

Circulating and Urinary Transforming Growth Factor β 1, Amadori Albumin, and Complications of Type 1 Diabetes

The EURODIAB Prospective Complications Study

NISH CHATURVEDI, MRCP¹
CASPER G. SCHALKWIJK, PHD²
HEIDEMARIE ABRAHAMIAN, MD³
JOHN H. FULLER, FRCP⁴

COEN D.A. STEHOUWER, PHD²
THE EURODIAB PROSPECTIVE
COMPLICATIONS STUDY GROUP

OBJECTIVE — Transforming growth factor (TGF)- β 1 is overexpressed in diabetes as a consequence of hyperglycemia and the creation of early glycated end products and may be responsible for the characteristic structural renal changes associated with diabetes. We sought to examine the role of both urinary and circulating TGF- β 1 and its promoter Amadori albumin in the vascular complications of type 1 diabetes.

RESEARCH DESIGN AND METHODS — The present article reports on a nested case-control study from the EURODIAB Prospective Complications Study of Europeans with type 1 diabetes. Case subjects ($n = 356$) were all individuals with one or more complications of diabetes; control subjects ($n = 185$) were all individuals with no evidence of complications.

RESULTS — Urinary TGF- β 1 and Amadori albumin were elevated in patients with micro- or macroalbuminuria. Standardized regression effects (SREs) for macroalbuminuria versus normoalbuminuria were 2.45 (95% CI 1.88–3.18, $P = 0.0001$ for urinary TGF- β 1) and 1.67 (1.34–2.07, $P = 0.001$ for Amadori albumin). The SRE for urinary TGF- β 1 remained statistically significant when adjusted for HbA_{1c}, Amadori albumin, and blood pressure. Circulating TGF- β 1 was elevated in individuals with proliferative retinopathy compared with individuals without retinopathy (SRE 1.29 [1.07–1.550], $P = 0.007$). This result was attenuated to 1.16 (0.95–1.43, $P = 0.2$) in the multivariate model, largely because of HbA_{1c}.

CONCLUSIONS — Elevated levels of urinary TGF- β 1 in macroalbuminuria were associated with elevations in Amadori albumin and HbA_{1c} and also in blood pressure. In contrast, only circulating TGF- β 1 was related to proliferative retinopathy, and HbA_{1c} largely accounted for this. These findings may indicate novel pathways for understanding mechanisms and therapeutic interventions.

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Improvements in glycemic control and the greater use of antihypertensive therapy should eventually have a beneficial impact on the incidence of severe nephropathy in type 1 diabetes. However,

early disease, such as microalbuminuria and macroalbuminuria, will continue to occur because glycemic control cannot wholly prevent the progression of albuminuria, and there is currently little evi-

From the ¹Department of Epidemiology and Public Health, Imperial College of Medicine at St. Mary's, London, U.K.; the ²Department of Medicine, Academic Hospital Vrije Universiteit, and the Institute for Cardiovascular Research, Vrije Universiteit, Amsterdam, the Netherlands; the ³Hospital Vienna Lainz, Vienna, Austria; and the ⁴Department of Epidemiology and Public Health, University College London, London, U.K.

Address correspondence and reprint requests to Professor Nish Chaturvedi, Department of Epidemiology and Public Health, Imperial College of Medicine at St. Mary's, Norfolk Place, London W2 1PG, U.K. E-mail: n.chaturvedi@ic.ac.uk

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Abbreviations: AER, albumin excretion rate; CVD, cardiovascular disease; ELISA, enzyme-linked immunosorbent assay; HSA, human serum albumin; SRE, standardized regression effect; TGF, transforming growth factor.

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

dence that antihypertensive use in normotensive normoalbuminuric patients is of clinical value (1). Both microalbuminuria and macroalbuminuria significantly increase the risk of morbidity and mortality from coronary heart disease and are strong predictors of subsequent severe renal disease (2). Prevention of the early stages of diabetic renal disease and reduction in progression should now be priorities, but these steps require a more complete understanding of the etiology to identify suitable targets for intervention.

Diabetic nephropathy is characterized by hypertrophy of the glomerular and tubulopithelial structures and thickening of the glomerular and tubular basement membrane, due largely to the effects of hyperglycemia (3). The cytokine transforming growth factor (TGF)- β 1 appears to be a key mediator for these changes (4). TGF- β 1 expression is enhanced in the presence of diabetes, either as a direct consequence of hyperglycemia or indirectly via the formation of early or advanced glycation end products (4). Hyperglycemia stimulates condensation reactions between glucose and proteins, and an early product of this reaction is an Amadori protein. Amadori albumin is the major early glycated protein, and such proteins may undergo further modifications to form advanced glycation end products.

TGF- β 1 also upregulates the expression of a serine/threonine kinase, hSGK, which indirectly stimulates the renin-angiotensin system (5). Angiotensin II is a potent stimulus for TGF- β 1 (6) and may be the mechanism by which hypertension results in renal damage. Angiotensin II may also further explain why blockade of the renin-angiotensin system is particularly effective in slowing progression of renal disease (7).

This defective extracellular matrix remodeling may also occur in other organs; therefore, it is reasonable to hypothesize that TGF- β 1 may also be involved in the

other complications of diabetes. One previous study of type 2 diabetes indicated that this was indeed the case, but it is not clear whether these observations were confounded by albuminuria (8). We therefore examined the role of TGF- β 1 and its possible inducers glucose and Amadori albumin in the complications of type 1 diabetes.

RESEARCH DESIGN AND METHODS

The EURODIAB Prospective Complications Study is a follow-up of the EURODIAB IDDM Complications Study (9). Baseline investigations (1988–1991) were performed on 3,250 men and women with type 1 diabetes drawn from 31 European centers. Sample selection was stratified by sex, age-group, and duration of diabetes to ensure sufficient representation in all categories. Type 1 diabetes was clinically defined as a diagnosis made before the age of 36 years, with a continuous need for insulin therapy within 1 year of diagnosis.

The follow-up (EURODIAB Prospective Complications Study) was performed on average 7–9 years later. Of the 3,250 patients, 1,880 (57.8%) returned for examination. Of these, full data on cardiovascular disease (CVD), microvascular complications, and neuropathy were available on 1,296 patients. Of those who were not reexamined, 437 had been recruited from centers not taking part in the follow-up, 101 had died, 465 provided morbidity data only, and 367 were untraced or otherwise unavailable.

All complications were again measured to a standard protocol (10). Two 24-h urine collections were performed (only one was performed at baseline) to measure the albumin excretion rate (AER). Aliquots were frozen and sent to London for analysis of urinary albumin with an immunoturbidimetric method using goat anti-human albumin antisera (Sanofi Diagnostics Pasteur, Minneapolis, MN) and human serum albumin (ORHA 20/21 grade human serum albumin [HSA]; Behring Diagnostics, Hoechst, U.K.) standards (11). Retinopathy was assessed from retinal photographs (two fields per eye) according to the EURODIAB protocol (12). CVD was defined as a positive medical history, bypass graft surgery, and/or major Q waves on a centrally coded electrocardiogram.

Blood samples were taken, fasting if possible, for measurement of lipids and

glycemic control. At a central laboratory, cholesterol and triglyceride levels were measured by enzymatic colorimetric tests (13), and HDL cholesterol was measured directly (14). HbA_{1c} was measured by a latex enhanced turbidimetric immunoassay (Roche, Welwyn Garden City, U.K.). The reference range for this assay was 4.2–6.2%.

Total TGF- β 1 was measured by an enzyme-linked immunosorbent assay (ELISA) development system (R&D Systems, Oxon, U.K.). To activate the latent plasma TGF- β 1 to the immunoreactive form, 0.1 ml plasma was acid-activated by 0.1 ml 2.5 N acetic acid/10 mol/l urea for 10 min at room temperature and neutralized by 0.1 ml 2.7 N NaOH/1 mol/l HEPES. For urine, 1 ml was activated by 0.2 ml 1 N HCl and neutralized by 0.2 ml 1.2 N NaOH/0.5 mol/l HEPES. Intra- and interassay variations were 5.0 and 7.0%, respectively. Urinary TGF- β 1 was indexed to urinary creatinine excretion. Amadori albumin was determined in a competitive ELISA as recently described (15).

Briefly, each well was coated with 100 μ l of 0.1 mg/ml glycated HSA as prepared by the incubation of HSA with 0.5 mol/l glucose for 4 weeks. This preparation was also used as standard in the competitive ELISA. Then, 50 μ l of the antibody conjugated with biotin (1:2,000) and 50 μ l of standard or a sample to be tested, diluted in PBS including 30 mg/ml HSA, was added to each well and incubated for 2 h. After three washes with PBS-Tween, the wells were incubated with streptavidin-peroxidase (CLB, Amsterdam, the Netherlands) for 1 h, followed by substrate development with 100 μ l tetramethylbenzidine. The reaction was stopped with 100 μ l 0.5 mol/l H₂SO₄. The extinction at 450 nm was measured with a multichannel spectrophotometer (SLT Microplate Reader; Wilten Bioteknika, Etten-Leur, the Netherlands). Plasma levels were expressed as Amadori albumin units, and 1 unit was defined as the antibody-reactive material equivalent to 1 μ g glycated HSA standard.

Statistical analysis

We used a nested case-control approach to maximize efficiency. Case subjects who had the greatest complication burden were selected to provide sufficient numbers for subgroup analyses. Control subjects who were completely free of

complications were selected. Thus, case subjects were individuals with CVD or proliferative retinopathy or macroalbuminuria at follow-up and individuals with microalbuminuria and some degree of retinopathy ($n = 356$). Control subjects were individuals who had no evidence of CVD, retinopathy, or neuropathy and were normoalbuminuric at follow-up ($n = 185$). This selection allowed us to compare people with and without single or multiple complications. Case and control subjects were unmatched, so that the impact of key variables, such as age, could still be assessed, and any adjustments were taken care of at the analysis stage. Of these 541 individuals, sufficient sampling material was available for 535 for Amadori albumin, 534 for circulating TGF- β 1, and 532 for urinary TGF- β 1. These numbers allow us to detect a difference between groups of at worst one-third of an SD, with 90% power and 5% significance.

Key risk factors in case and control subjects were compared, reporting crude means and SDs or geometric means and 25th and 75th percentiles for skewed variables. Urinary and circulating TGF- β 1 were log-transformed before analysis. Pearson correlation coefficients were first calculated for circulating and urinary TGF- β 1 and Amadori albumin and other risk factors for complications. Then mean values of circulating and urinary TGF- β 1 and Amadori albumin were estimated using ANOVA for each complication. Finally, standardized regression effects (SREs) were compared. These SREs were calculated by multiplying the β coefficient of each variable in a multivariate logistic regression model by its SD. This calculation allowed us to compare the impact of each factor directly with the other in standardized units. All SREs were first adjusted for age; adjustment for diabetes duration alone produced similar findings. There were no significant sex differences in these relationships; therefore, both sexes were combined, and no adjustment was made for sex.

RESULTS

Differences between case and control subjects

Case subjects were more likely to have an adverse risk factor profile and raised levels of urinary and circulating TGF- β 1 and

Table 1—Patient characteristics

Characteristic	Case subjects	Control subjects
n	356	185
Age (years)	41.7 ± 10.6	36.1 ± 8.1
Diabetes duration (years)	25.0 ± 9.0	15.5 ± 7.0
HbA _{1c} (%)	9.0 ± 1.6	7.7 ± 1.3
Systolic blood pressure (mmHg)	127 ± 21.5	115 ± 13.7
Diastolic blood pressure (mmHg)	75 ± 11.7	74 ± 10.9
AER (μg/min)	65.4 (9.21, 372)	6.2 (4.5, 8.4)
Amadori albumin (units/ml)	48.1 ± 13.7	42.7 ± 13.1
Urinary TGF-β1/creatinine (pg/mmol)	3.71 (2.34, 5.26)	2.36 (1.55, 3.46)
Circulating TGF-β1 (ng/ml)	6.17 (3.71, 9.00)	5.52 (3.29, 8.08)
Cholesterol (mmol/l)	5.5 ± 1.24	5.0 ± 1.10
HDL cholesterol (mmol/l)	1.60 ± 0.43	1.67 ± 0.45
Triglyceride (mmol/l)	1.23 (0.85, 1.58)	0.84 (0.66, 1.08)
Waist (cm)	86.0 ± 11.9	83.0 ± 10.9
Height (cm)	168 ± 9.4	170 ± 9.6
Weight (kg)	70.1 ± 12.5	69.3 ± 11.9
Waist-to-hip ratio	0.89 ± 0.13	0.88 ± 0.16

Data are means ± SD or geometric means (25th and 75th percentiles) for log-transformed data.

Amadori albumin than control subjects (Table 1).

Correlations with other risk markers of complications

Urinary and circulating TGF-β1 was not correlated (coefficient 0.05, P = 0.3, Table 2). Urinary TGF-β1 was correlated with diabetes duration, HbA_{1c}, systolic pressure, AER, Amadori albumin, and lipids and was correlated negatively with measures of body size. Circulating TGF-β1 was only correlated with HbA_{1c} and Amadori albumin. Amadori albumin, as anticipated, was correlated with HbA_{1c}, AER, and lipids and was correlated negatively with estimates of body size.

TGF-β1, Amadori albumin, and diabetic nephropathy

Mean urinary TGF-β1 and Amadori albumin were elevated in microalbuminuric and macroalbuminuric patients (Table 3). However, circulating TGF-β1 was only significantly raised in macroalbuminuric patients compared with normoalbuminuric patients.

The strength of the association between TGF-β1 and macroalbuminuria was second only to systolic blood pressure in the univariate analyses (Table 4, SRE 2.45 vs. 3.18). All univariate associations were attenuated in bivariate models. For example, the SRE for TGF-β1 was attenuated from 2.45 to 2.02 with the addition of

HbA_{1c}, and the univariate association between HbA_{1c} and macroalbuminuria was attenuated from 2.27 to 1.91 in the same model. In the multivariate model, the association between TGF-β1 and macroalbuminuria was severely attenuated, but by no means abolished, by the inclusion of HbA_{1c} and Amadori albumin; HbA_{1c} was the least affected by these inclusions. The impact of systolic blood

pressure was less strong than that of HbA_{1c} and was almost equivalent to that of Amadori albumin on TGF-β1 in the bivariate models. But in the multivariate model, blood pressure had an almost unchanged impact on macroalbuminuria compared with the univariate data, indicating that the impact of blood pressure on albuminuria is likely to be independent of the consequences of hyperglycemia.

TGF-β1, Amadori albumin, and diabetic retinopathy

In contrast to the findings on nephropathy, there was a striking difference in circulating TGF-β1 and Amadori albumin between patients with proliferative retinopathy and no retinopathy, which remained on adjustment for AER (Table 3). The association between urinary TGF-β1 and retinopathy could be accounted for by adjustment for AER. For proliferative retinopathy, the univariate association between circulating TGF-β1 was attenuated by the addition of the other covariates (Table 5). The SRE for Amadori albumin was barely altered with the addition of TGF-β1. However, the association between Amadori albumin and proliferative retinopathy was almost abolished with the addition of HbA_{1c}. In the multivariate model, associations between HbA_{1c}, and systolic blood pressure and

Table 2—Correlation coefficients between urinary and circulating TGF-β1 and Amadori albumin and risk factors for microvascular complications

	TGF-β1 urine*	TGF-β1 circulating	Amadori albumin
n	532	534	535
Age	0.07	-0.03	0.09 (0.04)
Diabetes duration	0.14 (0.002)	-0.01	0.12 (0.005)
HbA _{1c}	0.32 (<0.0001)	0.15 (0.0004)	0.43 (0.0001)
Systolic blood pressure	0.13 (0.003)	0.05	0.09 (0.03)
Diastolic blood pressure	0.02	0.08	-0.02
AER	0.37 (<0.0001)	0.07	0.22 (0.0001)
Amadori albumin	0.26 (<0.0001)	0.21 (<0.0001)	—
TGF-β1 urine	—	0.06	0.26 (0.0001)
TGF-β1 circulating	0.06	—	0.21 (0.0001)
Cholesterol	0.16 (0.0003)	0.08	0.16 (0.0002)
HDL cholesterol	0.07	0.04	0.16, (0.0002)
Triglyceride†	0.14 (0.01)	-0.03	0.13 (0.02)
Waist	-0.10 (0.02)	0.00	-0.07
Height	-0.20 (<0.0001)	-0.08	-0.13 (0.003)
Weight	-0.18 (<0.0001)	-0.08(0.05)	-0.11 (0.01)
Waist-to-hip ratio	-0.09 (0.03)	0.05	-0.06

Data in parentheses are P values. *Urinary TGF-β1 indexed to creatinine; †fasting triglyceride only restricted to n = 332 for urinary TGF-β1, n = 334 for circulating TGF-β1, and n = 335 for Amadori albumin.

Table 3—Mean age-adjusted values of circulating and urinary TGF- β 1 and Amadori albumin by albuminuria and retinopathy

	n	Urinary TGF- β 1/creatinine (pg/mmol)	Circulating TGF- β 1 (ng/ml)	Amadori albumin (units/ml)
Normoalbuminuria	326	2.6 (2.4–2.8)	5.6 (5.2–6.1)	43.2 (41.8–44.6)
Microalbuminuria	81	3.7 (3.2–4.3)	6.4 (5.5–7.5)	47.8 (44.9–50.6)
Macroalbuminuria	126	4.6 (4.1–5.2)	6.2 (5.5–7.0)	50.3 (48.1–52.6)
P for macro- vs. normoalbuminuria	—	0.0001	0.007	0.0001
P for micro- vs. normoalbuminuria	—	0.0001	0.5	0.004
No retinopathy	235	2.5 (2.3–2.7)	5.7 (5.2–6.2)	43.5 (41.8–45.2)
Background retinopathy	146	3.6 (3.2–4.1)	5.4 (4.8–6.1)	45.2 (43.0–47.3)
Proliferative retinopathy	154	3.8 (3.4–4.3)	6.7 (6.0–7.4)	49.3 (47.2–51.4)
P for trend	—	0.0001	0.02	0.0001
P for proliferative vs. none	—	0.06	0.02	0.0001
P for proliferative vs. none, adjusted for AER	—	0.9	0.04	0.04

Data are means (95% CI) unless otherwise indicated.

retinopathy, differed little from their univariate associations, but the relationship for circulating TGF- β 1 and Amadori albumin became statistically nonsignificant.

TGF- β 1, Amadori albumin, and CVD

There was no association between Amadori albumin and either urinary or circulating TGF- β 1 and CVD (data not shown).

TGF- β 1 levels and ACE inhibitor use

Circulating TGF- β 1 was higher in individuals on ACE inhibitors than in individuals not on any antihypertensive therapy (3.67 vs. 2.92 ng/ml, $P = 0.001$). A nonsignificant difference was observed for urinary TGF- β 1 (5.99 vs. 5.88 pg/mmol, $P = 0.9$). These associations may be confounded by nephropathy status. Strikingly, elevated levels of circulating TGF- β 1 were observed in individuals on ACE inhibitors in the normoalbuminuric and microalbuminuric range (5.87 vs. 5.58 and 7.46 vs. 6.05 ng/ml, respectively), whereas levels were lower in individuals on ACE inhibitors who had macroalbuminuria (5.58 vs. 7.03 ng/ml, respectively; $P = 0.04$ for interaction). Similar differences were observed for urinary TGF- β 1. Mean systolic blood pressure was 13 mmHg higher in normoalbuminuric patients on ACE inhibitors than in individuals not on treatment; this difference was 8 mmHg in microalbuminuric patients and 5 mmHg in macroalbuminuric patients. Adjustment for these blood pressure differences largely attenuated differences in both circulating and uri-

nary TGF- β 1 in normoalbuminuric and microalbuminuric patients but not in macroalbuminuric patients.

CONCLUSIONS— We show that both urinary TGF- β 1 and Amadori albumin are elevated in microalbuminuric and macroalbuminuric patients with type 1 diabetes. Separately, Amadori albumin and HbA_{1c} accounted for a similar proportion of the association between

TGF- β 1 and albuminuria, but their joint impact attenuated this association still further. This result indicates that HbA_{1c} effects TGF- β 1 via mechanisms in addition to its impact on Amadori albumin and on albuminuria via means that are not exclusive to TGF- β 1. This is not unexpected because it is likely that several other mechanisms account for the impact of hyperglycemia on diabetic renal disease.

Table 4—(SREs) for macroalbuminuria versus normoalbuminuria by key risk factors

	SRE	95% CI	P
Univariate			
HbA _{1c}	2.27	1.79–2.88	0.0001
Systolic blood pressure	3.18	2.37–4.27	0.0001
TGF- β 1 urine	2.45	1.88–3.18	0.0001
Amadori albumin	1.67	1.34–2.07	0.0001
Bivariate			
Systolic blood pressure and TGF- β 1 urine			
Systolic blood pressure	3.18	2.32–4.37	0.0001
TGF- β 1 urine	2.31	1.76–3.04	0.0001
HbA _{1c} and TGF- β 1 urine			
HbA _{1c}	1.91	1.49–2.45	0.0001
TGF- β 1 urine	2.02	1.54–2.65	0.0001
Amadori albumin and TGF- β 1 urine			
Amadori albumin	1.42	1.13–1.80	0.003
TGF- β 1 urine	2.26	1.73–2.94	0.0001
HbA _{1c} and Amadori albumin			
HbA _{1c}	2.10	1.59–2.64	0.0001
Amadori albumin	1.21	0.94–1.54	0.1
Multivariate, all variables in model			
Systolic blood pressure	3.24	2.32–4.52	0.0001
TGF- β 1 urine	1.86	1.40–2.48	0.0001
Amadori albumin	1.15	0.87–1.54	0.3
HbA _{1c}	1.87	1.38–2.53	0.0001

All SREs have been adjusted for age.

Table 5—Univariate and multivariate associations between risk factors and proliferative retinopathy (SREs)

	SRE	95% CI	P
Univariate			
Amadori albumin	1.45	1.20–1.75	0.0001
Circulating TGF- β 1	1.29	1.07–1.55	0.007
Systolic blood pressure	1.69	1.37–2.10	0.0001
HbA _{1c}	2.07	1.68–2.56	0.0001
Bivariate			
HbA _{1c} and Amadori albumin			
HbA _{1c}	1.98	1.57–2.49	0.0001
Amadori albumin	1.11	0.89–1.38	0.4
Amadori albumin and circulating TGF- β 1			
Amadori albumin	1.40	1.15–1.70	0.004
Circulating TGF- β 1	1.21	1.00–1.47	0.05
Circulating TGF- β 1 and HbA _{1c}			
Circulating TGF- β 1	1.20	0.98–1.46	0.08
HbA _{1c}	2.01	1.63–2.49	0.0001
Circulating TGF- β 1 and systolic blood pressure			
Circulating TGF- β 1	1.26	1.04–1.53	0.02
Systolic blood pressure	1.67	1.35–2.07	0.0001
Multivariate			
Circulating TGF- β 1, HbA _{1c} , and Amadori albumin			
Circulating TGF- β 1	1.19	0.97–1.45	0.1
HbA _{1c}	1.95	1.55–2.46	0.0001
Amadori albumin	1.08	0.87–1.35	0.3
Circulating TGF- β 1, HbA _{1c} , Amadori albumin, and systolic blood pressure			
Circulating TGF- β 1	1.16	0.95–1.43	0.2
HbA _{1c}	1.95	1.54–2.48	0.0001
Amadori albumin	1.07	0.85–1.34	0.6
Systolic blood pressure	1.66	1.32–2.08	0.0001

All SREs have been adjusted for age.

Cell culture studies show that a high glucose medium can enhance autocrine production and activation of TGF- β 1, and TGF- β 1 in turn can enhance glucose uptake through fibroblasts (16), indicating an amplification of the effect of hyperglycemia. In human studies of type 2 diabetes, both urinary and circulating TGF- β 1 have been found to be raised in individuals with proteinuria but not in individuals with microalbuminuria compared with control subjects (17). However, these previous studies were relatively small and may not have been powered to detect a difference for earlier stages of disease. Further, these studies have not generally accounted for the confounding effects of other risk factors and complications. Few data exist for type 1 diabetes and have so far not indicated elevations of urinary TGF- β 1 with albuminuria as we do here (18).

We also show that the association between Amadori albumin and macroalbuminuria could largely be explained by glycemic control because the univariate SRE was attenuated from 1.67 to 1.21 in the bivariate model with HbA_{1c}, with little attenuation to 1.15 in the multivariate model, indicating that much of the mechanisms of HbA_{1c} and Amadori albumin on diabetic nephropathy are shared and that at least part of that effect is mediated via TGF- β 1. There is now good mechanistic evidence demonstrating the role of Amadori albumin in the etiology of diabetic nephropathy. Mesangial cells can recognize and bind Amadori albumin in a dose-response fashion, and animal studies show that infusion of Amadori albumin produces renal glomerular changes that are similar to those observed in diabetes (19,20). That this nephropathic effect is mediated via TGF- β 1 is shown by

cell culture studies, in which incubation with glycated albumin resulted in overexpression of TGF- β 1 mRNA levels (21). Administration of anti-glycated albumin antibody in animal models reduces plasma levels of glycated albumin, reduces AER, and reverses glomerular pathology (22). Human studies have been conflicting, perhaps in part because of small numbers and confounding by other risk factors, but one of the largest previous studies clearly indicated that Amadori albumin levels were elevated in individuals with nephropathy compared with individuals without nephropathy (15,23–25).

Interestingly, the inclusion of both Amadori albumin and HbA_{1c} in our models could only account for around one-quarter of the association between TGF- β 1 and albuminuria, indicating that other mechanisms shape the association between TGF- β 1 and albuminuria. A clear candidate is hypertension, which alone was the strongest correlate of macroalbuminuria. Certainly the inclusion of blood pressure with TGF- β 1 had the same attenuating effect on the risk estimate of TGF- β 1 as Amadori albumin did, suggesting that raised blood pressure, an important risk factor for albuminuria, was in part working through TGF- β 1 to mediate its effects; however, clearly from the multivariate analyses, this was not the sole, or even the most important, mechanism. This finding can be accounted for by the observation of a stimulatory effect of TGF- β 1 on the renin angiotensin system (6) and the beneficial effect of angiotensin II receptor blockers on TGF- β 1 levels and renal pathology (26). Other mechanisms not studied here include activation of protein kinase C, other growth factors such as vascular endothelial growth factor and insulin-like growth factor, oxidative stress, and intraglomerular hemodynamic factors (27–29).

Interestingly, urinary TGF- β 1 was only lower in macroalbuminuric patients on ACE inhibitors compared with individuals on no treatment. In the normoalbuminuric and microalbuminuric categories, there appeared to be little difference or even a tendency to higher levels in individuals on ACE inhibitors. This was in part accounted for by the greater blood pressure differentials at lower albuminuric levels between individuals on treatment and those not on treatment. A separate explanation is that ACE inhibi-

tion suppresses TGF- β 1 receptor expression, which could reduce TGF- β 1 signaling and thus TGF- β 1-mediated effects (30).

In contrast, circulating TGF- β 1 was only elevated in the presence of macroalbuminuria, and not microalbuminuria, compared with normoalbuminuria, indicating that it may be less sensitive to renal changes. However, clear differences were observed between proliferative retinopathy and no retinopathy in circulating TGF- β 1, which unlike differences observed for urinary TGF- β 1, could not be accounted for by confounding with AER. The impact of TGF- β 1 on proliferative retinopathy remained largely unexplained by elevated blood pressure but could be largely accounted for by HbA_{1c}. Others (8) have indicated that circulating TGF- β 1 is elevated in nonproliferative retinopathy in type 2 diabetes. By inhibiting replacement of damaged cells, TGF- β 1 may contribute to capillary and pericyte loss in diabetic retinopathy (31). In contrast though, whereas total vitreous TGF- β 1 did not differ between individuals with proliferative retinopathy compared with control subjects, latent TGF- β 1 was substantially decreased in the former group, indicating that TGF- β 1 may also have an anti-mitogenic effect (32).

Animal studies show that overexpression of TGF- β 1 occurs within as little as 3 days of streptozotocin-induced diabetes and that this can be inhibited by insulin (33). The identification of such early markers of renal damage may have important implications for the treatment of early diabetic renal disease and for monitoring the success of therapy. Our findings also indicate that TGF- β 1 is an early marker of renal damage in type 1 diabetes. As may be expected from the current understanding of the metabolism of TGF- β 1, blockade of the renin angiotensin system and, separately, inhibitors of glycation end products reduce the overexpression of renal TGF- β 1 and have a beneficial effect on renal pathology in animal models (34). More unexpected is the finding that (again, in animal models only) lipid-lowering agents, specifically 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, also reduce the expression of TGF- β 1 and reduce albuminuria levels, although this appears not be associated with a reduction in lipid levels (35).

There are certain limitations to this

analysis. This is a cross-sectional study, and we can therefore only imply associations rather than causal relationships. However, our results are supported by *in vitro* and animal studies demonstrating the mechanisms of the associations between glucose, Amadori albumin, TGF- β 1, and diabetic complications. Further follow-up data on these and other patient groups would, of course, be invaluable in testing whether our findings are due to causal relationships. Unlike previous studies, a key strength here is the ability to account for confounding by other risk factors and complications, and the large sample size provides sufficient power for these analyses. There are concerns also about the use of plasma to measure circulating TGF- β 1. TGF- β 1 represents the major isoform of the TGF- β 1 superfamily in blood. It is present in platelet granules and is released on platelet activation. However, the degree of sample contamination by platelet release of TGF- β 1 in plasma samples appears to be relatively small (36), and because the protocol for sample collection and handling was identical across sites, any bias is likely to be nondifferential.

In conclusion, we demonstrate that urinary TGF- β 1 is closely correlated with the degree of albuminuria in type 1 diabetes and confirm that this effect is largely accounted for by hyperglycemia, Amadori albumin, and blood pressure. Circulating TGF- β 1, on the other hand, is more closely correlated with retinopathy, and its effect does not appear to be so closely associated with Amadori albumin. These findings underline new possibilities for both the monitoring and treatment of microvascular complications of type 1 diabetes and deserve further exploration.

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APPENDIX

Centers and staff involved in the study

B. Karamanos, A. Kofinis, K. Petrou, Hippokraton Hospital, Athens, Greece; R. Giorgino, F. Giorgino, G. Picca, A. Anga-

rano, G. De Pergola, Istituto di Clinica Medica, Endocrinologia e Malattie Metaboliche, Univesita di Bari, Bari, Italy; C. Ionescu-Tirgoviste, A. Coszma, Clinic of Diabetes, Nutrition, and Metabolic Diseases, Bucharest, Romania; M. Songini, A. Casu, M. Pedron, M. Fossarello, Department of Internal Medicine, *Ospedale San Michele, Cagliari, Italy; J.B. Ferriss, G. Grealy, D.O. Keefe, A. White, P.E. Cleary, Cork University Hospital, Cork, Ireland; M. Toeller, C. Arden, Diabetes Research Institute, Heinrich-Heine University, Dusseldorf, Germany; R. Rottiers, C. Tuytens, H. Priem, University Hospital of Gent, Gent, Belgium; P. Ebeling, M. Kylläinen, T. Kyostio-Renvall, University Hospital of Helsinki, Helsinki, Finland; B. Idzior-Walus, J. Sieradzki, K. Cyganek, Department of Metabolic Diseases, Jagiellonian University, Krakow, Poland; H.H.P.J. Lemkes, C. Roest, University Hospital of Leiden, Leiden, the Netherlands; J. Nunes-Correa, M.C. Rogado, L. Gardete-Correira, M.C. Cardoso, Portuguese Diabetic Association, Lisbon, Portugal; G. Michel, R. Wirion, S. Cardillo, Center Hospitalier, Luxembourg, Luxembourg; G. Pozza, R. Mangili, V. Asnaghi, R. Lattanzio, G. Galardi, Ospedale San Raffaele, Milan, Italy; E. Standl, B. Schaffler, H. Brand, A. Harms, City Hospital Schwabing, Munich, Germany; D. Ben Soussan, O. Verier-Mine, Center Hospitalier de Valenciennes, Valenciennes, France; J.H. Fuller, J. Holloway, L. Asbury, D.J. Betteridge, University College London, London, U.K.; G. Cathelineau, A. Bouallouche, B. Villatte Cathelineau, Hospital Saint-Louis, Paris, France; F. Santeusano, G. Rosi, V.D. Alessandro, C. Cagini, Istituto di Patologia Medica, Policlinico, Perugia, Italy; R. Navalesi, G. Penno, S. Bandinelli, R. Miccoli, Dipartimento di Endocrinologia e Metabolismo, Pisa, Italy; G. Ghirlanda, C. Saponara, P. Cotroneo, A. Manto, A. Minnella, Università Cattolica del Sacro Cuore, Rome, Italy; J.D. Ward, S. Tesfaye, S. Eaton, C. Mody, Royal Hallamshire Hospital, Sheffield, U.K.; M. Porta, P. Cavallo Perin, M. Borra, S. Giunti, Clinica Medica B, Patologia Medica, Ospedale Molinette, and Ospedale "Agnelli," Turin, Italy; N. Papanzoglou, G. Manes, General Hospital of Thessaloniki, Thessaloniki, Greece; M. Muggeo, M. Iagulli, Cattedra di Malattie del Metabolismo, Verona, Italy; K. Irsigler, H. Abrahamian, Hospital Vienna Lainz, Vienna, Austria; S. Walford, E.V.

Wardle, J. Sinclair, S. Hughes, New Cross Hospital, Wolverhampton, U.K.; and G. Roglic, Z. Metelko, Z. Resman, Vuk Vrhovac Institute for Diabetes, Zagreb, Croatia.

Steering committee members

J.H. Fuller (London), B. Karamanos, Chairman (Athens), A.-K. Sjolie (Aarhus), N. Chaturvedi (London), M. Toeller (Dusseldorf), G. Pozza (co-chairman; Milan), B. Ferriss (Cork), M. Porta (Turin), R. Rottiers (Gent), and G. Michel (Luxembourg).

Coordinating center

J.H. Fuller, N. Chaturvedi, J. Holloway, D. Webb, E. Asbury, University College London, London, U.K.

Central laboratory

G.-C. Viberti, R. Swaminathan, P. Lumb, A. Collins, S. Sankaralingham, Guy's and St Thomas' Hospital, London, U.K.

Retinopathy grading center

S. Aldington, T. Mortemore, H. Lipinski, Imperial College, London, U.K.

References

1. The EUCLID Study Group: Randomised placebo-controlled trial of lisinopril in normotensive patients with insulin-dependent diabetes and normoalbuminuria or microalbuminuria. *Lancet* 349:1787–1792, 1997
2. Rumble JR, Cooper ME, Soulis T, Cox A, Wu L, Youssef S, Jasik M, Jerums G, Gilbert RE: Vascular hypertrophy in experimental diabetes: a role in advanced glycation end products. *J Clin Invest* 99:1016–1027, 1997
3. Ziyadeh FN: The extracellular matrix in diabetic nephropathy. *Am J Kidney Dis* 22:736–744, 1993
4. Rocco MV, Chen Y, Goldfarb S, Ziyadeh FN: Elevated glucose stimulates TGF-beta gene expression and bioactivity in proximal tubule. *Kidney Int* 41:107–114, 1992
5. Lang F, Klingel K, Wagner CA, Stegen C, Warntges S, Friedrich B, Lanzendorfer M, Melzig J, Moschen I, Steuer S, Waldegger S, Sauter M, Paulmichl M, Gerke V, Risler T, Gamba G, Capasso G, Kandolf R, Hebert SC, Massry SG, Broer S: Deranged transcriptional regulation of cell-volume-sensitive kinase hSGK in diabetic nephropathy. *Proc Natl Acad Sci U S A* 97:7667–7669, 2000
6. Sun Y, Zhang JQ, Ramires FJ: Local angiotensin II and transforming growth factor-beta1 in renal fibrosis of rats. *Hypertension* 35:1078–1084, 2000
7. The ACE Inhibitors in Diabetic Nephropathy Trialist Group: Should all patients with type 1 diabetes mellitus and microalbuminuria receive angiotensin-converting enzyme inhibitors? A meta-analysis of individual patient data. *Ann Intern Med* 134:370–379, 2001
8. Pfeiffer A, Middleberg-Bisping K, Drewes C, Schatz H: Elevated plasma levels of transforming growth factor-beta 1 in NIDDM. *Diabetes Care* 19:1113–1117, 1996
9. The EURODIAB IDDM Complications Study Group: Microvascular and acute complications in insulin dependent diabetes mellitus: the EURODIAB IDDM Complications Study. *Diabetologia* 37:278–285, 1994
10. Chaturvedi N, Bandinelli S, Mangili R, Penno G, Rottiers RE, Fuller JH, on behalf of the EURODIAB PCS Group: Microalbuminuria in type 1 diabetes: rates, risk factors and glycaemic threshold: the EURODIAB PCS. *Kidney Int* 60:219–227, 2001
11. Kearney EM, Mount JN, Watts GF, Slavin BM, Kind PRN: Simple immunoturbidometric method for determining urinary albumin at low concentrations using centrifugal analyser. *J Clin Path* 40:465–468, 1987
12. Aldington SJ, Kohner EM, Meuer S, Klein R, Sjolie A-K, the EURODIAB IDDM Complications Study Group: Methodology for retinal photography and assessment of diabetic retinopathy: the EURODIAB IDDM Complications Study. *Diabetologia* 38:437–444, 1995
13. Fossati P, Prencipe L: Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. *Clin Chem* 28:2077–2080, 1982
14. Suguchi H, Uji Y, Okabe H, Irie T, Uekama K, Kayahara N, Miyauchi K: Direct measurement of high-density lipoprotein cholesterol in serum with polyethylene glycol-modified enzymes and sulfated alpha-cyclodextrin. *Clin Chem* 41:717–723, 1995
15. Schalkwijk CG, Ligtoet N, Twaalfhoven H, Jager A, Blaauwgeers HG, Schlingemann RO: Amadori-albumin in type 1 diabetic patients: correlation with markers of endothelial function, association with diabetic nephropathy and localisation in retinal capillaries. *Diabetes* 48:2446–2453, 1999
16. Kitagawa T, Masumi A, Akamatsu Y: Transforming growth factor-beta 1 stimulates glucose uptake and the expression of glucose transporter mRNA in quiescent Swiss mouse 3T3 cells. *J Biol Chem* 266:18066–18071, 1991
17. Hellmich B, Schellner M, Schatz H, Pfeiffer A: Activation of transforming growth factor-beta1 in diabetic kidney disease. *Metabolism* 49:353–359, 2000
18. Fagerudd JA, Groop PH, Honkanen E, Teppo AM, Gronhagen Riska C: Urinary excretion of TGF-beta 1, PDGF-BB and fibronectin in insulin-dependent diabetes mellitus patients. *Kidney Int Suppl* 63: S195–S197, 1997
19. Wu VY, Cohen MP: Evidence for a ligand receptor system mediating the biologic effects of glycated albumin by isolated microvessels: possible role in pathogenesis of diabetic microangiopathy. *Biochem Biophys Res Commun* 207:521–528, 1995
20. McVerry BA, Fisher C, Hopp A, Huehns ER: Production of pseudodiabetic renal glomerular changes in mice after repeated injections of glycosylated proteins. *Lancet* 1:738–740, 1980
21. Ziyadeh FN, Han DC, Cohen JA, Guo J, Cohen MP: Glycated albumin stimulates fibronectin gene expression in glomerular mesangial cells: involvement of the transforming growth factor-B system. *Kidney Int* 53:631–638, 1998
22. Cohen MP, Sharma K, Jin Y, Hud E, Wu VY, Tomaszewski J, Ziyadeh FN: Prevention of diabetic nephropathy in db/db mice with glycated albumin antagonists: a novel treatment strategy. *J Clin Invest* 95:2338–2345, 1995
23. Cavallo-Perin P, Chiambretti A, Calefato V, Tomalino M, Cecchini G, Gruden G, Pagano G: Urinary excretion of glycated albumin in insulin-dependent diabetic patients with micro- and macroalbuminuria. *Clin Nephrol* 38:9–13, 1992
24. Gragnoli G, Signorini AM, Tanganelli I: Non-enzymatic glycosylation of urinary proteins in type 1 (insulin-dependent) diabetes: correlation with metabolic control and the degree of proteinuria. *Diabetologia* 26:411–414, 1984
25. Sakai H, Jinde K, Suzuki D, Yagame M, Nomoto Y: Localization of glycated proteins in the glomeruli of patients with diabetic nephropathy. *Nephrol Dial Transplant* 11 (Suppl. 5):66–71, 1996
26. Kelly DJ, Skinner SL, Gilbert RE, Cox AJ, Cooper ME, Wilkinson-Berka JL: Effects of endothelin or angiotensin II receptor blockade on diabetes in transgenic (mRen-2)27 rat. *Kidney Int* 57:1882–1894, 2000
27. Derubertis FR, Craven PA: Activation of protein kinase C in glomerular cells in diabetes: mechanisms and potential links to the pathogenesis of diabetic glomerulopathy. *Diabetes* 43:1–8, 1994
28. Cortes P, Riser BL, Zhao X, Narins RG: Glomerular volume expansion and mesangial cell mechanical strain: mediators of glomerular pressure injury. *Kidney Int Suppl* 45:S11–S16, 1994
29. Flyvbjerg A, Marshall SM, Frystyk J, Hansen KW, Harris AG, Orskov H: Octreotide

- administration in diabetic rats: effects on renal hypertrophy and urinary albumin excretion. *Kidney Int* 41:805–812, 1992
30. Hill C, Logan A, Smith C, Gronbaek H, Flyvbjerg A: Angiotensin converting enzyme inhibitor suppresses glomerular transforming growth factor beta receptor expression in experimental diabetes in rats. *Diabetologia* 44:495–500, 2001
 31. Dosso AA, Brooks RA, Beltramo E, Molinatti PA, Kanse SM, Kohner EM, Porta M: A study of the effects of human blood derivatives and individual growth factors on [3H]thymidine uptake in bovine retinal pericytes and endothelial cells. *Acta Diabetol* 30:207–213, 1993
 32. Spranger J, Meyer-Schwickerath R, Klein M, Schatz H, Pfeiffer A: Deficient activation and different expression of transforming growth factor-beta isoforms in active proliferative diabetic retinopathy and neovascular eye disease. *Exp Clin Endocrinol Diabetes* 107:21–28, 1999
 33. Park IS, Kiyomoto H, Abboud SL, Abboud HE: Expression of transforming growth factor beta and type IV collagen in early streptozotocin-induced diabetes. *Diabetes* 46:473–480, 1997
 34. Guh JY, Yang ML, Yang YL, Chang CC, Chuang LY: Captopril reverses high-glucose induced growth effects on LLC-PK1 cells partly by decreasing transforming growth factor-beta receptor protein expressions. *J Am Soc Nephrol* 7:1207–1215, 1996
 35. Kim SI, Han DC, Lee HB: Lovastatin inhibits transforming growth factor-beta1 expression in diabetic rat glomeruli and cultured rat mesangial cells. *J Am Soc Nephrol* 11:80–87, 2000
 36. Wakefield LM, Letterio JJ, Chen T, Danielpour D, Allison RS, Pai LH, Denicoff AM, Noone MH, Cowan KH, O'Shaughnessy JA: Transforming growth factor-beta1 circulates in normal human plasma and is unchanged in advanced metastatic breast cancer. *Clin Cancer Res* 1:129–136, 1995