

# Markers of Endothelial Dysfunction and Inflammation in Type 1 Diabetic Patients With or Without Diabetic Nephropathy Followed for 10 Years

Association with mortality and decline of glomerular filtration rate

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**OBJECTIVE** — We evaluated the association of biomarkers of endothelial dysfunction and inflammation with all-cause mortality and cardiovascular mortality and morbidity and decline in glomerular filtration rate (GFR) in type 1 diabetic patients.

**RESEARCH DESIGN AND METHODS** — We prospectively followed 199 type 1 diabetic patients with diabetic nephropathy and 192 patients with persistent normoalbuminuria. Biomarkers were measured at baseline.

**RESULTS** — We constructed two Z scores: the mean inflammatory Z score combined C-reactive protein, interleukin-6, soluble intercellular adhesion molecule (sICAM-1), and secreted phospholipase A2 and the mean Z score for endothelial dysfunction combined soluble vascular cell adhesion molecule 1, plasminogen activator inhibitor-1, and sICAM-1. The mean Z score of inflammatory biomarkers was associated with mortality and the combined end point in patients with diabetic nephropathy after multivariate adjustment (hazard ratio 1.7 [95% CI 1.1–2.6];  $P = 0.025$  and 1.5 [1.1–2.2];  $P = 0.017$ ). The mean Z score for endothelial dysfunction biomarkers was associated with mortality in a model adjusting for age and sex in patients with diabetic nephropathy (1.6 [1.0–2.3];  $P = 0.031$ ). The mean Z score for endothelial dysfunction correlated with decline in GFR ( $r = -0.243$ ;  $P = 0.001$ ); the correlation persisted after multivariate adjustment (coefficient  $-1.38$  [95% CI  $-2.27$  to  $-0.50$ ];  $P = 0.002$ ).

**CONCLUSIONS** — Mean Z scores of inflammatory biomarkers are significantly associated with all-cause mortality and cardiovascular morbidity and mortality in patients with nephropathy after multivariate adjustment. These data suggest that the high risk of cardiovascular disease in type 1 diabetes may be explained in part by inflammatory activity. Mean Z score of endothelial dysfunction correlated after multivariate adjustment with the rate of decline in GFR.

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Patients with type 1 diabetes and nephropathy carry a high risk of cardiovascular disease (1–3). This risk cannot be fully explained by conventional risk factors.

Low-grade inflammation increases the invasion of monocytes into the vascular wall, leading to the formation of atherosclerosis and cardiovascular disease (4). The pathogenesis of vascular compli-

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**Abbreviations:** AUC, area under the receiver operating characteristic curve; GFR, glomerular filtration rate; hsCRP, high-sensitivity C-reactive protein; IL-6, interleukin-6; PAI-1, plasminogen activator inhibitor-1; sICAM-1, soluble intercellular adhesion molecule-1; sPLA2, secreted phospholipase A2; sVCAM-1, soluble vascular cell adhesion molecule-1; TGF- $\beta$ , transforming growth factor- $\beta$ .

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cations involves inflammation and endothelial dysfunction (5–8), but whether biomarkers of these processes are associated with prognosis in type 1 diabetes is not known.

In the Hoorn Study, endothelial dysfunction and inflammation explain much of the excess risk in patients with type 2 diabetes (9,10). In a large cross-sectional study (the EURODIAB Prospective Complications Study), type 1 diabetic patient markers of inflammation were associated with cardiovascular disease (11). In non-diabetic patients and in patients with type 2 diabetes, longitudinal studies have demonstrated that inflammatory activity precedes vascular complications (12). Until now, no longitudinal studies have been reported in type 1 diabetic patients. In our 10-year prospective follow-up study, we tested the hypothesis that markers of inflammation and endothelial dysfunction and transforming growth factor- $\beta$  (TGF- $\beta$ ) as a marker of fibrosis are associated with all-cause mortality and cardiovascular morbidity and mortality and that markers relate to progression of diabetic nephropathy.

## RESEARCH DESIGN AND METHODS

In 1993, we included 199 patients with diabetic nephropathy (fulfilled clinical criteria) (13) and 192 with persistent normoalbuminuria (urinary albumin excretion rate  $<30$  mg/24 h) in a prospective observational follow-up study. The inclusion criteria and selection of patients have been described previously (3). The two groups were matched for sex, age, and duration of diabetes.

The guidelines at Steno Diabetes Center recommended statins for type 1 diabetic patients with nephropathy in 2002. At baseline, no patients were treated with statins or angiotensin receptor blockers, but 9 patients (5%) with normoalbuminuria and 105 patients (53%) were treated with ACE inhibitors.

Patients were followed until 31 December 2003 or until death ( $n = 76$ ) or emigration ( $n = 3$ ). The study was approved by the ethics committee, and all patients gave informed consent.

### Baseline investigations

Investigations were performed after an overnight fast. No antihypertensive medication was ever prescribed in 24% of patients with nephropathy and 88% of the normoalbuminuric patients. All of the remaining patients were asked to stop their antihypertensive and diuretic treatment 8 days before the examination; however, 34 and 4% of patients in the nephropathy and normoalbuminuria group, respectively, had taken antihypertensive medication on the day of the examination.

Heart rate variation was assessed by expiration/inspiration variation in heart rate (14). The patient, in a supine position, was asked to breathe deeply at the rate of 6 breaths/min for 1 min while being monitored by electrocardiogram. The maximum and minimum heart rates during each breathing cycle were measured, and the mean of the differences was calculated.

Diabetic retinopathy was assessed by fundus photography. Patients were interviewed using the World Health Organization cardiovascular questionnaire. Smoking was defined as individuals smoking  $\geq 1$  cigarettes/cigars/pipes per day; all others were classified as nonsmokers.

### Laboratory analyses

Analyses of urinary albumin concentration, serum creatinine, and glomerular filtration rate (GFR) have been described elsewhere (3). GFR was measured during follow-up approximately every year. Only patients with a minimum of three measurements were used to assess the rate of decline in kidney function (15).

Analyses of the biomarkers were performed at a central laboratory by C.S. High-sensitivity C-reactive protein (hsCRP) was determined by enzyme immunoassays (normal range 0.13–3.0 mg/l) as described previously (16). Commercially available enzyme-linked immunosorbent assay kits were used for measurements of plasma soluble vascular cell adhesion molecule-1 (sVCAM-1) (range for assay 538–1,286 ng/ml), soluble intercellular adhesion molecule-1 (sICAM-1) (range 98–647 ng/ml), interleukin-6 (IL-6), secreted phospholipase A2 (sPLA2), and plasminogen activator inhibitor-1 (PAI-1). (Quantikine High

Sensitivity; R&D Systems, Oxon, U.K.). Total TGF- $\beta$  was measured by an ELISA Development system (R&D Systems). The laboratory analysis of the biomarkers was done on frozen ( $-80^{\circ}\text{C}$ ) samples.

### Follow-up examination and end points

Patients were traced in the national register in January 2004. If a subject had died before 31 December 2003, the death certificate was obtained and the cause of death was adjudicated by two observers masked to nephropathy status. Of the 315 patients alive in 2003, 274 patients (87%) participated in the follow-up examination (121 patients with nephropathy and 153 patients with normoalbuminuria).

The primary end point was all-cause mortality, and the secondary end point was a composite end point of cardiovascular death, nonfatal myocardial infarction, percutaneous coronary intervention, coronary artery bypass grafting, nonfatal stroke, amputation as a result of ischemia, and vascular surgery for peripheral atherosclerotic disease (17). Cardiovascular death was classified as all deaths for which an unequivocal noncardiovascular cause was not established (18).

All patients alive at the follow-up examination were evaluated for end points in their patient file. Nonfatal events were confirmed from hospital files.

### Statistical analysis

At baseline, urinary albumin excretion rate, triglycerides, creatinine, and biomarkers were non-normally distributed and therefore were log transformed to normal distribution and are given as medians (range). All other values are means  $\pm$  SD. For normally distributed variables, comparison between groups was performed by unpaired Student's  $t$  test or ANOVA. A  $\chi^2$  test was used to compare noncontinuous variables.

To combine biomarkers, we constructed two mean Z scores: the mean inflammatory Z score and the mean Z score for endothelial dysfunction biomarkers. A Z score was calculated as (value in individual minus the mean value in the study population)/SD; the value thus ranges from approximately  $-2.5$  to  $+2.5$  (5). For each individual, a mean Z score was calculated combining biomarkers. The mean inflammatory Z score = (Z score hsCRP + Z score IL-6 + Z score sICAM-1 + Z score sPLA2)/4, and the mean Z score for endothelial dysfunction = (Z score

PAI-1 + Z score sVCAM-1 + Z score sICAM-1)/3. Because sICAM-1 is considered an inflammatory marker and a marker of endothelial dysfunction, it was included in both mean Z scores. TGF- $\beta$  was analyzed per se as a continuous variable. In Kaplan-Meier analysis, groups were compared by log-rank tests.

A multiple Cox regression analysis was performed with variables significantly ( $P < 0.1$ ) associated in a univariate analysis with all-cause mortality. Prespecified were smoking, age, and sex. To avoid overfitting the model, only one parameter for kidney function, lipids, and blood pressure was used, chosen by the highest overall  $\chi^2$  score. In all models, the following variables were used: age, sex, systolic blood pressure, smoking, total cholesterol, A1C, urinary albumin excretion, heart rate variation, and TGF- $\beta$  and either mean Z score of inflammatory biomarkers or the mean Z score of endothelial dysfunction biomarkers. In analysis of the specific biomarkers, the two mean Z scores and TGF- $\beta$  were not in the model. For the "mechanistic model," age and sex were the only adjusting variables in the model, together with the specific biomarker or mean Z score.

Progression in diabetic nephropathy was assessed as the change in GFR with time. Linear regression analysis using all measured GFR values during follow-up in each patient versus time was used to determine the rate of decline in GFR (slope) for each patient. The correlation between rate of decline in GFR and Z scores and log TGF- $\beta$  was tested with a Pearson's test for correlation.

Hazard ratios (HRs) for log-transformed variables are expressed as HR for a change of 1 SD in the log-transformed variable. Two-tailed  $P$  values  $\leq 0.05$  were considered significant. All calculations were made with SPSS 12.0 (SPSS, Chicago, IL).

**RESULTS**—Table 1 shows baseline variables. At baseline, patients with diabetic nephropathy had higher IL-6, sVCAM-1, PAI-1, and TGF- $\beta$ , whereas hsCRP, sPLA2, and sICAM-1 did not differ between groups. IL-6, sICAM-1, sVCAM-1, and PAI-1 were higher in nonsurvivors at baseline ( $P < 0.05$ ); other biomarkers did not differ significantly between survivors and nonsurvivors.

At baseline, both mean Z scores were correlated with known cardiovascular risk factors. Urinary albumin excretion, heart rate variation, A1C, total cholesterol, and systolic blood pressure were

Table 1—Baseline data in 391 type 1 diabetic patients with and without diabetic nephropathy and in patients who were alive versus patients who were dead at follow-up

	Normoalbuminuria	Nephropathy	P value	Patients alive at follow-up	Patients dead at follow-up	P value
n	192	199		315	76	
Sex (male/female)	118/74	122/77	NS	189/126	51/25	0.255
Age (years)	43 ± 10	41 ± 9	NS	39 ± 9	45 ± 9	<0.001
Duration of diabetes (years)	27 ± 8	28 ± 8	NS	27 ± 8	29 ± 9	<0.001
BMI (kg/m <sup>2</sup> )	23.6 ± 2.5	24.0 ± 3.3	NS	23.9 ± 2.9	23.4 ± 3.0	0.164
A1C (%)	8.5 ± 1.1	9.5 ± 1.5	<0.001	9.4 ± 1.5	10.0 ± 1.6	<0.001
GFR (ml/min per 1.73 m <sup>2</sup> )	—	74 ± 34	—	81 ± 32*	60 ± 32*	<0.001
UAER (mg/24 h)†	8 (1–30)	796 (16–14,565)	—	19 (1–8,824)	768 (2–14,545)	<0.001
S-creatinine (μmol/l)	76 (40–116)	103 (54–684)	<0.001	81 (40–587)	115 (54–684)	<0.001
Systolic blood pressure (mmHg)	132 ± 18	151 ± 23	<0.001	146 ± 21	162 ± 22	<0.001
Diastolic blood pressure (mmHg)	76 ± 10	86 ± 13	<0.001	85 ± 12	88 ± 13	0.001
Hemoglobin (mmol/l)				8.3 ± 1.0	7.9 ± 1.3	<0.001
Smoking (%)	43	50	NS	43‡	55‡	0.065
History of stroke/MI	4 (2)	21 (11)	<0.001	13 (4)	12 (16)	<0.001
Retinopathy§	67/106/19	0/62/137	<0.001	64/138/113	5/28/43	<0.001
Total cholesterol (mmol/l)	4.8 ± 1.0	5.6 ± 1.2	<0.001	5.4 ± 1.2	6.2 ± 1.2	<0.001
HDL cholesterol (mmol/l)	1.56 ± 0.5	1.46 ± 0.5	<0.001	1.47 ± 0.55	1.46 ± 0.54	0.903
LDL cholesterol (mmol/l)	2.82 ± 0.9	3.54 ± 1.1	<0.001	3.1 ± 1.0	3.8 ± 1.1	<0.001
Heart rate variation (bpm)	13 (0–55)	6 (0–50)	<0.001	11 (0–55)	5 (0–50)	<0.001
Triglycerides (mmol/l)	0.77 (0.3–3.1)	1.22 (0.3–9.9)	<0.001	1.0 (0.9–1.1)	1.5 (1.3–1.7)	<0.001
Inflammatory Z score	−0.11 ± 0.68	0.10 ± 0.62	0.001	−0.04 ± 0.66	0.18 ± 0.63	0.008
Endothelial Z score	−0.22 ± 0.53	0.21 ± 0.63	<0.001	−0.06 ± 0.60	0.24 ± 0.65	<0.001
hsCRP (mg/dl)	2.50 (1.75–3.25)	2.60 (1.87–3.31)	0.85	2.48 (1.89–3.06)	2.83 (1.71–3.95)	0.266
IL-6 (pg/ml)	2.30 (1.78–2.82)	3.09 (2.57–3.61)	0.033	2.63 (2.20–3.07)	2.98 (2.39–3.57)	0.006
sPLA-2 (μg/ml)	5.56 (4.89–6.23)	5.64 (5.01–6.28)	0.86	5.62 (5.10–6.15)	5.52 (4.60–6.45)	0.744
sICAM-1 (ng/ml)	713 (675–750)	745 (709–782)	0.223	712 (684–741)	799 (735–863)	0.004
sVCAM-1 (ng/ml)	914 (874–954)	1,084 (1,033–1,136)	<0.001	986 (948–1023)	1,060 (980–1,139)	0.045
PAI-1 (μg/ml)	55.8 (50.6–60.9)	84.8 (76.9–92.8)	<0.001	68.6 (63.1–74.0)	80.9 (68.6–93.3)	0.039
TGF-β1 (ng/ml)	14.4 (13.2–15.7)	17.4 (15.6–19.2)	0.008	15.8 (14.5–17.0)	16.9 (14.4–19.3)	0.274

Data are n (%), means ± SD, or median (range). \*Only patients with diabetic nephropathy. †Urinary albumin excretion rate (UAER): some patients with previously persistent albuminuria receiving antihypertensive medication had 300 mg/24 h. Values are the mean of three 24-h urine collections. ‡Percentage of patients alive in 2003 smoked at baseline in 1993. §Retinopathy: nil/simplex/proliferative. ||Z score (value in the individual minus the mean value in the study population)/SD; a value that thus ranged from approximately −2.5 to +2.5.

correlated with the mean Z score for endothelial dysfunction biomarkers and with the mean Z score for inflammatory biomarkers (except heart rate variation) ( $P < 0.01$ ). Age did not correlate with any of the mean Z scores. TGF-β correlated at baseline with both mean Z scores and cholesterol ( $P < 0.01$ ) but not with other risk factors.

### All-cause mortality

During follow-up 60 patients (30%) with nephropathy died versus 16 (8%) of normoalbuminuric patients (log-rank test  $P < 0.0001$ ). Twenty-five patients (42%) with diabetic nephropathy died from cardiovascular causes versus 7 patients (38%) with normoalbuminuria. Thirty patients (50%) died from end-stage renal disease.

When dividing patients into tertiles according to mean Z scores, we observed

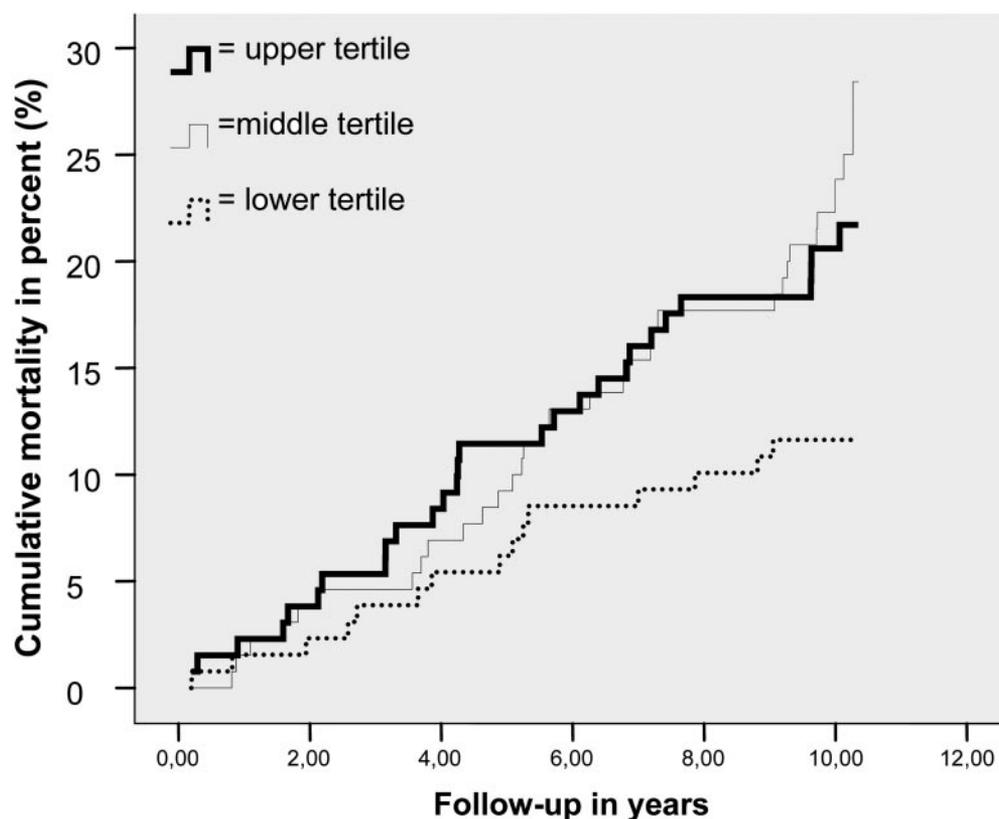
that patients in the lowest tertile had the lowest mortality; 15, 28, and 33 patients died in the lowest, middle, and highest tertile, respectively, of the mean Z score of endothelial dysfunction ( $P$  value for differences between the highest and lowest tertile = 0.033). Correspondingly, 18, 23, and 35 patients died in the three tertiles of the mean Z score of inflammatory biomarkers ( $P = 0.028$ ). Figure 1 shows patients divided into tertiles of the mean Z score for inflammatory biomarkers; the figure for endothelial dysfunction is not shown but is similar.

In an unadjusted Cox regression analysis, the mean Z score for inflammatory biomarkers was significantly associated with death in all patients, but after adjustment for cardiovascular risk factors the result was no longer significant (HR 1.33 [95% CI 0.83–2.15];  $P = 0.241$ ). In an analysis of patients with diabetic ne-

phropathy per se, the Z score for low-grade inflammation was significantly associated with death in unadjusted (1.9 [1–3.2.8];  $P = 0.001$ ) and in adjusted analysis (HR 2.13 [1.17–3.88];  $P = 0.014$ ).

The mean Z score for endothelial dysfunction was associated with death in all patients in unadjusted analysis and in patients with diabetic nephropathy per se; however, after adjustment the association disappeared.

In a mechanistic model with adjustment for only age and sex, the results were similar to those for the unadjusted model, with the same biomarkers giving significant association to the outcome. The area under the receiver operating characteristic curve (AUC) for all-cause mortality for all traditional risk factors used in the Cox model in all patients was 0.845. When the Z score for inflammation or the Z score for



**Figure 1**—Cumulative mortality in all patients divided into tertiles after mean Z score of inflammatory biomarkers (log-rank test  $P = 0.028$ ).

endothelial dysfunction was added, there was no change in AUC. In patients with diabetic nephropathy the AUC for all risk factors mentioned was 0.815, and in patients with normoalbuminuria it was 0.789. There was again no change when the Z scores were added. TGF- $\beta$  was not associated with death in the group as a whole nor when the two groups were analyzed per se.

If all patients were traced in July 2007, a total of 112 patients had died. However, these deaths did not change the analysis on biomarkers substantially.

### Cardiovascular mortality and morbidity

During follow-up 79 patients (40%) with diabetic nephropathy reached the combined end point of cardiovascular death or major cardiovascular event versus 19 patients (10%) with persistent normoalbuminuria (log-rank test  $P < 0.0001$ ) (Table 2). The 79 patients affected experienced a total of 107 events.

The mean Z score for inflammatory biomarkers was associated with the combined end point in all patients in unadjusted analysis (HR 1.5 [1.1–2.0];  $P = 0.006$ ); after adjustment the results were borderline significant (1.50 [1.0–2.25];  $P = 0.051$ ). In patients with diabetic ne-

phropathy the results were significant even after adjustment for risk factors.

The mean Z score for endothelial dysfunction was analysis associated with the combined end point in unadjusted Cox regression only when all patients combined were analyzed (HR 1.7 [1.2–2.3],  $P = 0.001$ ), and the association disappeared after adjusting for cardiovascular risk factors. In a mechanistic model with adjustments only for age and sex, the results were similar to those for the unadjusted model.

There was no change in AUC when either of the Z scores were added on top of risk factors used in the Cox model. TGF- $\beta$  was not associated with the combined cardiovascular end point in the group as whole or when patients were analyzed separately.

### Progression of renal disease

Progression of diabetic nephropathy measured as rate of decline in GFR correlated with the mean Z score for endothelial dysfunction ( $r = -0.243$ ;  $P = 0.001$ ); however, there was no correlation between rate of decline in GFR and the mean Z score for inflammation or TGF- $\beta$ . The correlation between the mean Z score for endothelial dysfunction and rate of decline in GFR persisted even after adjust-

ment for urinary albumin excretion, baseline GFR, systolic blood pressure, A1C, cholesterol, age, sex, and hemoglobin (coefficient  $-1.38$  [95% CI  $-2.27$  to  $-0.50$ ];  $P = 0.002$ ), meaning that per 1 unit increase in mean Z score for endothelial dysfunction the decline in GFR increases at 1.38 ml/min per 1.73 m<sup>2</sup> per year.

**CONCLUSIONS**— This is the first prospective study to evaluate the association of biomarkers of low-grade inflammation and endothelial dysfunction with mortality and decline of GFR in type 1 diabetes. The study had three main results. First, in patients with diabetic nephropathy, low-grade inflammation (estimated from a Z score of four markers) was associated with all-cause mortality and the combined cardiovascular end point after adjustment for cardiovascular risk factors. Second, in unadjusted analyses, endothelial dysfunction (estimated from a Z score of three markers) was also associated with these end points. Third, endothelial dysfunction correlated significantly with the rate of decline of GFR, a measure of progression of diabetic nephropathy.

Inflammation and endothelial dysfunction are thought to be key processes

Table 2—Cox regression analysis of biomarkers as predictors of all-cause mortality and the combined end point of cardiovascular mortality and morbidity

	All-cause mortality			
	Unadjusted analysis		Adjusting for risk factors*	
	Nephropathy	Normoalbuminuria	Nephropathy	Normoalbuminuria
Inflammatory Z score	1.9 (1.3–2.8); 0.001	0.5 (0.2–1.2); 0.11	2.13 (1.17–3.88); 0.014	0.44 (0.16–1.17); 0.099
Endothelial Z score	1.6 (1.0–3.0); 0.031	0.9 (0.4–9.5); 0.87	0.71 (0.38–1.32); 0.28	1.43 (0.41–4.95); 0.57
TGF-β (ng/ml)	1.2 (0.5–3.0); 0.67	1.2 (0.1–9.5); 0.87	1.22 (0.44–3.37); 0.71	2.14 (0.22–20.8); 0.51
hsCRP (mg/dl)	1.5 (0.9–2.5); 0.087	0.5 (0.2–1.2); 0.14	1.30 (0.77–2.17); 0.33	0.51 (0.20–1.28); 0.15
IL-6 (pg/ml)	2.8 (1.4–5.5); 0.003	0.1 (0.01–0.9); 0.037	2.16 (0.94–5.00); 0.07	0.08 (0.01–0.69); 0.022
sPLA-2 (ng/ml)	1.5 (0.6–3.7); 0.391	0.4 (0.07–2.5); 0.34	2.33 (0.70–7.74); 0.17	0.49 (0.075–3.21); 0.46
sICAM-1 (ng/ml)	12.7 (2.3–70.8); 0.004	1.6 (0.5–52.4); 0.80	6.47 (1.05–40.05); 0.045	1.05 (0.03–32.81); 0.98
sVCAM-1 (ng/ml)	1.88 (0.24–14.84); 0.548	0.5 (0.01–50.9); 0.77	0.45 (0.04–5.31); 0.52	0.03 (0.004–2.84); 0.58
PAI-1 (ng/ml)	1.29 (0.54–3.06); 0.569	0.74 (0.1–5.5); 0.77	1.04 (0.42–2.58); 0.94	0.51 (0.06–4.11); 0.53

	HR in unadjusted analysis		HR after adjusting for risk factors*	
	Combined cardiovascular mortality and morbidity			
	Nephropathy	Normoalbuminuria	Nephropathy	Normoalbuminuria
Inflammatory Z score	1.5 (1.1–2.1); 0.021	1.0 (0.5–1.9); 0.96	1.94 (1.19–3.16); 0.008	1.02 (0.41–2.55); 0.96
Endothelial Z score	1.2 (0.8–1.7); 0.35	1.2 (0.6–2.8); 0.61	0.62 (0.36–1.08); 0.09	0.80 (0.27–2.39); 0.68
TGF-β (ng/ml)	1.54 (0.70–3.38); 0.29	1.02 (0.15–6.88); 0.98	1.8 (0.8–3.8); 0.16	0.9 (0.2–5.7); 0.94
hsCRP (mg/dl)	1.31 (0.86–1.99); 0.21	0.93 (0.43–2.03); 0.86	1.33 (0.86–2.06); 0.20	1.03 (0.44–2.43); 0.94
IL-6 (pg/ml)	3.03 (1.7–5.53); <0.0001	0.51 (0.11–2.41); 0.39	2.62 (1.34–5.13); 0.005	0.47 (0.12–1.86); 0.28
sPLA-2 (ng/ml)	1.12 (0.49–2.54); 0.79	1.77 (0.39–8.15); 0.46	1.60 (0.62–4.14); 0.33	1.79 (0.37–8.67); 0.47
sICAM-1 (ng/ml)	2.05 (0.42–9.94); 0.37	1.25 (0.05–32.9); 0.9	3.30 (0.61–17.86); 0.16	0.32 (0.12–0.87); 0.50
sVCAM-1 (ng/ml)	0.84 (0.13–5.32); 0.85	0.12 (0.001–10.2); 0.35	0.31 (0.04–2.50); 0.27	0.022 (0.00–2.04); 0.09
PAI-1 (ng/ml)	1.50 (0.70–3.21); 0.30	4.37 (0.81–23.7); 0.09	1.49 (0.68–3.30); 0.32	5.92 (0.88–39.84); 0.07

Values are HR (95% CI); P value. \* In the multivariate Cox regression analysis the values are adjusted for age, sex, systolic blood pressure, smoking, total cholesterol, A1C (%), urinary albumin excretion, heart rate variation, TGF-β, and either Z score of inflammation or Z score of endothelial dysfunction. However, in analysis of the single biomarkers all other biomarkers were taken out of the regression analysis.

in atherothrombosis (4). Indeed, in type 2 diabetes, biomarkers have already been shown in a longitudinal study to progress over time and to precede vascular complications (12). Our data on inflammation are in line with these concepts. The results for endothelial dysfunction were less clear but should not be interpreted to indicate that endothelial dysfunction is not involved in the development of cardiovascular disease. In fact, endothelial dysfunction was strongly correlated with cardiovascular risk factors at baseline, in agreement with previous data (11), and to mortality outcome in unadjusted analyses. Because cardiovascular risk factors cause endothelial dysfunction, the fully adjusted model is most likely over-adjusted. Thus, a reasonable interpretation is that our results are compatible with a role for endothelial dysfunction, as has been suggested before (19,20). Additional reasons why the association between endothelial dysfunction and mortality may have been underestimated include the fact that we were able to include only three biomarkers (data on more specific endothelial biomarkers such as soluble E-selectin and von Willebrand factor were not available) and that we were unable to include a more direct estimate of endothelial nitric oxide availability, e.g., by measuring flow-mediated endothelium-dependent vasodilatation (19,21).

We find that endothelial dysfunction correlates with progression of renal disease throughout the follow-up period, indicating that endothelial dysfunction is an active part of the pathophysiological pathway causing further progression of the glomerulosclerosis and is not just active in initiating disease. This is a very important and novel finding in patients with type 1 diabetes. Endothelial dysfunction has previously been shown to predict progression of renal disease in patients with type 2 diabetes (22). Intervention studies toward endothelial dysfunction in regard to decline in kidney function must be the next step.

Our study had some limitations. First, we show results both for groups per se and in all patients together. The original design was a case-control design with intention to follow and compare the two groups. We believe that there is a significant difference in the development of end points and perhaps even in the pathophysiological pathway leading to disease. As a result, in the normoalbuminuric group, the study may lack power to show

the influence of biomarkers. Second, samples for analyses were taken at baseline only, and we therefore could not evaluate the time course of changes in biomarker levels. Third, the AUCs did not change after addition of the mean Z scores on top of traditional risk factors. Actually, the AUCs were remarkably high with the traditional risk factors alone. Even though this result limits the clinical value of the mean Z scores, it does not negate the value of the study with regard to providing insights into the pathophysiological role of inflammation and endothelial dysfunction. Fourth, our results on TGF- $\beta$  were negative, even though, from animal models, TGF- $\beta$  is believed to act as a mediator of renal fibrosis (23) and antibodies against TGF- $\beta$  seem to be as effective as antifibrotic therapy in renal diseases, at least when combined with renin-angiotensin system blockade (24). However, we cannot exclude a local effect of TGF- $\beta$  in the kidney. TGF- $\beta$  levels were significantly higher at baseline in patients with diabetic nephropathy, and perhaps most of the role of TGF- $\beta$  is played before the clinical diagnosis of diabetic nephropathy is evident. Finally, we used Z scores to estimate inflammation and endothelial function. An important assumption in the use of such Z score is that all variables included are about equally reflective of the underlying process. On the other hand, the idea to combine biomarkers resolves some of the problem with the sometimes large biological variability of each measure (25). Another major advantage of the mean Z score is that it circumvents issues raised by multiple testing of single markers. Conversely, if the Z score is not significantly related to the outcome one must be very cautious with attaching much value to other, single-marker observations.

With the new knowledge of the association of biomarkers with hard end points in type 1 diabetes added by our study, together with prognostic data in type 2 diabetic patients (9,12) and a recent post hoc analysis of the Irbesartan in Patients with Type 2 Diabetes and Microalbuminuria (IRMA 2) trial showing that irbesartan reduces inflammatory activity in patients with type 2 diabetes and microalbuminuria (22), the next step is trials with intention to alter inflammation and endothelial dysfunction as suggested in a statement by the American Heart Association (26). Patients with type 1 diabetes and nephropathy provide a group in whom intervention toward inflammation

and endothelial dysfunction could be targeted as we show Z scores of inflammation and endothelial dysfunction to be associated with strong baseline risk factors, and, even though the biomarkers did not add prognostic value on top of known cardiovascular risk factors, they do provide insights to the pathophysiological pathway of cardiovascular disease.

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