Plasma Connective Tissue Growth Factor Is an Independent Predictor of End-Stage Renal Disease and Mortality in Type 1 Diabetic Nephropathy

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OBJECTIVE — We evaluated the predictive value of baseline plasma connective tissue growth factor (CTGF) in a prospective study of patients with type 1 diabetes.

RESEARCH DESIGN AND METHODS — Subjects were 198 type 1 diabetic patients with established diabetic nephropathy and 188 type 1 diabetic patients with persistent normoalbuminuria. Follow-up time was 12.8 years. Prediction of end-stage renal disease (ESRD) and mortality by plasma CTGF was analyzed in conjunction with conventional risk factors.

RESULTS — Plasma CTGF was higher in patients with nephropathy than in patients with normoalbuminuria (median 381 [interquartile range 270–630] vs. 235 [168–353] pmol/l). In patients with nephropathy, elevated plasma CTGF was an independent predictor of ESRD (covariate-adjusted hazard ratio [HR] 1.6 [95% CI 1.1–2.5]) and correlated with the rate of decline in glomerular filtration rate (GFR) (cumulative $R = 0.46$). Area under the receiver operating characteristic curve for prediction of ESRD was 0.72. Plasma CTGF above a cutoff level of 413 pmol/l predicted ESRD with a sensitivity of 73% and a specificity of 63% and was associated with a higher rate of decline in GFR (mean ± SD 5.4 ± 4.9 vs. 3.3 ± 3.5 ml/min per 1.73 m^2 per year). Moreover, in patients with nephrotic range albuminuria (>3 g/day), plasma CTGF was the only predictor of ESRD (covariate-adjusted HR 4.5 [2.0–10.4]). Plasma CTGF was an independent predictor also of overall mortality (covariate-adjusted HR 1.4 [1.1–1.7]). In contrast, in normoalbuminuric patients, plasma CTGF did not correlate with clinical parameters and did not predict outcome.

CONCLUSIONS — Plasma CTGF contributes significantly to prediction of ESRD and mortality in patients with type 1 diabetic nephropathy.

Diabetes Care 31:1177–1182, 2008

Diabetic nephropathy is the most important cause of end-stage renal disease (ESRD) and contributes significantly to mortality, mainly through increases in cardiovascular disease (1). However, the course of diabetic nephropathy remains unpredictable, and the pathogenesis of progression is not completely understood.

Connective tissue growth factor (CTGF) was first identified in conditioned media of endothelial cells as a 36- to 38-kDa polypeptide containing chemotactic activity toward fibroblasts (2). CTGF has been acknowledged as a key factor in extracellular matrix production and other profibrotic activity mediated by transforming growth factor-β1 (3). Other biological functions of CTGF include angiogenesis, chondrogenesis, osteogenesis, and cell adhesion, migration, proliferation, and differentiation (4).

Recently, CTGF has emerged as an important factor in diabetic nephropathy. In renal cells, CTGF is induced by high glucose, and it is critically involved in diabetes-associated changes such as extracellular matrix synthesis, cell migration, and epithelial-to-mesenchymal transition (5–8). Furthermore, upregulation of CTGF has been observed in human and experimental diabetic nephropathy (6,9–12), whereas the structure and function of the kidney are largely preserved in diabetic mice with hemizygous CTGF deletion and in diabetic mice treated with CTGF antibody or CTGF antisense oligonucleotides (13–15).

CTGF is a secreted protein and can be detected in biological fluids. Previous small studies have reported that both urinary CTGF excretion and plasma CTGF levels are elevated in patients with diabetic nephropathy (16–19). Recently, we have shown in a large cross-sectional study of patients with type 1 diabetes that urinary CTGF excretion is associated with urinary albumin excretion (UAE) and associated inversely with glomerular filtration rate (GFR), both important clinical markers for severity of renal disease (20). In aggregate, these data suggest that CTGF might be a useful marker of renal deterioration in patients with diabetic nephropathy.

Because all previous clinical studies addressing CTGF as a biomarker were performed in cross-sectional designs, the possible prognostic value of CTGF levels in diabetic nephropathy still has remained elusive. Therefore, we set out to evaluate whether plasma CTGF might

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Received for publication 31 December 2007 and accepted in revised form 7 March 2008.

Published ahead of print at http://care.diabetesjournals.org on 14 March 2008. DOI: 10.2337/dc07-2469.

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R.G. has received research support grants and consulting fees from FibroGen.

Abbreviations: CTGF, connective tissue growth factor; ESRD, end-stage renal disease; GFR, glomerular filtration rate; ROC, receiver operating characteristic; UAE, urinary albumin excretion.

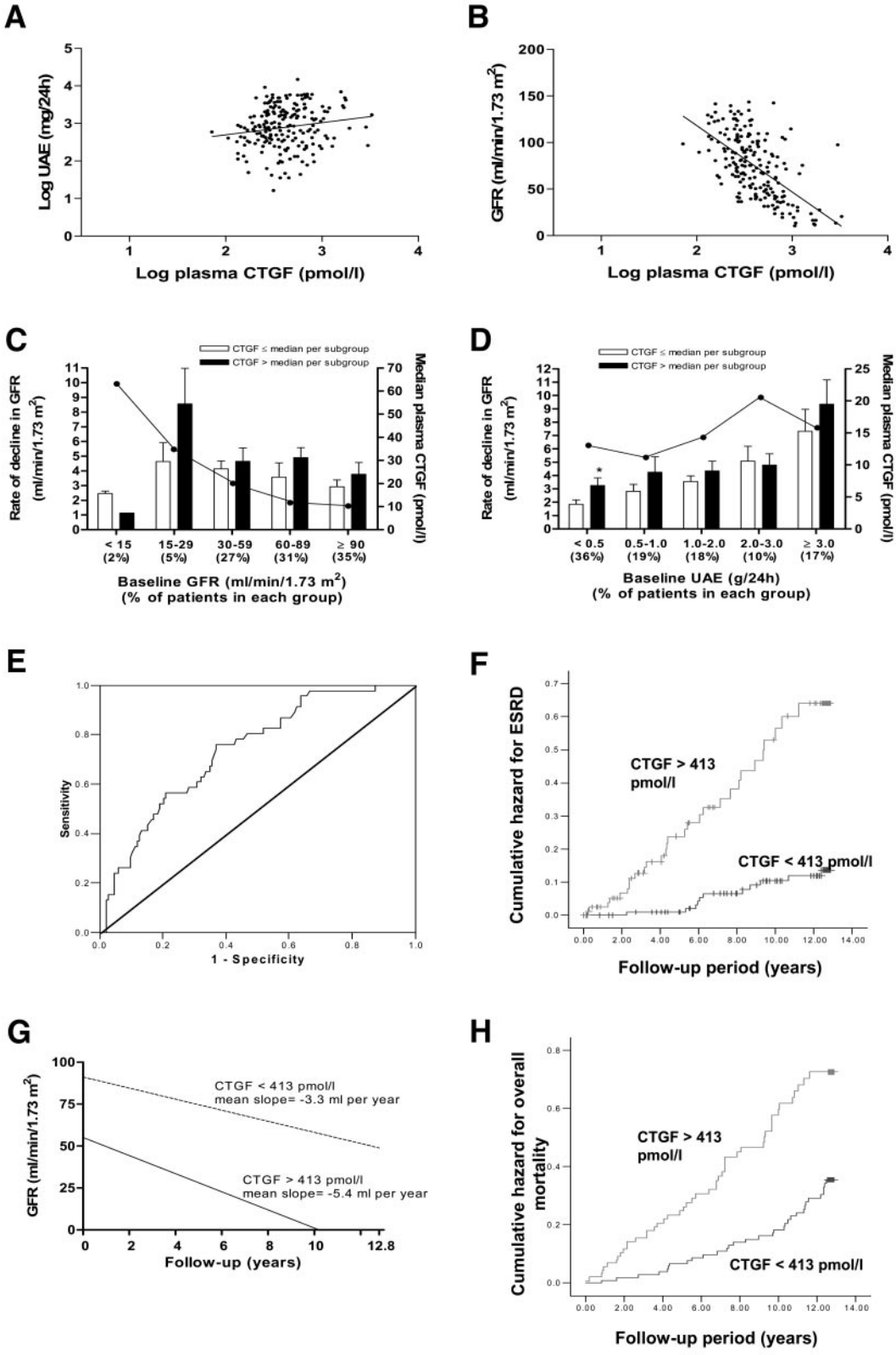

Figure 1—*Baseline plasma CTGF in patients with diabetic nephropathy.* A: *Log plasma CTGF correlates with log UAE* ($R = 0.16$, $P = 0.02$). B: *Log plasma CTGF correlates inversely with GFR* ($R = -0.58$, $P < 0.001$). C: *Analysis per KDOQI subgroup for chronic kidney disease. Within each subgroup, patients with plasma CTGF above the median tend to have a higher rate of decline in GFR.* D: *Analysis per subgroup for UAE. Within each subgroup, patients with plasma CTGF above the median tend to have a higher rate of decline in GFR.**$P < 0.05$ *versus group with plasma CTGF below the median.* E: *Area under the ROC curve for prediction of ESRD by plasma CTGF is 0.74. With a cutoff value for plasma CTGF of 413 pmol/l, ESRD is predicted with a sensitivity of 73% and a specificity of 63%.* F: *Kaplan-Meier curve for prediction of ESRD. Cumulative hazard for ESRD is higher in patients with plasma CTGF >413 pmol/l than in patients with plasma CTGF <413 pmol/l. Log-rank test,* $P < 0.001$. G: *Graphic*

predict loss of GFR, ESRD, and mortality in a prospective study of 386 type 1 diabetic patients with and without diabetic nephropathy during a follow-up period of 12.8 years.

RESEARCH DESIGN AND METHODS

— Patients with type 1 diabetic nephropathy attending the outpatient clinic of Steno Diabetes Center in 1993 were invited to participate in a case-control study (21,22). Type 1 diabetes was considered present if the age at onset of diabetes was ≤35 years and time to definite insulin therapy was ≤1 year. Patients were categorized as having diabetic nephropathy if they had persistent albuminuria (>300 mg/day) in at least two of three consecutive 24-h urine collections, diabetic retinopathy, and no other kidney or renal tract disease (23). Patients with equally long-lasting type 1 diabetes (>15 years) and persistent normoalbuminuria (<30 mg/day), who were matched for sex, age, and duration of diabetes, served as control subjects. Thus, 198 patients with nephropathy and 188 patients with normoalbuminuria were included in the study. The study was approved by the local ethics committee, in accordance with the Declaration of Helsinki, and all patients gave their informed written consent.

Investigations were performed in the morning after an overnight fast. Blood pressure was measured twice after at least 10 min of rest in the supine position. UAE was measured by an enzyme immunoassay from 24-h urine collections. Plasma creatinine was assessed by a kinetic Jaffé method. GFR was measured after a single intravenous injection of ^{51}Cr-EDTA (3.7 MBq) by following the plasma clearance of the tracer for 4 h (24). Linear regression of yearly GFR measurements in each individual was used to estimate rate of decline in GFR. In normoalbuminuric patients, GFR was estimated by the Modification of Diet in Renal Disease equation (25).

Diabetic retinopathy was assessed by fundus photography and graded as nil, simplex, or proliferative retinopathy. Patients were interviewed using the World Health Organization cardiovascular questionnaire. Major cardiovascular events were diagnosed as a history of stroke and/or myocardial infarction. Smoking was defined as smoking ≥1 cigarettes/cigars/pipes a day.

In a prospective observational study design, patients were followed up until 1 November 2006 or until death (n = 99) or emigration (n = 3). If a patient had died, the date of death was recorded, and the cause of death was obtained from the death certificate. Additional available information from necropsy reports was included. All deaths were classified as cardiovascular deaths unless an unequivocal noncardiovascular cause was established. Information about the date of ESRD was obtained from patient records or discharge letters from other hospitals. ESRD was defined as the need for dialysis or renal transplantation.

Enzyme-linked immunosorbent assay for plasma CTGF

CTGF was measured in plasma samples drawn at study entry that had been stored at −80°C at the Steno Diabetes Center. Storage time and freeze-thaw cycles of all samples were identical. CTGF was determined by a sandwich enzyme-linked immunosorbent assay using monoclonal antibodies against two distinct epitopes on the N-terminal part of human CTGF (FibroGen, South San Francisco, CA) as described previously (18,20).

Statistical analysis

Normally distributed variables are expressed as means ± SD. UAE, plasma creatinine, and plasma CTGF were logarithmically transformed before analysis and are expressed as medians (interquartile range).

Comparisons between groups were performed by unpaired Student's t test or Mann-Whitney test. Multiple logistic regression analysis was used to identify the contribution of parameters to risk of diabetic nephropathy. Pearson and Spearman correlations and forward stepwise regression analysis were used to identify parameters that correlated with rate of decline in GFR. All time-to-event variables were analyzed using log-rank tests and were displayed on Kaplan-Meier plots according to levels being above or below the cutoff value, as determined by the receiver operating characteristic (ROC) curve. The cutoff value with the most discriminative value was defined as the point of the ROC curve closest to the left upper corner ($d = \sqrt{[(1 - \text{specificity})^2 + (1 - \text{sensitivity})^2]}$).

Cox proportional hazards regression models with forward selection were used to evaluate the contribution of baseline covariates to ESRD and overall mortality. For this, continuous variables were standardized for 1-SD difference. In the Cox regression model for ESRD, baseline covariates that were associated with rate of decline in GFR ($P < 0.1$) were entered into the model. These covariates were plasma CTGF, sex, duration of diabetes, UAE, GFR, and systolic blood pressure. In the Cox regression models for cardiovascular and overall mortality, the following prespecified baseline covariates were entered into the model: plasma CTGF, sex, age, smoking, UAE, GFR, A1C, history of cardiovascular event, and systolic blood pressure. Results are given as hazard ratios (HRs) with 95% CI.

In all cases, $P < 0.05$ was considered significant (two-tailed). All calculations were performed using SPSS (version 12.0; SPSS, Chicago, IL).

RESULTS

Plasma CTGF is increased in patients with diabetic nephropathy

General characteristics and baseline parameters of patients are summarized in Table 1. Plasma CTGF was higher in patients with diabetic nephropathy than in patients with normoalbuminuria (median 381 [interquartile range 270–630] vs. 235 [168–353] pmol/l; $P < 0.001$). In patients with nephropathy, plasma CTGF correlated with UAE ($R = 0.16$, $P = 0.02$) and correlated inversely with GFR ($R = -0.58$, $P < 0.001$) (Fig. 1*A* and *B*). In these patients, plasma CTGF was higher in those receiving antihypertensive medication (n = 151) compared with those not receiving antihypertensive medica-

illustration of the relation between plasma CTGF and GFR at baseline and rate of decline in GFR during 12.8 years of follow-up. The mean rate of decline in GFR is higher in patients with high plasma CTGF (>413 pmol/l; ——, 5.4 ± 4.9 ml/min per 1.73 m^2 per year) than in patients with low plasma CTGF (<413 pmol/l; – – –, 3.3 ± 3.5 ml/min per 1.73 m^2 per year). Regression lines were computed from all available data points. The x*-intercept value of 10.2 years for the high plasma CTGF group indicates mean time to ESRD.* H: *Kaplan-Meier curve for prediction of overall mortality. Cumulative hazard for overall mortality is higher in patients with plasma CTGF >413 pmol/l than in patients with plasma CTGF <413 pmol/l. Log-rank test,* P < *0.001.*

Table 1—*Baseline parameters of 386 type 1 diabetic patients with or without diabetic nephropathy*

	Nephropathy	Normoalbuminuria	*P* value
General patient characteristics			
n (% men)	198 (62)	188 (61)	0.84
Age (years)	41.0 ± 9.5	42.5 ± 9.9	0.14
Duration of diabetes (years)	27.7 ± 8.0	26.8 ± 8.5	0.26
BMI (kg/m^2)	24.0 ± 3.3	23.7 ± 2.5	0.20
Retinopathy (nil/simplex/proliferative)	0/61/137	66/103/19	<0.001
A1C (%)	9.6 ± 1.5	8.5 ± 1.1	<0.001
Renal function			
UAE (mg/24 h)	794 (342–2,050)	8 (5–13)	—
Plasma creatinine (μmol/l)	103 (82–134)	76 (69–83)	<0.001
GFR (ml/min per 1.73 m^2)	74 ± 33	94 ± 16	<0.001
Cardiovascular characteristics			
Systolic blood pressure (mmHg)	151 ± 23	132 ± 18	<0.001
Diastolic blood pressure (mmHg)	86 ± 13	76 ± 10	<0.001
Smokers	99 (50)	81 (43)	0.17
History of myocardial infarction	10 (5.1)	2 (1.1)	0.036
History of stroke	14 (7.1)	1 (0.5)	0.001
CTGF levels			
Plasma CTGF (pmol/l)	381 (270–630)	235 (168–353)	<0.001

Data are *n* (%), means ± SD, or median (interquartile range).

tion ($n = 47$) (588 [289–697] vs. 333 [197–422] pmol/l; $P = 0.01$), but this difference disappeared after adjustment for GFR. In patients with normoalbuminuria, plasma CTGF did not correlate with any of the clinical parameters.

Plasma CTGF contributes to risk of diabetic nephropathy

After adjustment for duration of diabetes, BMI, systolic blood pressure, A1C, and GFR, a standardized increase of plasma CTGF resulted in 2.0-fold increased chance of diabetic nephropathy (odds ratio [OR] 2.0 [95% CI 1.5–2.8]). This result was comparable with the ORs for diabetic nephropathy of increased A1C (2.2 [1.6–2.9]) and systolic blood pressure (1.7 [1.5–2.9]) after adjustment for duration of diabetes, GFR, plasma CTGF, and systolic blood pressure or A1C, respectively.

Baseline plasma CTGF correlates with rate of decline in GFR

In patients with nephropathy, the mean rate of decline in GFR was 4.3 ml/min per 1.73 m^2 per year. With rate of decline in GFR as a dependent variable, UAE was identified as the regression parameter with the strongest correlation ($R = 0.43$, $P < 0.001$). Applying significance cutoff at $P < 0.05$, baseline plasma CTGF was the next and only parameter significantly contributing to an increase in this correlation, resulting in a cumulative R of 0.46 ($P = 0.001$). Addition of other parameters did not significantly increase correlation with rate of decline in GFR. In particular, the effect of plasma CTGF concentration on this regression was independent of baseline GFR.

Similarly, in separate analysis of Kidney Disease Outcomes Quality Initiative (KDOQI) subgroups for chronic kidney disease, patients with plasma CTGF above the median of their particular subgroup tended to have a higher rate of decline in GFR, but this trend did not reach statistical significance (Fig. 1*C*). Also within subgroups stratified for UAE, a similar trend of a higher rate of decline in GFR was observed in patients with higher plasma CTGF levels (Fig. 1*D*).

Baseline plasma CTGF predicts ESRD in diabetic nephropathy

At baseline, 6 of 198 patients with diabetic nephropathy had already developed ESRD. These patients were excluded from further prospective analyses. Area under the ROC curve for prediction of ESRD by plasma CTGF was 0.72, by UAE was 0.73, and by systolic blood pressure was 0.68. The optimal cutoff value for plasma CTGF (413 pmol/l) predicted ESRD with a sensitivity of 73% and a specificity of 63% (Fig. 1*E*).

During follow-up, 40 of 192 patients with nephropathy and none of the patients without nephropathy developed ESRD. Within the nephropathy group, development of ESRD occurred in a larger proportion of patients with plasma CTGF >413 pmol/l ($P < 0.001$) (Fig. 1*F*). The rate of decline in GFR was higher in these patients than in those with plasma CTGF <413 pmol/l (5.4 ± 4.9 vs. 3.3 ± 3.5 ml/min per 1.73 m^2 per year; $P < 0.001$) (Fig. 1*G*).

In all patients with diabetic nephropathy, the covariate-adjusted HR of plasma CTGF for ESRD was 1.6 (95% CI 1.1–2.5) ($P = 0.03$). In patients with nephrotic range albuminuria (>3 g/day), 17 of 32 patients developed ESRD, consistent with previous studies in type 1 and type 2 diabetes (26,27). In this subgroup, plasma CTGF was the only independent predictor of ESRD, with a covariate-adjusted HR of 4.5 (2.0–10.4) ($P < 0.001$) (Table 2). In patients with non-nephrotic range albuminuria, the only independent predictor of ESRD was GFR (data not shown).

Baseline plasma CTGF predicts mortality in diabetic nephropathy

Mortality in patients with nephropathy was 40%, of which 54% was due to cardiovascular mortality. In patients with diabetic nephropathy, area under the ROC curve for prediction of both overall mor-

Table 2—*Cox proportional hazards model for ESRD of baseline risk factors associated with rate of decline in GFR in patients with diabetic nephropathy*

	HR (95% CI)	*P* value
All patients with diabetic nephropathy ($n = 198$)		
GFR (per 1-SD decrease = 34 ml/min per 1.73 m^2)	3.13 (1.90–5.15)	<0.001
Sex (male vs. female)	2.52 (1.23–5.14)	0.01
UAE (per 1-SD increase = 3.6-fold)	2.08 (1.44–3.01)	<0.001
Plasma CTGF (per 1-SD increase = 1.9-fold)	1.62 (1.05–2.50)	0.03
Patients with nephrotic range albuminuria ($n = 33$)		
Plasma CTGF (per 1-SD increase = 1.9-fold)	4.53 (1.96–10.44)	<0.001

Adjusted for systolic blood pressure, duration of diabetes, and all other variables of this table.

Table 3—*Cox proportional hazards model of baseline risk factors for overall mortality in patients with diabetic nephropathy*

	HR (95% CI)	*P* value
Sex (male vs. female)	2.03 (1.18–3.48)	0.011
A1C (per 1-SD increase = 1.5%)	1.47 (1.16–1.86)	0.001
Systolic blood pressure (per 1-SD increase = 22 mmHg)	1.41 (1.10–1.80)	0.007
Plasma CTGF (per 1-SD increase = 1.9-fold)	1.39 (1.11–1.74)	0.005
Age (per 1-SD increase = 9.5 years)	1.37 (1.10–1.69)	0.004

Adjusted for smoking, history of major cardiovascular event, UAE, GFR, and all variables of this table.

tality and cardiovascular mortality by plasma CTGF was 0.66. A cutoff value for plasma CTGF of 413 pmol/l predicted overall mortality with optimal sensitivity and specificity (59 and 63%, respectively). A cutoff value for plasma CTGF of 419 pmol/l predicted cardiovascular mortality with optimal sensitivity and specificity (67 and 61%, respectively). Both cardiovascular mortality and overall mortality were higher in patients with plasma CTGF above either of these cutoff values ($P < 0.001$) (overall mortality shown in Fig. 1*H*).

Significant independent baseline predictors of overall mortality in patients with nephropathy were sex, plasma CTGF, A1C, systolic blood pressure, and age. The covariate-adjusted HR of plasma CTGF for prediction of overall mortality was 1.4 (95% CI 1.1–1.7) ($P = 0.005$) (Table 3). In the subpopulation of nephrotic range albuminuric patients, this HR was 2.3 (1.2–4.4) ($P = 0.01$). As for cardiovascular mortality, this was predicted independently by history of major cardiovascular event, systolic blood pressure, and GFR, but not by plasma CTGF.

In normoalbuminuric patients, overall mortality was 11%, of which 42% was due to cardiovascular mortality. Although plasma CTGF at baseline was higher in normoalbuminuric patients who died during follow-up compared with those who were still in the study (median 274 [interquartile range 202–392] vs. 230 [164–343] pmol/l, $P = 0.034$), plasma CTGF was an independent predictor of neither overall mortality nor cardiovascular mortality in patients with normoalbuminuria.

CONCLUSIONS — The major findings in this study are that the plasma CTGF level correlates with rate of decline in GFR and that it is an independent predictor of both ESRD and mortality in patients with type 1 diabetic nephropathy.

Baseline plasma CTGF was higher in patients with diabetic nephropathy than in patients with normoalbuminuria. This result is in accordance with our previous observations in a smaller study, in which plasma CTGF levels were increased in 10 patients with diabetic nephropathy (18). Although the correlations between plasma CTGF and UAE ($R = 0.16$) and between plasma CTGF and GFR ($R = -0.58$) are relatively weak, the association of diabetic nephropathy with plasma CTGF is of strength similar to its association with the established risk factors A1C and systolic blood pressure. Of interest, the OR for diabetic nephropathy of elevated plasma CTGF is of magnitude comparable to that of increased urinary CTGF excretion observed in a previous study (OR = 2.0 and 2.3, respectively) (20). However, because urine and plasma samples have not been available from the same patients thus far, the relation between CTGF levels in plasma and urine remains to be determined in future studies.

In normoalbuminuric patients, renal function remained well preserved, and progression to ESRD was not observed during the follow-up period. However, in patients with diabetic nephropathy, renal function deteriorated progressively, and 21% developed ESRD. Consistent with previous reports, rate of decline in patients with overt diabetic nephropathy was most strongly associated with UAE ($R = 0.43$) (28,29). Of interest, addition of plasma CTGF increased this correlation to a cumulative R of 0.46, whereas no such increase was observed with addition of any other baseline parameter, including baseline GFR. Accordingly, patients with high plasma CTGF levels had a steeper slope of decline in GFR than those with low plasma CTGF (cutoff 413 pmol/l).

Baseline plasma CTGF was identified as an independent parameter, but its association with decline in renal function was much stronger in patients with severe proteinuria than in those with mild proteinuria. Separate analysis of patients with nephrotic range albuminuria revealed that plasma CTGF was the only independent predictor of ESRD, whereas differences even in GFR or UAE no longer predicted outcome in this subgroup. On the other hand, despite overlapping plasma CTGF levels, no such correlation or predictive value was found in patients with normoalbuminuria. It thus appears that plasma CTGF has unique potential as a prognostic biomarker of renal function decline, especially in diabetic patients with severe proteinuria.

Previously, we observed that urinary CTGF excretion is elevated only in macroalbuminuric and not in microalbuminuric and normoalbuminuric patients (20). Together with the well-established profibrotic effects of CTGF on tubular epithelial cells (8,30), these observations suggest that progressive loss of renal function might relate to excess plasma CTGF leaking into the urine in patients with severe proteinuria. It would be interesting to study whether levels of CTGF in unselected normoalbuminuric or microalbuminuric patients could identify subjects at high risk for progression of albuminuria, but this could not be investigated in the present study as the normoalbuminuric control group was selected for having long diabetes duration and thus low risk of progression of albuminuria. A microalbuminuric group was not available for this study.

In summary, addition of plasma CTGF to conventional risk factor assessment significantly improves prediction of ESRD and mortality in patients with overt type 1 diabetic nephropathy. Its unique predictive value for disease progression in patients with diabetic nephropathy, in particular those with heavy proteinuria, suggests that plasma CTGF might have clinical application as a biomarker. In addition, our findings lend further support to the notion that CTGF is an important pathogenic factor in progression of human diabetic nephropathy, consistent with previous observations in preclinical models.

Acknowledgments— This study was supported by the Dutch Kidney Foundation (C05.2144) and the Netherlands Organization for Scientific Research (017.003.037).

We thank Lotte Wieten for technical assistance and Jack Wetzels for helpful discussions.

Part of this study were presented in abstract form at Renal Week 2007: American Society of Nephrology Annual Meeting, San Francisco, California, 31 October–5 November 2007.

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