Cellular Basis of Diabetic Nephropathy

1. Study Design and Renal Structural–Functional Relationships in Patients With Long-Standing Type 1 Diabetes

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This study was designed to elucidate the cellular basis of risk of or protection from nephropathy in patients with type 1 diabetes. Entry criteria included diabetes duration of ≥8 years (mean duration, 22.5 years) and glomerular filtration rate (GFR) >30 ml · min^{-1} · 1.73 m^{-2}. Patients were classified, on the basis of the estimated rate of mesangial expansion, as "fast-track" (upper quintile) or "slow-track" (lower quintile). A total of 88 patients were normoalbuminuric, 17 were microalbuminuric, and 19 were proteinuric. All three groups had increased glomerular basement membrane (GBM) width and mesangial fractional volume [Vv(Mes/glom)], with increasing severity from normoalbuminuria to microalbuminuria to proteinuria but with considerable overlap among groups. Vv(Mes/glom) (r = 0.75, P < 0.001) and GBM width (r = 0.63, P < 0.001) correlated with albumin excretion rate (AER), whereas surface density of peripheral GBM per glomerulus [Sv(PGBM/glom)] (r = 0.50, P < 0.001) and Vv(Mes/glom) (r = −0.48, P < 0.001) correlated with GFR. Vv(Mes/glom) and GBM width together explained 59% of AER variability. GFR was predicted by Sv(PGBM/glom), AER, and sex. Fast-track patients had worse glycemic control, higher AER, lower GFR, more hypertension and retinopathy, and, as expected, worse glomerular lesions than slow-track patients. Thus, there are strong relationships between glomerular structure and renal function across the spectrum of AER, but there is considerable structural overlap among AER categories. Given that normoalbuminuric patients may have advanced glomerulopathy, the selection of slow-track patients based on glomerular structure may better identify protected patients than AER alone. *Diabetes* 51:506–513, 2002

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Received for publication 9 August 2001 and accepted in revised form 26 October 2001.

AIIRB, angiotensin II type 1 receptor blocker; ACEI, angiotensin-converting enzyme inhibitor; AER, albumin excretion rate; DBP, diastolic blood pressure; DN, diabetic nephropathy; ECM, extracellular matrix; EM, electron microscopy; GBM, glomerular basement membrane; GFR, glomerular filtration rate; HPLC, high-performance liquid chromatography; MBP, mean blood pressure; MC, mesangial cell; Mes, mesangium; MES, mesangial expansion score; MM, mesangial matrix; PGBM, peripheral glomerular basement membrane; SBP, systolic blood pressure; SF, skin fibroblast; TBM, tubular basement membrane.

Only a subset of patients with type 1 diabetes develop clinical diabetic nephropathy (DN) (1,2). Although related to glycemia, DN risk is, in large part, genetically determined (1,3). Family studies have shown strong concordance for the risk of DN (3–6), as well as concordance for the severity and patterns of diabetic glomerular structural lesions among sibling pairs with type 1 diabetes (7), consistent with major gene effects in the pathogenesis of DN. Skin fibroblasts (SF), after multiple in vitro passages, demonstrate behaviors that correspond to DN risk. SF behaviors are also highly concordant in sibling pairs who have diabetes (8) and have been shown to be concordant for both DN risk and lesions (7).

The central abnormality in DN is renal extracellular matrix (ECM) accumulation, especially in the mesangium (Mes) (9). Increase in glomerular basement membrane (GBM) and tubular basement membrane (TBM) width and Mes matrix (MM) expansion, all representing ECM accumulation, are hallmarks of DN. Because renal lesions can develop for years despite normal kidney function (10–12), kidney biopsy is important in understanding early factors in DN risk. At later stages, when lesions are more advanced (13), forces that drive renal functional decline may be separate from factors that are responsible for earlier diabetic lesions (14–16).

This study aimed to detect cellular markers of DN risk in SF and thereby to formulate pathogenetic hypotheses for the mechanisms involved in ECM accumulation and to identify candidate DN genes (17). This study of highly characterized patients with type 1 diabetes, while also evaluating renal functional parameters, used renal structure factored for diabetes duration as the key determinant for the categorization of the DN risk. This strategy allows uniform classification despite variations in diabetes duration and elimination of patients with normal renal function but rapidly developing renal lesions from a group selected to be at low risk of developing DN.

The design of the cellular studies is based on the partitioning of 125 patients with long-standing type 1 diabetes into two groups: the upper quintile, with rapid development of lesions, and the lower quintile, with slow development of DN lesions. These groups were chosen to

maximize the ability to detect differences in cellular variables associated with DN risk with the aim that these cellular differences could add to the predictive value of currently available tests, such as the albumin excretion rate (AER). This article describes the study design and the characteristics of the total cohort from which the patients with rapid ("fast-track") and slow ("slow-track") development of DN lesions were selected. In addition, we describe the renal structural–functional relationships in this large cohort of patients with long-standing type 1 diabetes as well as the clinical and glomerular structural characteristics of fast-track and slow-track patients.

RESEARCH DESIGN AND METHODS

Patients. Patients with type 1 diabetes who had kidney biopsies performed between 1991 and 2000 either as part of their evaluation for pancreas transplantation or as members of a study of renal structure and function in sibling pairs with type 1 diabetes were eligible for this study. To be included, patients needed to have adequate tissue from kidney and skin biopsies. Additional eligibility criteria were diabetes duration of at least 8 years so that duration was adequate to access the rate of development of DN lesions, serum creatinine <2.0 mg/dl, and glomerular filtration rate (GFR) >30 ml · min^{-1} · 1.73 m^{-2} to avoid the study of end-stage DN. Patients with other kidney diseases were excluded. None of these patients were included in previous publications of renal structural–functional relationships in type 1 diabetes. In addition, electron microscopy (EM) morphometry was carried out by currently used methods (10) and not by those initially used in our laboratory (13). A total of 25 age- and sex-matched normal subjects who had skin biopsies performed were used as control subjects for the cellular studies. These studies were approved by the Committee for the Use of Human Subjects in Research of the University of Minnesota. Informed consent was obtained from all participants before each study. The patients were admitted in the General Clinical Research Center (GCRC) at the University of Minnesota, where renal function studies, percutaneous kidney biopsy, and skin biopsy were performed.

Kidney function studies. Blood pressure levels were assessed by trained observers using an oscillometric automatic monitor while the patients were in the GCRC. The mean value of multiple measurements was used to calculate systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean blood pressure (MBP). Hypertension was defined as blood pressure levels ≥130/85 mmHg (18) or use of antihypertensive medication. HbA_{1c} was measured by high-performance liquid chromatography (HPLC). Serum and urinary creatinine were measured by an automated method using the Jaffe reaction. AER was assessed from three 24-h sterile urine collections by a fluorometric assay (19). Patients were classified as normoalbuminuric (AER <20 μg/min), microalbuminuric (AER 20–200 μg/min), or proteinuric (>200 μg/min) depending on at least two of three AER measurements being in the same range. Patients who were known to be microalbuminuric or proteinuric before antihypertensive treatment was begun were classified according to their pretreatment AER values. The median AER value for each patient was used for the analyses. AER data were not available for one patient. GFR was estimated by iothalamate clearance using four timed urine and blood collections or by Iohexol plasma clearance. These two methods of GFR determination are highly correlated (20). The mean of three 24-h creatinine clearances carefully performed by the GCRC nursing staff was taken as a GFR estimate in patients studied several years ago. We previously demonstrated that GCRC creatinine clearances are highly correlated with classic inulin clearances (21).

Retinal studies. Retinopathy was assessed by indirect funduscopy and classified as absent, background, or proliferative. Retinopathy was not evaluated in two patients.

Renal structural studies. Percutaneous kidney biopsy was performed with ultrasound guidance under local anesthesia. Renal tissue was processed for light microscopy and EM.

Morphometric analyses.

Tissue processing. EM tissues were processed as detailed elsewhere (10). Briefly, kidney tissue was fixed in 2.5% glutaraldehyde in Millonig's buffer and embedded in Polybed 812. Ultrathin sections were examined with a JEOL 100CX electron microscope (Tokyo, Japan). A calibration grid was photographed with each glomerulus. Between 10 and 20 evenly spaced micrographs were obtained 11,000× for measurement of GBM width and for Mes composition. Micrographs at 3,900× were constructed into a montage of the entire glomerular profile for measurements of the fractional volume of glomeruli occupied by Mes [Vv(Mes/glom)] and the surface density of the peripheral GBM [Sv(PGBM/glom)].

EM measurements. Having at least two nonsclerotic glomeruli per biopsy in the EM blocks was an entry criterion for this study. Glomeruli were evaluated for *1*) GBM width by the orthogonal intercept method (22); *2*) Vv(Mes/glom) on the low-magnification montages by point counting (10); *3*) Mes components using a grid over the high-magnification micrographs, where points falling on MM and mesangial cell (MC) were noted, and percent of glomerulus occupied by MM and MC were calculated (9); and *4*) Sv(PGBM/glom), estimated on the low-magnification montages using intercept counting (10). The rate of development of DN was determined by the estimated rate of mesangial expansion, using mesangial expansion score (MES), defined as [measured Vv(Mes/glom) − mean normal Vv(Mes/glom)]/diabetes duration in years × 100. Reference values for glomerular structural parameters were derived from 76 age- and sex-matched normal living kidney transplant donors. These subjects (33 men) were 37.6 ± 12.1 years of age (19–64 years). The mean ± 2 SD of the measurements obtained in these patients were used to define the reference range for glomerular structure.

Cellular studies. Skin biopsy was performed with a 3-mm punch at the kidney biopsy site in diabetic patients and in a similar location in control subjects.

Patients were ranked, for the purpose of the cellular studies, according to their MES. Patients with diabetes and slow or rapid development of DN lesions were designated slow-track or fast-track, respectively. Fast-track patients are the highest quintile and slow-track patients the lowest quintile of estimated MES.

Statistical analyses. On the basis of preliminary studies, it was estimated that 25 patients per group (fast-track patients, slow-track patients, and control subjects) would provide 80% power (assuming a 0.05 two-sided probability of type I error) of detecting group differences in the SF mRNA expression levels of genes that could be related to DN.

Results are presented as means ± SD. AER, not normally distributed, is presented as median and range. Values for AER were logarithmically transformed before analysis. Patients were classified according to their AER category. ANOVA was used to compare continuous variables between control subjects and normoalbuminuric, microalbuminuric, and proteinuric patients. Fisher's least significant difference procedure was used to perform multiple comparisons between groups; tests were made at the 0.05 level of significance only when the overall F test was significant. Discrete variables were compared by χ^2. Pearson's correlation coefficient was used to evaluate the relationships between the variables studied. Multiple linear regression analyses were used to identify the predictors of AER, GFR, Vv(Mes/glom), and MES. Student's t tests and χ^2 tests were used to compare clinical and structural parameters (MES) between fast-track and slow-track patients. ANOVA and Fisher's least significant difference procedure were used to compare structural variables between control subjects and fast-track and slow-track patients. $P < 0.05$ was considered statistically significant.

RESULTS

Total cohort of patients with long-standing type 1 diabetes. The mean age of the 125 patients (53 men) was 37.6 ± 9.3 years, the mean age at diabetes onset was 15.1 ± 9.4 years, and the diabetes duration was 22.5 ± 10.0 years. HbA_{1c} at the time of biopsy was 8.5 ± 1.6%. Median AER was 8.9 μg/min (1.8–4,630 μg/min). Of the 125 patients, 88 were normoalbuminuric, 17 were microalbuminuric, 19 were proteinuric, and 1 could not be classified. GFR ranged from 33 to 166 ml · min^{-1} · 1.73 m^{-2}. Retinopathy was present in 83 of 123 patients (67%); 38 (31%) of 123 had proliferative changes. Hypertension was present in 56 of the 125 patients (45%); 31 were receiving antihypertensive drugs at the time of the studies, and 21 angiotensin-converting enzyme inhibitors (ACEI) or angiotensin II type 1 receptor blockers (AIIRB).

Demographic and clinical characteristics of patients with long-standing type 1 diabetes. Demographic and clinical characteristics of the 124 patients with type 1 diabetes classified according to their AER category are summarized in Table 1. Sex distribution and age were not different among normoalbuminuric, microalbuminuric, and proteinuric patients with type 1 diabetes. Normoalbu-

TABLE 1
Demographic and clinical characteristics of patients with long-standing type 1 diabetes

	AER categories			
	Normoalbuminuria	Microalbuminuria	Proteinuria	*P* value
Sex (M/F)	37/51	8/9	8/11	NS
Age (years)	38 ± 9	34 ± 8	39 ± 9	NS
Age at diabetes onset (years)	17 ± 10	11 ± 7	10 ± 5	0.001
Diabetes duration (years)	21 ± 10	23 ± 11	29 ± 7	0.005
HbA_{1c} (%)	8.1 ± 1.4	9.3 ± 2.1	9.6 ± 1.4	<0.0001
SBP (mmHg)	122 ± 11	124 ± 13	138 ± 14	<0.0001
DBP (mmHg)	70 ± 8	72 ± 7	78 ± 8	0.001
MBP (mmHg)	87 ± 8	89 ± 8	97 ± 9	<0.0001
Hypertension (yes/no)	27/61	11/6	18/1	<0.0001
Antihypertensive therapy (yes/no)	8/80	6/11	17/2	<0.001
RAS blocker (yes/no)	5/83	5/12	11/8	<0.0001
Serum creatinine (mg/dl)	0.87 ± 0.18	0.99 ± 0.26	1.31 ± 0.28	<0.0001
GFR ($ml \cdot min^{-1} \cdot 1.73\ m^{-2}$)	112 ± 23	102 ± 31	66 ± 22	<0.0001
AER* (μg/min)	6.2 (1.8–18.2)	30.9 (5.7–164.8)	839.0 (41.4–4,630.0)	<0.0001
Retinopathy (none/background/proliferative)	35/38/13	3/7/7	1/0/18	<0.0001

Data are *n*, mean ± SD, or median (range). RAS, renin-angiotensin system. *AER values reflect the median of three measurements at time of kidney biopsy.

minuric patients were older than microalbuminuric ($P = 0.008$) or proteinuric ($P = 0.001$) patients at diabetes onset. Normoalbuminuric patients had shorter diabetes duration ($P = 0.001$) than proteinuric patients, but duration was not significantly different between normoalbuminuric and microalbuminuric patients ($P = 0.391$) or between microalbuminuric and proteinuric patients ($P = 0.073$). HbA_{1c} was lower in normoalbuminuric than in microalbuminuric ($P = 0.003$) or proteinuric ($P = 0.0002$) patients, whereas microalbuminuric and proteinuric patients had similar HbA_{1c} values. Hypertension was more frequent in proteinuric than in normoalbuminuric ($P < 0.0001$) or microalbuminuric patients ($P < 0.037$) and also more frequent in microalbuminuric than in normoalbuminuric patients ($P < 0.012$). Proteinuric patients had higher SBP and DBP than normoalbuminuric ($P < 0.001$ for both comparisons) or microalbuminuric patients ($P = 0.002$ and $P = 0.027$, respectively). Renin-angiotensin system blockers (ACEI or AIIRB) were used in the same frequency by normoalbuminuric, microalbuminuric, or proteinuric hypertensive patients. As expected, serum creatinine was higher and GFR lower in proteinuric versus normoalbuminuric or microalbuminuric patients ($P < 0.001$ for all comparisons). Retinopathy in normoalbuminuric patients was less frequent than in proteinuric patients ($P = 0.003$) and tended to be less frequent than in microalbuminuric patients ($P = 0.10$). There was a significant increase in the prevalence of proliferative retinopathy in proteinuric compared with normoalbuminuric ($P < 0.0001$) or microalbuminuric ($P < 0.001$) patients.

Glomerular structure of patients with long-standing type 1 diabetes. Two to six glomeruli (3.2 ± 0.7) were evaluated per patient. There was a wide range of glomerular lesions among these patients with long-standing type 1 diabetes, varying from measurements in the normal range to advanced disease (Table 2). GBM width was increased in 74.4% of diabetic patients ($P < 0.0001$ versus control subjects). Vv(Mes/glom) varied from normal (0.14–0.26) to nearly three times normal and was also greater than in control subjects ($P < 0.0001$). All mesangial components, MM ($P < 0.0001$), MC ($P < 0.0001$), and MM/(MM+MC) ($P < 0.001$), were increased in the glomeruli of these patients with diabetes compared with control subjects, and Sv(PGBM/glom) was decreased when compared with control subjects ($P < 0.0001$). Table 2 shows the glomerular structural values according to AER categories. GBM width (Fig. 1), Vv(Mes/glom) (Fig. 2), Vv(MM/glom), and Vv[MM/(MM+MC)] were increased and Sv(PGBM/glom) was decreased in each of the AER categories of patients with diabetes compared with control subjects (Table 2). Vv(MC/glom) was greater in proteinuric ($P < 0.0001$) and microalbuminuric patients ($P = 0.009$) versus control subjects, whereas the comparison of control with normoalbuminuric patients ($P = 0.13$) did not

TABLE 2
Glomerular structural characteristics of nondiabetic control subjects and patients with long-standing type 1 diabetes

	Control subjects	AER categories			*P*
		Normoalbuminuria	Microalbuminuria	Proteinuria	
GBM width (nm)	332 ± 46	465 ± 100	602 ± 157	700 ± 141	<0.0001
Vv(Mes/glom)	0.20 ± 0.03	0.28 ± 0.07	0.34 ± 0.09	0.50 ± 0.12	<0.0001
Vv(MM/glom)	0.09 ± 0.02	0.16 ± 0.05	0.19 ± 0.08	0.31 ± 0.09	<0.0001
Vv(MC/glom)	0.08 ± 0.02	0.09 ± 0.02	0.10 ± 0.03	0.14 ± 0.06	<0.0001
Vv[MM/(MM+MC)]	0.51 ± 0.08	0.63 ± 0.07	0.64 ± 0.11	0.70 ± 0.10	<0.0001
Sv(PGBM/glom) ($\mu m^2/\mu m^3$)	0.13 ± 0.02	0.11 ± 0.02	0.10 ± 0.03	0.06 ± 0.03	<0.0001
MES	NA	0.39 ± 0.32	0.66 ± 0.48	1.12 ± 0.61	<0.0001

Data are means ± SD. NA, not applicable in nondiabetic control subjects because MES factors for diabetes duration.

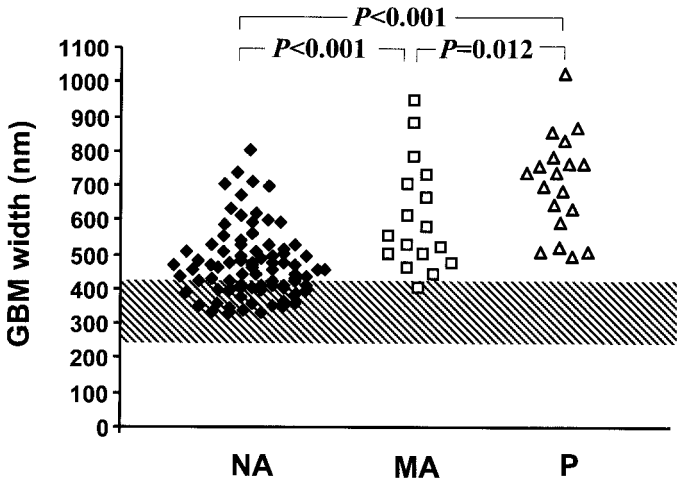

FIG. 1. GBM width in 88 normoalbuminuric (NA), 17 microalbuminuric (MA), and 19 proteinuric (P) patients with type 1 diabetes. The hatched area represents the mean ± 2 SD in a group of 76 age-matched normal control subjects. All groups are different from control subjects.

reach statistical significance. GBM width increased progressively with increasing AER class from normoalbuminuria to microalbuminuria ($P < 0.0001$) or proteinuria ($P < 0.0001$) and from microalbuminuria to proteinuria ($P = 0.002$). Vv(Mes/glom) also increased progressively from normoalbuminuric to microalbuminuric ($P = 0.003$) or to proteinuric ($P < 0.001$) patients and was also greater in proteinuric versus microalbuminuric patients ($P < 0.0001$). The mesangial components, MM and MC, were also increased in proteinuric versus normoalbuminuric ($P < 0.0001$ for all comparisons) and microalbuminuric ($P < 0.0001$ and $P = 0.0005$, respectively) patients. MM was greater in microalbuminuric versus normoalbuminuric ($P = 0.007$) patients. Vv[MM/(MM+MC)] was increased in proteinuric compared with normoalbuminuric patients ($P = 0.002$). Sv(PGBM/glom) decreased with increasing in AER levels ($P < 0.0001$) but was not different between normoalbuminuric and microalbuminuric patients. MES increased progressively from normoalbuminuria to microalbuminuria ($P = 0.01$) or proteinuria ($P <$

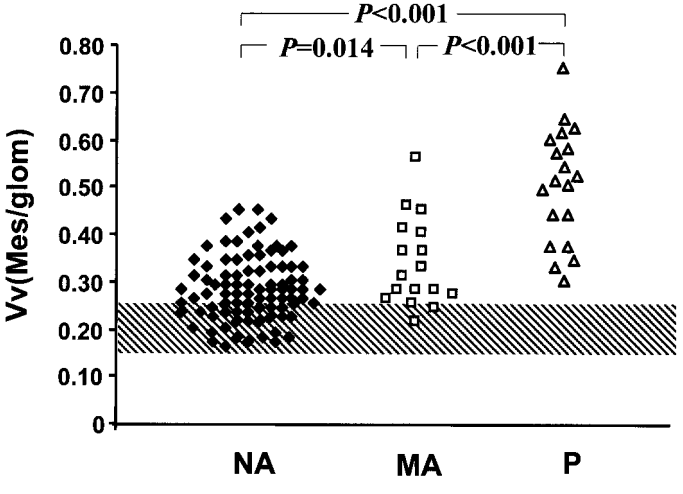

FIG. 2. Vv(Mes/glom) in 88 normoalbuminuric (NA), 17 microalbuminuric (MA), and 19 proteinuric (P) patients with type 1 diabetes. The hatched area represents the mean ± 2 SD in a group of 76 age-matched normal control subjects. All groups are different from control subjects.

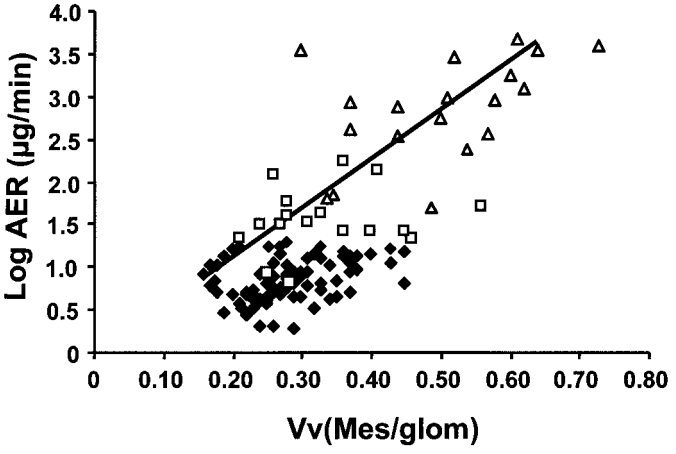

FIG. 3. Correlation between Vv(Mes/glom) and AER in 124 patients with type 1 diabetes. ♦, Normoalbuminuric patients; ■, microalbuminuric patients; △, proteinuric patients. $r = 0.75$, $P < 0.001$.

0.0001) and from microalbuminuria to proteinuria ($P = 0.001$). Despite highly statistically significant differences, there was substantial overlap in glomerular structure among the normoalbuminuric, microalbuminuric, and proteinuric groups (Figs. 1 and 2).

Structural–functional relationships of patients with long-standing type 1 diabetes. AER was inversely correlated with age at diabetes onset ($r = -0.32$, $P < 0.001$) and GFR ($r = -0.47$, $P < 0.001$) and directly correlated with diabetes duration ($r = 0.30$, $P < 0.001$), MBP ($r = 0.48$, $P < 0.001$), HbA_{1c} ($r = 0.35$, $P < 0.001$), and serum creatinine ($r = 0.56$, $P < 0.001$). Conversely, GFR was inversely correlated with diabetes duration ($r = -0.29$, $P = 0.001$), MBP ($r = -0.21$, $P = 0.019$), and serum creatinine ($r = -0.60$, $P < 0.001$). Clinical variables were also related to glomerular structure. Age was inversely related to GBM width ($r = -0.27$, $P = 0.003$), whereas age at diabetes onset and diabetes duration were related to all structural variables. Age at onset was directly related to Sv(PGBM/glom) and inversely related to the other structural variables, and diabetes duration was inversely related to Sv(PGBM/glom) and directly related to the other structural variables. SBP, DBP, and MBP were related to all glomerular structural parameters. MBP correlated with Vv(Mes/glom) ($r = 0.32$, $P < 0.001$), Vv(MM/glom) ($r = 0.31$, $P < 0.001$), GBM width ($r = 0.23$, $P = 0.009$), and MES ($r = 0.23$, $P = 0.009$). When the 21 patients who were receiving ACEI or AIIRB were excluded from these analyses, SBP but not DBP remained significantly correlated with Vv(Mes/glom) ($r = 0.26$, $P = 0.007$) and Vv(MM/glom) ($r = 0.24$, $P = 0.013$). Vv(Mes/glom) ($r = 0.75$, $P < 0.001$; Fig. 3) and GBM width ($r = 0.63$, $P < 0.001$; Fig. 4) correlated with AER across the entire range, from normoalbuminuria to proteinuria. Sv(PGBM/glom) had a negative correlation with AER ($r = -0.62$, $P < 0.001$). The glomerular fractional volume of the mesangium components (MM [$r = 0.71$, $P < 0.001$] and MC [$r = 0.50$, $P < 0.001$]) and MES ($r = 0.63$, $P < 0.001$) were also correlated with AER. All parameters of glomerular structure were related to GFR. The strongest relationships between GFR and glomerular structure were with Vv(Mes/glom) ($r = -0.48$, $P < 0.001$), Vv(MM/glom) ($r = -0.53$, $P < 0.001$), and Sv(PGBM/glom) ($r = 0.50$, $P < 0.001$; Fig. 5). The

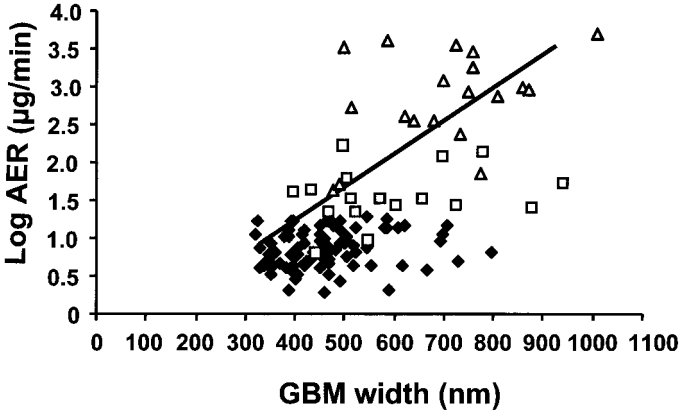

FIG. 4. Correlation between GBM width and AER in 124 patients with type 1 diabetes. ◇, Normoalbuminuric patients; ■, microalbuminuric patients; △, proteinuric patients. $r = 0.63$, $P < 0.001$.

relationships between glomerular structure and AER [GBM width ($r = 0.53$, $P < 0.001$), Vv(Mes/glom) ($r = 0.61$, $P < 0.001$), Vv(MM/glom) ($r = 0.57$, $P < 0.001$), Vv(MC/glom) ($r = 0.49$, $P < 0.001$), and Sv(PGBM/glom) ($r = -0.42$, $P < 0.001$)] or GFR [GBM width ($r = -0.35$, $P < 0.001$), Vv(Mes/glom) ($r = -0.33$, $P = 0.001$), Vv(MC/glom) ($r = -0.19$, $P = 0.049$), Vv(MM/glom) ($r = -0.38$, $P < 0.001$), and Sv(PGBM/glom) ($r = 0.36$, $P < 0.001$)] were similar when patients who were receiving ACEI or AIIRB were excluded from the analyses. The strong relationships between AER and glomerular structural parameters were present only when patients from all AER categories were included. When only patients within a given AER category were considered, these relationships were weaker in the normoalbuminuric [Vv(Mes/glom) ($r = 0.34$, $P = 0.001$), Vv(MM/glom) ($r = 0.29$, $P = 0.007$), Vv(MC/glom) ($r = 0.29$, $P = 0.008$), and Sv(PGBM/glom) ($r = -0.25$, $P = 0.021$)] and proteinuric [Vv(Mes/glom) ($r = 0.50$, $P = 0.03$), Vv(MM/glom) ($r = 0.48$, $P = 0.039$), and Sv(PGBM/glom) ($r = -0.52$, $P = 0.023$)] groups and absent in the microalbuminuric group.

In multiple linear regression analysis, Vv(Mes/glom) and GBM width explained 59% of the variability in AER ($P < 0.001$). When clinical parameters were added to the model, 65% of AER variability ($P < 0.001$) was explained by Vv(Mes/glom), GBM width, and MBP, and this was independent of renin-angiotensin system blockade. The other variables studied, HbA_{1c} and sex, were not significant and were excluded from the model. The same results were obtained when hypertension instead of MBP was used as an independent variable ($r^2 = 0.64$, $P < 0.001$). Also, 33% of the variability in GFR was predicted by Sv(PGBM/glom), AER, and sex ($P < 0.0001$), whereas Vv(Mes/glom), GBM width, diabetes duration, HbA_{1c}, and MBP were not independent GFR predictors. The same results were obtained when age instead of diabetes duration was included as an independent variable.

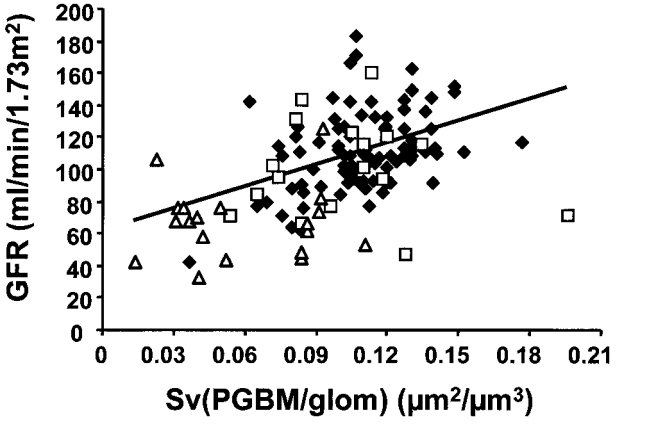

FIG. 5. Correlation between Sv(PGBM/glom) and GFR in 125 patients with type 1 diabetes. ◇, Normoalbuminuric patients; ■, microalbuminuric patients; △, proteinuric patients. $r = 0.48$, $P < 0.001$.

Clinical variables also predicted glomerular structure. Thus, AER and GFR predicted Vv(Mes/glom) ($r^2 = 0.58$, $P < 0.001$), whereas AER, age, and retinopathy (present/absent) predicted MES ($r^2 = 0.45$, $P < 0.001$).

Demographic, clinical, and glomerular structural characteristics of fast-track and slow-track patients with type 1 diabetes. Fast-track patients were not different from slow-track patients regarding sex, age, age at diabetes onset, and diabetes duration (Table 3). Fast-track patients had higher HbA_{1c}, DBP, MBP, serum creatinine, and AER and lower GFR than slow-track patients. All but two slow-track patients were normoalbuminuric, whereas 16 of the 25 fast-track patients were microalbuminuric (5 patients) or proteinuric (11 patients). Hypertension was present in 24% of the slow-track and in 76% of the fast-track patients. Proliferative retinopathy was present in only 2 of 25 slow-track versus 13 of 25 fast-track patients ($P < 0.001$; Table 3). All glomerular structural parameters in Table 4 were different from normal in the fast-track patients (all $P < 0.0001$). In contrast, whereas GBM width ($P < 0.0001$), Vv(MM/glom) ($P = 0.019$), and Vv[MM/(MM+MC)] ($P < 0.0001$) were increased in slow-track patients compared with control subjects, Vv(Mes/glom) (Fig. 6) and Sv(PGBM/glom) were not different from normal. As expected, fast-track patients had much more advanced glomerular lesions than slow-track patients (Table 4; Fig. 6). MES was, by definition, greater in fast-track than in slow-track patients.

DISCUSSION

This is the largest single-center study using uniform tissue processing and measurement methods to evaluate structural–functional relationships in patients with long-standing type 1 diabetes and wide ranges of renal structure and function. Grouped according to their AER class, ages were similar but proteinuric and microalbuminuric patients were younger at diabetes onset than normoalbuminuric patients, whereas duration was longer in the proteinuric group. These results could be interpreted as consonant with published evidence against the hypothesis that the prepubertal years of diabetes are protected from nephropathy (23). However, the rate of development of lesions may not be linear over time, and longitudinal biopsy studies of children and adults will be necessary to answer this question.

This study confirmed the relationships of glycemia to the risk of diabetic complications (24); at the same time, the results suggest that other variables are of equal or greater importance in influencing nephropathy risk. Although blood pressure values were similar in normoalbuminuric and microalbuminuric patients, the prevalence of

TABLE 3
Demographic and clinical characteristics of fast-track and slow-track patients with type 1 diabetes

	Fast-track	Slow-track	*P*
Sex (M/F)	10/15	13/12	NS
Age (years)	35 ± 7	38 ± 10	NS
Age at diabetes onset (years)	14 ± 7	18 ± 11	NS
Diabetes duration (years)	22 ± 8	20 ± 11	NS
HbA_{1c} (%)	9.2 ± 1.7	7.8 ± 1.2	0.002
SBP (mmHg)	128 ± 15	122 ± 10	NS
DBP (mmHg)	76 ± 8	71 ± 7	0.033
MBP (mmHg)	93 ± 10	88 ± 7	0.043
Hypertension (yes/no)	19/6	6/19	<0.001
Antihypertensive therapy (yes/no)	15/10	2/23	<0.001
RAS blocker (yes/no)	9/16	2/23	0.017
Serum creatinine (mg/dl)	1.01 ± 0.31	0.90 ± 0.10	0.006
GFR (ml · min^{-1} · 1.73 m^{-2})	90.5 ± 31.9	110.6 ± 14.2	0.007
AER* (μg/min)	51.1 (4.9–4,630)	5.7 (2.6–54.6)	0.006
Retinopathy (none/background/proliferative)	4/8/13	12/11/2	0.037

Data are *n*, means ± SD, or median (range). RAS, renin-angiotensin system. *AER values reflect the median of three measurements at time of kidney biopsy.

hypertension and antihypertensive treatment was greater in the microalbuminuric patients, potentially masking blood pressure differences. Blood pressures were highest, as expected (13), in proteinuric patients, despite that almost all patients were receiving antihypertensive therapy. Correlations, albeit weak, were seen between blood pressure and glomerular structure in the entire cohort, confirming earlier studies (25). Several studies have explored the relationships between blood pressure and the development of DN. The risk of developing microalbuminuria (26) and proteinuria (27) seems to be increased in patients with higher levels of blood pressure. Also, the use of antihypertensive drugs, particularly ACEIs, may slow the progression of renal disease in patients with type 1 diabetes (28,29), and similar results were obtained with AIIRB in type 2 diabetes (30–32).

Some debate still remains as to whether diabetic glomerular lesions are present in normoalbuminuric patients with type 1 diabetes (33,34). This study, evaluating a new subset of patients, confirmed that normoalbuminuric patients can have advanced glomerular lesions as well as other clinical findings of renal disease, including low GFR and hypertension (10,35). This could explain why some normoalbuminuric patients with long-standing type 1 diabetes progress to proteinuria (36–38). In fact, initial results of a 5- to 17-year follow-up study of normoalbuminuric patients with long-standing diabetes showed that those who progressed to microalbuminuria or proteinuria had worse glomerular lesions at baseline than those who remained normoalbuminuric (39). Also, in longitudinal studies of microalbuminuric patients with type 1 diabetes, Bangstad et al. (40) found that GBM width at baseline biopsy was predictive ($r^2 = 0.67$, $P < 0.0001$) of AER after 6 years of follow-up, whereas Vv(Mes/glom) was a significant but less precise predictor. Although these longitudinal renal structural studies suggest that biopsies could add to the predictive value of AER for diabetic nephropathy risk, much more work would need to be done before this could be clinically applicable. Moreover, given the impracticability of broad clinical adoption of renal biopsy and EM morphometry to large patient populations, the studies described here seek to identify cellular markers of risk that could improve predictive precision.

As described here and elsewhere (10), there was a wide range of glomerular lesions in microalbuminuric patients, and, despite having more advanced lesions than normoalbuminuric patients, there was considerable overlap between these two groups for all glomerular parameters studied. Considering these findings, it is not surprising that

TABLE 4
Glomerular structural characteristics of fast-track and slow-track patients with type 1 diabetes

	Fast-track	Slow-track	*P* value
GBM width (nm)	685 ± 168	440 ± 77	<0.0001
Vv(Mes/glom)	0.47 ± 0.12	0.21 ± 0.03	<0.0001
Vv(MM/glom)	0.28 ± 0.09	0.11 ± 0.02	<0.0001
Vv(MC/glom)	0.13 ± 0.05	0.07 ± 0.02	<0.0001
Vv[MM/(MM+MC)]	0.68 ± 0.08	0.60 ± 0.07	0.0005
Sv(PGBM/glom) ($\mu m^2/\mu m^3$)	0.07 ± 0.03	0.12 ± 0.02	<0.0001
MES	1.27 ± 0.44	0.02 ± 0.17	NA

Data are means ± SD. NA, not applicable, different by definition.

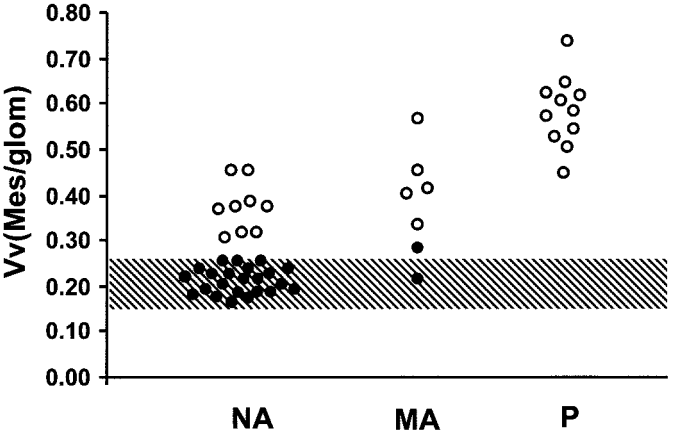

FIG. 6. Vv(Mes/glom) in fast-track (○) and slow-track (●) patients with type 1 diabetes. The hatched area represents the means ± 2 SD in a group of 76 age-matched normal control subjects.

only ~30–45% of microalbuminuric patients with long-standing type 1 diabetes will progress to proteinuria during 6–10 years of follow-up (36–38,41), whereas ~20% of long-standing normoalbuminuric patients will progress to microalbuminuria or proteinuria during this follow-up interval (36–38,42,43). Conversely, microalbuminuric patients more frequently had hypertension and proliferative retinopathy compared with normoalbuminuric patients, and, for many patients, microalbuminuria is a late indicator of DN risk. Glomerular lesions, as expected, were even more advanced in proteinuric patients and decreased GFR, hypertension, and retinopathy were more frequently observed in these patients, paralleling the high risk of progression to end-stage renal disease and blindness in these patients.

There were strong correlations between structure and AER across the entire range from normoalbuminuria to proteinuria, and a large part of the AER variability was explained by two glomerular structural variables, Vv(Mes/glom) and GBM width. The strength of these relationships, occurring despite the known day-to-day variability in AER, suggests that functional manifestations of DN may appear earlier than heretofore appreciated and that progressive changes in AER, even within the normoalbuminuric range, could be significant (10,11,38,39). Higher values of AER, still in the normoalbuminuric range, have been associated with increased risk of progression to microalbuminuria and proteinuria (42,44–46). We also observed that there was an increase in AER levels with worsening of glomerular structure within the normoalbuminuric range, albeit that the correlations of AER in this range with glomerular mesangial structure were weaker. However, some variability in AER remained unexplained by our studies and other structural variables, such as percentage of glomerular sclerosis, arteriolar hyalinosis and interstitial fibrosis, epithelial cell structure, and glomerular capillary wall biochemistry, need to be studied in these patients. GFR also correlated with structural variables, although only ~35% of GFR variability was explained by structural and clinical parameters. Finally, clinical variables were able to predict approximately half of Vv(Mes/glom) or MES variability.

Fast-track and slow-track patients were classified on the basis of their rate of mesangial expansion (MES). Mesangial fractional volume, as confirmed in the present study (13), is the glomerular structural parameter most closely related to the functional manifestations of DN. This study confirms that Vv(Mes/glom) is not only highly correlated with AER but also the strongest independent predictor of AER. Moreover, sequential biopsy studies (11) have shown that changes in AER over 5 years are associated with changes in Vv(Mes/glom) but not with other parameters.

Although the intent of the selection of patients in the upper and lower quintiles of MES distribution was to construct groups with different DN risk, some slow-track patients were microalbuminuric and some fast-track patients were normoalbuminuric. This is not surprising, because there was a wide range of diabetes duration among patients (8–60 years), and some normoalbuminuric patients have advanced lesions whereas some microalbuminuric patients have mild lesions. As discussed here and elsewhere (38), normoalbuminuria is not a precise predictor of safety from DN and microalbuminuria is not a precise predictor of DN risk in patients with long-standing type 1 diabetes. Because fast-track and slow-track patients were similar in diabetes duration, classification by MES did result in the creation of two groups that did not overlap in Vv(Mes/glom). As expected, fast-track patients had worse glycemic control and more frequently had hypertension and proliferative retinopathy than slow-track patients. Despite no increase in Vv(Mes/glom) in slow-track patients compared with control subjects, this group had an increase in the proportion of the glomerulus [Vv(MM/glom)] and of the mesangium made up by MM. Moreover, ~30% increase in GBM width was observed in slow-track patients when compared with control values. These results suggest that certain structural glomerular changes occur at a very slow rate in all patients with type 1 diabetes, and these may have little prognostic significance. However, more severe GBM thickening and mesangial expansion associated with reduced filtration surface are strongly associated with the clinical manifestations of DN and with risk for end-stage renal disease.

In conclusion, this article describes clinical, renal functional, and renal structural characteristics of 125 patients with long-standing type 1 diabetes who represent the cohort from which 25 fast-track and 25 slow-track patients were selected, on the basis of rate of mesangial expansion, for studies of cellular markers of DN risk (17). The overlap in renal structural and functional variables between the groups classified as normoalbuminuric, microalbuminuric, and proteinuric is consistent with recent observations indicating that AER alone is a strong but imprecise indicator of DN risk. The use of rate of mesangial expansion (MES) for classification into fast-track and slow-track groups has the advantage of allowing the inclusion of patients with varying diabetes duration in the cellular studies. This strategy also helps to identify, for the purpose of the cellular studies, normoalbuminuric patients who may be at risk of DN by virtue of having advanced DN lesions.

ACKNOWLEDGMENTS

This work was supported by grants from National Institutes of Health (DK-13083 and DK-54638) and National Center for Research Resources (M01-RR00400).

M.L.C. was initially supported by the Foundation for the Coordination of Higher Education and Graduate Training (CAPES) from the Brazilian Government and is currently supported by a research fellowship grant from the Juvenile Diabetes Foundation International. C.H. is supported by a mentor-based fellowship grant from the American Diabetes Association.

We thank John Basgen and Thomas Groppoli for performing the morphometric studies, Michele Watrin for patient recruitment, and Susan Kupcho-Sandberg for AER measurements.

REFERENCES

1. Krolewski AS, Warram JH, Rand LI, Kahn CR: Epidemiologic approach to the etiology of type I diabetes mellitus and its complications. *N Engl J Med* 317:1390–1398, 1987
2. Andersen AR, Christiansen JS, Andersen JK, Kreiner S, Deckert T: Diabetic nephropathy in type 1 (insulin-dependent) diabetes: an epidemiological study. *Diabetologia* 25:496–501, 1983
3. Borch-Johnsen K, Norgaard K, Hommel E, Mathiesen ER, Jensen JS,

Deckert T, Parving HH: Is diabetic nephropathy an inherited complication? *Kidney Int* 41:719–722, 1992
4. Quinn M, Angelico MC, Warram JH, Krolewski AS: Familial factors determine the development of diabetic nephropathy in patients with IDDM. *Diabetologia* 39:940–945, 1996
5. Seaquist ER, Goetz FC, Rich S, Barbosa J: Familial clustering of diabetic kidney disease: evidence for genetic susceptibility to diabetic nephropathy. *N Engl J Med* 320:1161–1165, 1989
6. Pettitt DJ, Saad MF, Bennett PH, Nelson RG, Knowler WC: Familial predisposition to renal disease in two generations of Pima Indians with type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia* 33:438–443, 1990
7. Fioretto P, Steffes MW, Barbosa J, Rich SS, Miller ME, Mauer M: Is diabetic nephropathy inherited? Studies of glomerular structure in type 1 diabetic sibling pairs. *Diabetes* 48:865–869, 1999
8. Trevisan R, Fioretto P, Barbosa J, Mauer M: Insulin-dependent diabetic sibling pairs are concordant for sodium-hydrogen antiport activity. *Kidney Int* 55:2383–2389, 1999
9. Steffes MW, Bilous RW, Sutherland DE, Mauer SM: Cell and matrix components of the glomerular mesangium in type I diabetes. *Diabetes* 41:679–684, 1992
10. Fioretto P, Steffes MW, Mauer M: Glomerular structure in nonproteinuric IDDM patients with various levels of albuminuria. *Diabetes* 43:1358–1364, 1994
11. Fioretto P, Steffes MW, Sutherland DE, Mauer M: Sequential renal biopsies in insulin-dependent diabetic patients: structural factors associated with clinical progression. *Kidney Int* 48:1929–1935, 1995
12. Berg UB, Torbjornsdotter TB, Jaremko G, Thalme B: Kidney morphological changes in relation to long-term renal function and metabolic control in adolescents with IDDM. *Diabetologia* 41:1047–1056, 1998
13. Mauer SM, Steffes MW, Ellis EN, Sutherland DE, Brown DM, Goetz FC: Structural-functional relationships in diabetic nephropathy. *J Clin Invest* 74:1143–1155, 1984
14. Jacobsen P, Rossing K, Rossing P, Tarnow L, Mallet C, Poirier O, Cambien F, Parving HH: Angiotensin converting enzyme gene polymorphism and ACE inhibition in diabetic nephropathy. *Kidney Int* 53:1002–1006, 1998
15. Parving HH, Jacobsen P, Tarnow L, Rossing P, Lecerf L, Poirier O, Cambien F: Effect of deletion polymorphism of angiotensin converting enzyme gene on progression of diabetic nephropathy during inhibition of angiotensin converting enzyme: observational follow up study. *Br Med J* 313:591–594, 1996
16. Brenner BM: Hemodynamically mediated glomerular injury and the progressive nature of kidney disease. *Kidney Int* 23:647–655, 1983
17. Huang C, Kim Y, Rich S, Miller ME, Caramori ML, Fish AJ, Mauer M: Alterations of Na^+/H^+ exchanger (NHE-1) mRNA expression in type 1 diabetic patients with rapid vs. slow development of diabetic nephropathy lesions (Abstract). *J Am Soc Nephrol* 11:116A, 2000
18. The sixth report of the Joint National Committee on prevention, detection, evaluation, and treatment of high blood pressure. *Arch Intern Med* 157:2413–2446, 1997
19. Chavers BM, Simonson J, Michael AF: A solid phase fluorescent immunoassay for the measurement of human urinary albumin. *Kidney Int* 25:576–578, 1984
20. Rocco MV, Buckalew VM Jr, Moore LC, Shihabi ZK: Measurement of glomerular filtration rate using nonradioactive Iohexol: comparison of two one-compartment models. *Am J Nephrol* 16:138–143, 1996
21. Ellis EN, Steffes MW, Goetz FC, Sutherland DE, Mauer SM: Glomerular filtration surface in type I diabetes mellitus. *Kidney Int* 29:889–894, 1986
22. Jensen EB, Gundersen HJ, Østerby R: Determination of membrane thickness distribution from orthogonal intercepts. *J Microsc* 115:19–33, 1979
23. Ellis EN, Warady BA, Wood EG, Hassanein R, Richardson WP, Lane PH, Howard C, Kemp SF, Aceto T, Garibaldi L, Wiegmann TB, Savin VJ: Renal structural-functional relationships in early diabetes mellitus. *Pediatr Nephrol* 11:584–591, 1997
24. The Diabetes Control and Complications (DCCT) Research Group: Effect of intensive therapy on the development and progression of diabetic nephropathy in the Diabetes Control and Complications Trial. *Kidney Int* 47:1703–1720, 1995
25. Mauer SM, Sutherland DE, Steffes MW: Relationship of systemic blood pressure to nephropathology in insulin-dependent diabetes mellitus. *Kidney Int* 41:736–740, 1992
26. Poulsen PL, Hansen KW, Mogensen CE: Ambulatory blood pressure in the transition from normo- to microalbuminuria: a longitudinal study in IDDM patients. *Diabetes* 43:1248–1253, 1994
27. Orchard TJ, Forrest KY, Kuller LH, Becker DJ: Lipid and blood pressure treatment goals for type 1 diabetes: 10-year incidence data from the Pittsburgh Epidemiology of Diabetes Complications Study. *Diabetes Care* 24:1053–1059, 2001
28. 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
29. Lewis EJ, Hunsicker LG, Bain RP, Rohde RD: The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy. The Collaborative Study Group. *N Engl J Med* 329:1456–1462, 1993
30. Brenner BM, Cooper ME, de Zeeuw D, Keane WF, Mitch WE, Parving HH, Remuzzi G, Snapinn SM, Zhang Z, Shahinfar S: Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. *N Engl J Med* 345:861–869, 2001
31. Lewis EJ, Hunsicker LG, Clarke WR, Berl T, Pohl MA, Lewis JB, Ritz E, Atkins RC, Rohde R, Raz I: Renoprotective effect of the angiotensin-receptor antagonist irbesartan in patients with nephropathy due to type 2 diabetes. *N Engl J Med* 345:851–860, 2001
32. Parving HH, Lehnert H, Brochner-Mortensen J, Gomis R, Andersen S, Arner P: The effect of irbesartan on the development of diabetic nephropathy in patients with type 2 diabetes. *N Engl J Med* 345:870–878, 2001
33. Mogensen CE: Microalbuminuria, blood pressure and diabetic renal disease: origin and development of ideas. *Diabetologia* 42:263–285, 1999
34. Østerby R: Glomerular structural changes in type 1 (insulin-dependent) diabetes mellitus: causes, consequences, and prevention. *Diabetologia* 35:803–812, 1992
35. Lane PH, Steffes MW, Mauer SM: Glomerular structure in IDDM women with low glomerular filtration rate and normal urinary albumin excretion. *Diabetes* 41:581–586, 1992
36. Forsblom CM, Groop PH, Ekstrand A, Groop LC: Predictive value of microalbuminuria in patients with insulin-dependent diabetes of long duration. *Br Med J* 305:1051–1053, 1992
37. Rudberg S, Persson B, Dahlquist G: Increased glomerular filtration rate as a predictor of diabetic nephropathy—an 8-year prospective study. *Kidney Int* 41:822–828, 1992
38. Caramori ML, Fioretto P, Mauer M: The need for early predictors of diabetic nephropathy risk: is albumin excretion rate sufficient? *Diabetes* 49:1399–1408, 2000
39. Caramori ML, Fioretto P, Mauer M: Long-term follow-up of normoalbuminuric longstanding type 1 diabetic patients: progression is associated with worse baseline glomerular lesions and lower glomerular filtration rate (Abstract). *J Am Soc Nephrol* 10:126A, 1999
40. Bangstad HJ, Østerby R, Hartmann A, Berg TJ, Hanssen KF: Severity of glomerulopathy predicts long-term urinary albumin excretion rate in patients with type 1 diabetes and microalbuminuria. *Diabetes Care* 22: 314–319, 1999
41. Rossing P, Hougaard P, Borch-Johnsen K, Parving H-H: Progression of microalbuminuria in type 1 diabetes: 10 years observation and follow-up (Abstract). *Diabetologia* 43 (Suppl. 1):A254, 2000
42. Mathiesen ER, Ronn B, Storm B, Foght H, Deckert T: The natural course of microalbuminuria in insulin-dependent diabetes: a 10-year prospective study. *Diabetes Med* 12:482–487, 1995
43. Chiarelli F, Catino M, Tumini S, de Martino M, Mezzetti A, Verrotti A, Vanelli M: Increased Na+/Li+ countertransport activity may help to identify type 1 diabetic adolescents and young adults at risk for developing persistent microalbuminuria. *Diabetes Care* 22:1158–1164, 1999
44. Microalbuminuria Collaborative Study Group, United Kingdom: Risk factors for development of microalbuminuria in insulin dependent diabetic patients: a cohort study. *Br Med J* 306:1235–1239, 1993
45. Mogensen CE, Poulsen PL: Epidemiology of microalbuminuria in diabetes and in the background population. *Curr Opin Nephrol Hypertens* 3:248–256, 1994
46. Royal College of Physicians of Edinburgh Diabetes Register Group: Near-normal urinary albumin concentrations predict progression to diabetic nephropathy in type 1 diabetes mellitus. *Diabetes Med* 17:782–791, 2000