC) was analyzed to study the value of the UKPDS CHD risk engine for the prediction of carotid atherosclerosis (upper vs. lower two tertiles of IMT). Calculations were performed using online provided software (http//:www.dtu.ox.ac.uk). Intertertile cutoff points of IMT were <0.82 mm (n = 52), 0.82–0.94 mm (n = 52), and >0.94 mm (n = 52). Values for the AUC as obtained by the UKPDS risk engine were compared with the AUC after additional inclusion of AER and urinary adiponectin. Statistical comparisons of the AUC ROC curves were performed using "roccomp" command provided by the statistical software Stata/SE 8.2 for Windows (Stata Corporation, College Station, TX). $P < 0.05$ was considered to be statistically significant.

RESULTS

Immunohistochemistry of adiponectin in human kidneys and determination of urinary adiponectin isoforms by Western blot. A strong staining for adiponectin was detected on the endothelium of intrarenal arteries/arterioles and on the endothelial surface of glomerular and peritubular capillaries in all nondiabetic kidneys, and representive findings are shown in Fig. 1*A*. In patients with early diabetic nephropathy, the homogeneous glomerular staining pattern of adiponectin was markedly decreased, whereas adiponectin staining in intrarenal arteries/arterioles remained unchanged (Fig. 1*B*). In type 2 diabetic kidney biopsies, adiponectin was found in tubular casts, indicating urinary excretion of the protein (Fig. 1*C*). In patients at advanced stages of diabetic nephropathy with overt glomerulosclerosis, glomerular staining for adiponectin was almost completely lost (not shown).

Next, we studied whether adiponectin is specifically detectable in urine of patients with diabetic nephropathy and either current normoalbuminuria or microalbuminuria and healthy control subjects and whether different isoforms of adiponectin are present in these samples. In Western blot analyses of urine samples from patients with diabetic nephropathy, the strongest signals were found at ~75 kDa, most likely representing the low–molecular weight adiponectin trimer (68 kDa). An additional signal at ~25 kDa represents the adiponectin monomer (28 kDa) (Fig. 1*D*) as previously shown for serum adiponectin (17). In healthy control subjects, antigens representing adiponectin dimers (56 kDa) and monomers were found; however, overall intensity of these signals was much lower compared with the diabetic patients studied (Fig. 1*D*).

Association of serum and urinary adiponectin levels with cardiovascular risk factors. Characteristics of control subjects and patients with type 2 diabetes and bivariate analyses of associations between different variables and serum or urinary adiponectin are shown in Table 1. Serum adiponectin was significantly lower and urinary adiponectin levels significantly higher in patients with type 2 diabetes than in control subjects (mean serum adiponectin: 7.0 ± 4.9 vs. 10.3 ± 6.6 μg/ml, $P = 0.033$; mean urinary adiponectin: 7.68 ± 14.26 vs. 2.91 ± 3.85 μg/g creatinine, $P = 0.008$; Fig. 1*E*). Type 2 diabetic patients were more often men, nonsmokers, and had higher age, BMI and WHR compared with control subjects. As would be expected, type 2 diabetes was associated with significantly increased values of traditional cardiovascular risk factors, such as LDL, triglycerides, systolic and diastolic blood pressure, MAP, FPG, A1C, and hsCRP values (Table 1). Kidney function was not significantly different between the two groups (type 2 diabetes, creatinine clearance: 109.6 ± 40.7 vs. control subjects: 119.3 ± 41.2 ml/min, $P = 0.146$), whereas serum cystatin C levels and levels of albuminuria were significantly higher in patients with type 2 diabetes than in control subjects, reflecting the early functional alterations in diabetic nephropathy (mean cystatin C: 0.79 ± 0.31 vs. 0.57 ± 0.13 mg/l, $P < 0.001$; median AER: 43.9 [21.5–103.1] vs. <10 mg/24 h, $P < 0.001$; Table 1). Bivariate correlations showed significant associations between serum adiponectin and sex, age, WHR, HDL, triglycerides, FPG, creatinine clearance, and hsCRP in patients with type 2 diabetes. For urinary adiponectin, there were no significant correlations with components of the metabolic syndrome like blood lipids or WHR, yet there were significant associations with increased age,

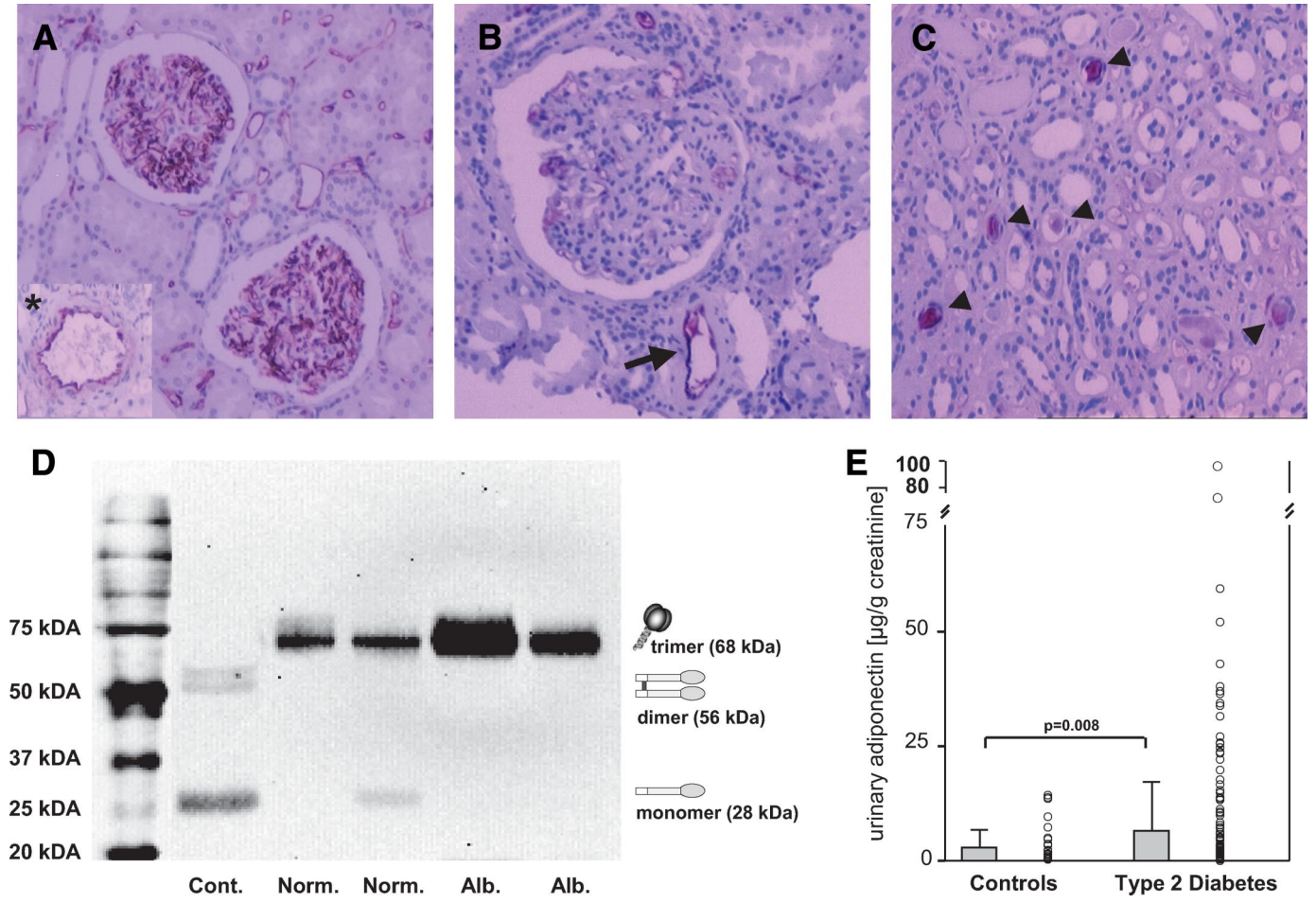

FIG. 1. Adiponectin in human kidneys and urinary adiponectin excretion. Immunohistochemical analyses (ABC-POD method) of human kidney tissues for adiponectin. *A*: Nondiabetic kidneys demonstrate strong endothelial adiponectin staining lining the glomerular and intertubular capillaries as well as intrarenal arteries/arterioles (*inserted panel*). *B*: In patients with early diabetic nephropathy, adiponectin staining was considerably decreased in glomerular capillaries, showing an inhomogeneous, segmental pattern. However, adiponectin was still present at the endothelial surface of intrarenal arteries/arterioles (arrow). *C*: Positive adiponectin staining of tubular casts (arrowheads) in patients with diabetic nephropathy. *D*: Identification of adiponectin in urine. Urine from healthy individuals (Cont.), type 2 diabetic patients with normoalbuminuria (Norm.), and with microalbuminuria (Alb.) was analyzed by Western blotting and representive findings are presented. In type 2 diabetic patients, bands were found running at ~75 kDa, most likely representing the adiponectin trimer (68 kDa). An additional weak band running at ~25 kDa represents the adiponectin monomer (28 kDa). In healthy control subjects, only adiponectin dimers (56 kDa) and monomers were found, and signal intensity was clearly decreased compared with the diabetic patients studied. *E*: Dots represent individual urinary adiponectin levels in 156 patients with type 2 diabetes and 40 healthy control subjects. Mean urinary adiponectin (±SE) was significantly higher in patients with type 2 diabetes compared with control subjects ($P = 0.008$; gray bars). (A high-quality digital representation of this figure is available in the online issue.)

duration of diabetes, and patients treated with ACE inhibitors or angiotensin receptor blockers (Table 1).

Twenty-one (13.5%) of the participants with type 2 diabetes were found to have increased urinary adiponectin but no current albuminuria (Table 2, group 2). This subgroup of patients did not differ significantly from patients without adiponectinuria (group 1) or current albuminuria (group 3) with respect to age, glycemic control, and renal function (Table 2). However, individuals in this group had significantly increased IMT compared with patients without adiponectinuria (0.95 ± 0.10 vs. 0.84 ± 0.14 mm; $P < 0.001$) and patients with current albuminuria (0.95 ± 0.10 vs. 0.89 ± 0.15 mm; $P < 0.05$), although the latter had longer diabetes duration (13.6 [7.0–18.6] vs. 9.5 [5.8–17.3] years: $P < 0.05$) and increased FPG levels (8.67 ± 2.97 vs. 6.94 ± 2.67 mmol/l; $P < 0.01$) (Table 2).

Associations of carotid atherosclerosis with urinary and serum adiponectin and other CVD risk factors in patients with type 2 diabetes. In bivariate correlation analysis IMT was significantly and positively associated with urinary adiponectin ($r = 0.479$, $P < 0.001$) and negatively associated with serum adiponectin levels ($r = -0.202$, $P = 0.017$). Among other cardiovascular risk factors, age ($r = 0.277$, $P = 0.001$), diabetes duration ($r = 0.227$, $P = 0.007$), systolic blood pressure ($r = 0.171$, $P = 0.048$), and LDL cholesterol ($r = 0.212$, $P = 0.012$) were significantly associated with IMT.

In multivariable linear regression analyses with IMT as dependent variable, age ($\beta = 0.239$, $P = 0.012$), LDL cholesterol ($\beta = 0.231$, $P = 0.009$), and diabetes duration ($\beta = 0.197$, $P = 0.026$) were independently associated with the extent of carotid atherosclerosis (Table 3). Urinary adiponectin had the strongest association with IMT in this model ($\beta = 0.360$, $P < 0.001$). We performed subgroup analyses investigating a potential linear relationship between urinary adiponectin and IMT, and patients were divided into quartiles of increasing urinary adiponectin. A stepwise increase in urinary adiponectin was associated with increased IMT (urinary adiponectin <1.05 µg/g creatinine: IMT mean [95% CI] 0.79 (0.75–0.83) mm; urinary adiponectin 1.05–1.89 µg/g creatinine: IMT 0.85 (0.80–0.91) mm; urinary adiponectin 1.90–7.20 µg/g creatinine: IMT 0.89 (0.85–0.93)

TABLE 2
Carotid IMT and characteristics of type 2 diabetic patients with and without adiponectinuria and albuminuria

	1 Adiponectinuria (−) Albuminuria (−)	2 Adiponectinuria (+) Albuminuria (−)	3 Albuminuria (+)
n	43	21	92
Age (years)	60.8 ± 8.9	61.4 ± 7.1	60.7 ± 7.0
Sex (male)	33 (76.7)	14 (66.7)	71 (77.2)
BMI (kg/m²)	32.3 ± 5.0	32.7 ± 6.6	33.6 ± 6.4
FPG (mmol/l)	7.08 ± 1.55	6.94 ± 2.67	8.67 ± 2.97*†
A1C (%)	6.9 ± 0.8	7.2 ± 1.6	7.4 ± 1.2†
Creatinine clearance (ml/min)	111.5 ± 46.2	109.1 ± 29.5	107.9 ± 41.0
Serum adiponectin (μg/ml)	10.6 ± 7.4	11.4 ± 6.1	10.0 ± 6.7
Duration of diabetes (years)	10.0 (5.0–13.0)	9.5 (5.8–17.3)	13.6 (7.0–18.6)†§
Carotid IMT (mm)	0.84 ± 0.14	0.95 ± 0.10‖	0.89 ± 0.15†§

Data are means ± SD, *n* (%), or median (interquartile range). Adiponectinuria is defined as adiponectin excretion >mean + 2 SD (95th percentile) of control urinary adiponectin excretion. Albuminuria is defined as AER >30 mg/24 h. *$P < 0.01$ for group 3 group 2; †$P < 0.05$ for group 3 vs. group 1 (by independent *t* test or Mann-Whitney *U* test); §$P < 0.05$; ‖$P < 0.001$ for group 2 vs. group 1.

mm; urinary adiponectin >7.20 μg/g creatinine: IMT 0.98 (0.95–1.02) mm; $P < 0.001$ by ANOVA; Fig. 2).

Predictive value of urinary adiponectin in comparison with urinary albumin for extent of carotid atherosclerosis. We performed ROC analyses in models including traditional cardiovascular risk factors adding AER and urinary adiponectin levels to quantify their power for the prediction of carotid IMT (upper vs. lower two tertiles) in patients with type 2 diabetes. Carotid IMT as well as the UKPDS CHD risk engine have previously been shown to predict CVD risk in patients with type 2 diabetes (19); in this study, the UKPDS CHD risk engine score and IMT were significantly correlated ($r = 0.509$, $P < 0.001$). Therefore, the risk factors represented in the UKPDS CHD risk engine (age at diabetes diagnosis, diabetes duration, sex, smoking status, systolic blood pressure, A1C, and total and HDL cholesterol) were chosen in a basis model for ROC analysis. The UKPDS CHD risk engine factors reached an AUC of 0.700 (95% CI 0.607–0.792, Fig. 3). Although urinary albumin levels were significantly associated with IMT in bivariate analysis ($r = 0.202$, $P < 0.010$), further addition of AER to the basis model did not significantly alter the predictive value for carotid IMT (AUC 0.702 [95% CI 0.610–0.795], Fig. 3). Inclusion of urinary adiponectin added significant predictive value for prediction of IMT compared with the basis model of the UKPDS CHD risk engine factors (AUC 0.800 [95% CI 0.724–0.872], $P = 0.007$, Fig. 3).

TABLE 3
Independent predictors of IMT in patients with type 2 diabetes

	Common carotid artery IMT		
	β	*t*	*P*
Age (years)	0.239	2.562	0.012†
Sex (male)	−0.118	−1.114	0.268
WHR	0.142	1.274	0.206
HDL cholesterol (mmol/l)	−0.239	−1.497	0.138
LDL cholesterol (mmol/l)	0.231	2.649	0.009*
hsCRP (mg/l)†	−0.034	−0.393	0.696
A1C (%)†	0.089	1.031	0.305
AER (mg/24h)†	0.034	0.385	0.701
Current smoker (yes)	0.107	1.292	0.200
MAP, 24 h (mmHG)	−0.104	−1.219	0.226
Diabetes duration (years)†	0.197	2.269	0.026‡
Serum adiponectin (μg/ml)†	−0.128	−1.362	0.177
Urinary adiponectin (μg/g creatinine)†	0.360	4.005	<0.001§
r	0.665		
r^2	0.442		

*$P < 0.01$; †log-transformed variables; ‡$P < 0.05$; §$P < 0.001$.

DISCUSSION

This is the first study evaluating urinary adiponectin as a novel marker for vascular damage. The results of this study imply that urinary adiponectin may emerge as a pathophysiologically plausible and valuable marker for prevalent micro- and macrovascular disease in type 2 diabetes.

The American guidelines recommend an office-based assessment as the initial step in predicting CVD risk in primary prevention utilizing multifactorial statistical models (20). However, a certain proportion of patients will be

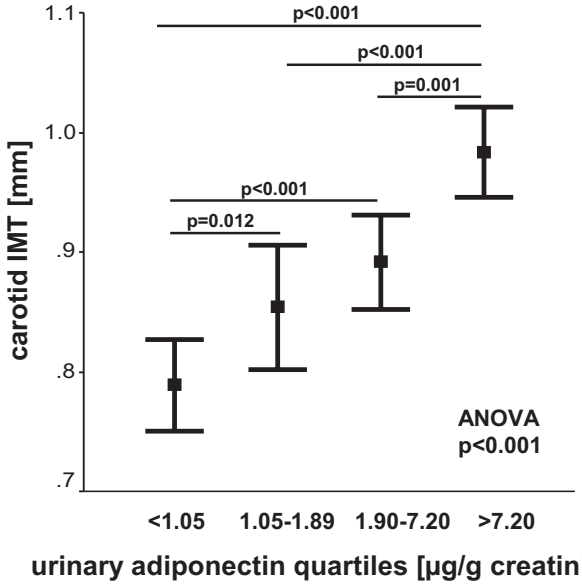

FIG. 2. Associations between urinary adiponectin levels and carotid atherosclerosis in type 2 diabetes. A stepwise increase in urinary adiponectin levels was associated with a significant increase in IMT (ANOVA: $P < 0.001$). Compared with patients in the lowest urinary adiponectin quartile (<1.05 μg/g creatinine), those with urinary adiponectin excretion levels 1.05–1.89, 1.90–7.20, and >7.20 μg/g creatinine had a significantly increased extent of carotid IMT (all $P < 0.02$). Comparisons between two sets of patients were performed by independent *t* test. The central box represents the means ± SE.

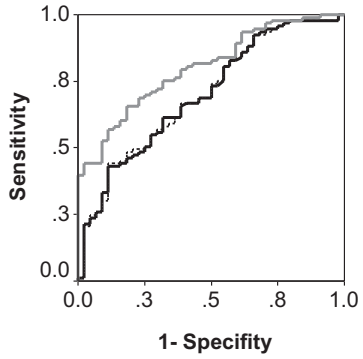

FIG. 3. ROC curves of the UKPDS risk engine, albuminuria and urinary adiponectin excretion, and predictive values for the extent of carotid atherosclerosis. Prediction of carotid atherosclerosis extent was based on discrimination between subjects with low or moderate (lower two tertiles of common carotid artery IMT) and severe (upper IMT tertile) carotid wall irregularities. The AUC for the UKPDS CHD risk engine was 0.700 (95% CI 0.607–0.792); bold black line, basis model. Further addition of the AER to the basis model did not significantly alter the predictive value for IMT (AUC 0.702 [95% CI 0.610–0.795]; dashed line). On the other hand, inclusion of urinary adiponectin added significant value for the prediction of IMT compared with the basis model of the UKPDS CHD risk engine factors (AUC 0.800; gray line).

misclassified using this traditional risk prediction, and CVD risk has particularly been underestimated in patients with diabetes (21). Apart from albuminuria, atherogenic dyslipidemia and to a lesser extent long-term glucose control (that are included in the UKPDS risk engine), novel biochemical risk markers were found to be of minor importance for the overall prediction of CVD risk (2,6,7).

In this study, urinary adiponectin was superior to AER in the prediction of increased IMT. Measurement of urinary albumin excretion is currently utilized as a screening test for the presence of diabetes-related kidney disease. Furthermore, microalbuminuria is a marker of endothelial dysfunction and predicts CVD in patients with type 2 diabetes (22,23). However, risk prediction by assessment of urinary albumin levels reveals some important limitations. A diabetes duration of >6 years may precede the first appearance of urinary albumin (24), and vascular changes start long before the first appearance of albumin in urine and even before the diagnosis of diabetes (25,26). Hence, closing this diagnostic gap is highly desired. In this study, 13.5% of the type 2 diabetic patients were shown to have a significant adiponectinuria without current albuminuria. Despite similar age and glycemic control, this subgroup of patients had a significantly increased IMT compared with participants without adiponectinuria or current albuminuria (Table 2). In accordance with the hypothesis, this implies that adiponectinuria could be an early marker of endothelial damage on a prealbuminuric level and that urinary adiponectin has the potential to exceed the predictive value of urinary albumin for CVD risk evaluation. However, although Table 2 shows significant differences between groups 2 and 3, the subgroup of patients with adiponectinuria only (group 2) is small and differences between the two groups will have to be studied in larger cohorts. One possible explanation for the superiority of urinary adiponectin compared with urinary albumin for prediction of increased IMT in this study could be originated from previously published experimental and clinical data. The prevalence of microalbuminuria in patients with type 2 diabetes is estimated at about 20%, and about 30% in those subjects >55 years of age (27). Hence, it appears that applicability of microalbuminuria for CVD risk evaluation in type 2 diabetes per se is limited. Moreover, in large prospective cohort studies, the increased risk for CVD started from urinary albumin levels well below the cutoff for micoalbuminuria (25), and, thus, a considerable percentage of high-risk patients will be missed by screening for albuminuria utilizing current cut off values. This has recently been supported by data in the Finnish Diabetic Nephropathy (FinnDiane) Study, in which a significant number of type 1 diabetic patients at high risk for premature death had normoalbuminuria (28).

The association between IMT and urinary adiponectin shown here was independent of circulating adiponectin levels. Moreover, urinary adiponectin levels did not significantly correlate with most of the traditional cardiovascular risk factors in bivariate analyses. This is an advantageous precondition to achieve significant additional value in risk prediction models. Although serum adiponectin is strongly associated with the components of the metabolic syndrome and closely linked to metabolic risk factors like WHR, HDL cholesterol, and triglycerides, urinary adiponectin seems to indicate vascular damage and is therefore associated with older age and duration of type 2 diabetes in this study.

The finding of extensive staining for adiponectin in control kidneys, lining glomerular capillaries, and intrarenal arterioles was somehow unexpected, because in rodents, adiponectin accumulation could only be detected in injured vessels (29). Although histological examination of tumor distant regions from resected kidney tissue, serving as "healthy" control deserves cautious interpretation our data indicate that binding of adiponectin to the glomerular endothelium could be essential for kidney homeostasis. This idea is further supported by two independent experimental studies (13,30) in which adiponectin-deficient ($Adip^{-/-}$) mice treated with adiponectin showed a reduction of albuminuria, improvement of podocyte function, and decreased urinary and glomerular markers of oxidant stress. Thus, we speculate that the ubiquitous distribution of adiponectin in nondiabetic glomerular capillaries in this study and its subsequently increasing appearance in urine, associated with loss of glomerular adiponectin in diabetic nephropathy, might reflect earlier vascular damage than the vascular leakage detected by albuminuria. On the other hand, adiponectin was still present at the endothelial surface of intrarenal arterioles in patients with diabetic nephropathy that may be because of different functions of adiponectin in these tissues (13,29,30). The role of adiponectin in the human glomerulum needs to be addressed in future studies because its excretion could be a more pathophysiological marker of vascular stress and may precede the onset of microalbuminuria and renal failure in type 2 diabetes. Consistent with this hypothesis, we found a significant lower accumulation of adiponectin in glomerular capillaries of patients with type 2 diabetes. This could reflect an increased urinary loss of the adipokine and might be explained by damage to the glomerular capillary wall resulting in a significant loss of endothelial binding sites for adiponectin.

We recognize the limitations of the present study. The cross-sectional design of our study warrants cautious interpretation of the results, and further investigations in large prospective trials with defined CVD end points are necessary to substantiate these findings. As all patients of our study were selected by a previous history of microalbuminuria, future studies will have to address whether the proposed association between urinary adi-

ponectin and atherosclerosis can be confirmed in patients at advanced stages of diabetic nephropathy, as well as in nonalbuminuric diabetic patients. Conclusions can only be drawn for the high-risk group of type 2 diabetic patients in this study. Moreover, because the number of histological samples examined in this study is low, additional studies are clearly needed to further elucidate the distribution pattern of glomerular adiponectin in different renal pathologies to substantiate the potential role of adiponectin for glomerular homeostasis in humans.

In conclusion, measurement of urinary adiponectin may emerge as a novel and easy-to-obtain method for the clinical assessment of vascular stress and CVD risk in type 2 diabetes that needs to be validated in larger prospective studies and different samples including diabetic patients without a history of microalbuminuria.

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