Type 2 Diabetic Patients with Nephropathy Show Structural–Functional Relationships that Are Similar to Type 1 Disease

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Abstract. Glomerular structural–functional relationships were investigated in 21 type 2 diabetic patients with proteinuria. Structural parameters were quantified using both light and electron microscopy and standard stereologic techniques. Data were also available on 14 nondiabetic subjects. Mesangial and matrix volume fractions and glomerular basement membrane (GBM) width were increased in type 2 patients when compared with nondiabetic subjects (mean ± SD: 0.45 ± 0.13 *versus* 0.18 ± 0.03, $P < 0.001$; 0.28 ± 0.09 *versus* 0.10 ± 0.02, $P < 0.001$; and 665 ± 138 *versus* 361 ± 51 nm, $P < 0.001$, respectively). An increase in mesangial volume fraction was associated with high levels of proteinuria and low creatinine clearance ($r = 0.64$, $P = 0.002$; $r = -0.58$, $P = 0.006$, respectively). GBM width and mesangial foot process width (FPW_{mes}) also correlated with proteinuria ($r = 0.58$, $P = 0.006$; $r = 0.60$, $P = 0.004$, respectively). Volume fraction of interstitium correlated with creatinine clearance ($r = -0.58$, $P = 0.006$). Patients had previously been defined by light microscopy as having either diffuse or nodular glomerulosclerosis; those with nodules had larger mesangial and matrix volume fractions and more proteinuria than those classified as diffuse (mean ± SD: 0.51 ± 0.12 *versus* 0.36 ± 0.08, $P = 0.007$; 0.32 ± 0.08 *versus* 0.21 ± 0.05, $P = 0.003$; median, range: 4.3, 1.1 to 9.6 *versus* 1.1, 0.9 to 12.7 g/24 h, $P = 0.027$). Creatinine clearance did not differ significantly between the groups. Type 2 diabetic patients with proteinuria have established glomerulopathy, which is more advanced in those with nodular glomerulosclerosis. Creatinine clearance correlated with both mesangial and interstitial expansion, whereas proteinuria correlated only with glomerular pathology. These results suggest that type 2 patients with advanced nephropathy have structural–functional relationships similar to type 1, consistent with a common pathogenesis, and strongly support an important role of the tubulointerstitium in the role of renal impairment.

It is well recognized that declining renal function with increasing albumin excretion is characteristic of nephropathy in both type 1 and type 2 diabetes. However, most studies analyzing glomerular structure have concentrated on the structural–functional relationships in people with type 1 diabetes (1–3), even though the lesions characteristic of diabetic glomerulopathy are known to occur in both type 1 and type 2 patients. It has been suggested that there is more heterogeneity in type 2 glomerulopathy with up to 63% of proteinuric patients showing nondiabetic lesions (4); however, others have suggested that this proportion is much less in unselected biopsy series (5).

A pilot study of 47 patients was designed to test the safety of irbesartan (an angiotensin II receptor antagonist) and amlodipine for a major trial studying the effects of irbesartan on morbidity and mortality in hypertensive type 2 diabetic patients with diabetic nephropathy. Thirty-six patients underwent renal biopsy to confirm a diagnosis of diabetic glomerulopathy. The light microscopic appearances have been reported and showed differences in the clinical features between those with nodular *versus* diffuse glomerulosclerosis (6). We report here the results of morphometric analysis performed at the electron microscopic level in the patients in whom there were at least three analyzable glomeruli, and describe the relationships between ultrastructural appearances and renal function.

Materials and Methods

Study Subjects

Forty-seven patients with type 2 diabetes, hypertension, and proteinuria were entered into the study from 10 collaborating clinics. The inclusion and exclusion criteria were defined to select a population of type 2 diabetic patients whose proteinuria was likely to be the result of diabetic nephropathy. Inclusion criteria were a 24-h urine protein excretion ≥500 mg/24 h, a serum creatinine <266 μmol/L (3.0 mg/dl), and either already receiving antihypertensive treatment or having a diastolic BP 90 to 110 mmHg and/or systolic BP 140 to 185 mmHg. Type 2 diabetes was defined by the absence of a history of ketoacidosis and the presence of one of the following conditions: hyperglycaemia requiring treatment with an oral hypoglycemic agent, hyperglycemia requiring treatment with insulin and fasting C-peptide level of >0.1 pmol/ml that at least doubles 90 min post-mixed meal (*e.g.*, Sustacal), or treatment with diet and fasting plasma glucose ≥7.8 mmol/L (140 mg/dl) on two occasions. All patients satisfying

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these criteria during a single examination were eligible for the study. Patients were excluded if they were less than 20 yr old at the onset of diabetes, had uncontrolled diabetes mellitus ($HbA_{1c} > 10.5\%$), had lens opacities that precluded visualization of the retina, or had a history of cardiovascular disease, congestive heart failure, arrhythmias, or cerebrovascular disease. Women of childbearing potential were also excluded.

This study was performed in accordance with the guidelines proposed in the Declaration of Helsinki. Approval was obtained from local independent review bodies, and informed signed consent was obtained from each patient.

The patients were randomized to receive either irbesartan or amlodipine for 14 wk. Percutaneous renal biopsies were performed between weeks 12 and 14 on 36 of the 47 patients. Eleven patients did not consent to be biopsied. The biopsies were performed at this stage to ensure that the patient's BP was under optimal control during the biopsy procedure. It was considered extremely unlikely that 12 wk of therapy on an angiotensin II receptor antagonist or calcium channel blocker would have any effect on the pathology of the kidney. Twenty-one patients had sufficient tissue for morphometric analysis using electron microscopy.

Data were also available on renal biopsies obtained from 14 nondiabetic kidney donors (six male) at the time of transplantation (mean age 37 yr; range, 20 to 60). These biopsies had been analyzed previously by the same observer using the same methodology. Light microscopy slides stained with Masson's trichrome, for the measurement of interstitium, were available on a separate group of nondiabetic kidney donors (mean age 49 yr; range, 22 to 68).

Clinical Methods

Blood pressure was measured in the clinic after the patient had sat quietly for at least 10 min. The sitting BP is the mean of three readings taken 1 min apart using a calibrated mercury sphygmomanometer.

All urine passed during a 24-h period was collected for creatinine clearance and urine protein excretion. Values are derived from the average of two collections during the enrollment period. At the completion of each 24-h collection, a blood sample for serum creatinine was obtained for the calculation of creatinine clearance. All laboratory parameters were analyzed centrally. Creatinine concentration was determined by the modified Jaffé rate-blanked alkaline picrate method. Urine protein concentration was determined by the benzethonium chloride method.

HbA_{1c} was calculated from total glycated hemoglobin determined by ion capture methodology. The normal range was $<6.5\%$.

Laboratory Methods

Biopsy material was processed for light, fluorescence, and electron microscopy. Paraffin-embedded tissue was sectioned and treated with a variety of histologic stains. The total biopsy series examined by light microscopy has been described in detail elsewhere (6). Sections stained with Masson's trichrome and periodic acid-Schiff (PAS) were available for this study.

Tissue for electron microscopy was fixed in glutaraldehyde, postfixed in osmium tetraoxide, and embedded in Epon. Semithin (1 μm) sections were taken through the tissue block at 10-μm intervals and stained with 1% toluidine blue. Open, nonoccluded glomeruli were identified by light microscopy, and the first three such glomeruli were sampled for electron microscopy by taking ultrathin sections at 50 μm, or multiples thereof, from the baseline section of the block. This systematic sectioning ensured that glomeruli were sampled independently of their size and resulted in three to five profiles per glomerulus. For each biopsy, the second glomerular profile from each of the three glomeruli was stained with uranyl acetate and lead citrate and examined using a Philips CM100 electron microscope.

Light Microscopy

Light microscopy was used to determine the percentage of occluded glomeruli and the volume fraction of interstitium in cortex, and to obtain an estimate of glomerular volume.

The percentage of occluded glomeruli was estimated from a combination of the serially sectioned blocks stained with toluidine blue and the single sections stained with PAS. A glomerulus was said to be occluded when there were no clearly identifiable capillaries within the profile. At least 15 glomeruli per biopsy had to be counted for the measurement to be considered valid. The actual number of glomeruli counted for each biopsy ranged from 15 to 76 (mean 35).

Interstitial volume fraction was estimated by point counting on the sections stained with Masson's trichrome. The interstitium was defined as the portion of cortex not composed of glomeruli, tubules, arteries, arterioles, or large veins, but including capillaries and small veins (7). A grid of coarse and fine points (ratio 1:4) was superimposed on the section at a magnification of $\times$360 using a drawing tube attachment. Coarse points landing on cortex and fine points landing on interstitium (as defined above), glomeruli, and vessels (arteries, arterioles, and large veins) were counted. The interstitium was expressed as a fraction of the cortex and also as a fraction of the cortex minus the glomeruli and vessels, *i.e.*, the tubulointerstitium.

Glomerular volume was estimated from the PAS-stained sections by the method described by Weibel and Gomez (8,9). Only complete, nonoccluded glomeruli were sampled. The Weibel and Gomez estimate requires a sample size of at least 15 glomeruli (8) and preferably >30 (10). Unfortunately, few of the 21 biopsies met these criteria, as only eight biopsies had a sample size of at least 15 glomeruli, and only one had more than 30. Glomerular volume was also estimated from the 1-μm toluidine blue sections by the Cavalieri principle (8,9). However, to obtain a valid estimate, a sample size of at least five complete glomeruli is required (11), which was only fulfilled by three biopsies.

Electron Microscopy

Each glomerulus was photographed and the resulting series of overlapping micrographs was put together to form a montage of the entire profile at a final magnification of approximately $\times$2000. High-power micrographs were obtained by entering the glomerulus randomly and systematically sampling 25 to 30% of the glomerular tuft area (2,12). These micrographs were printed at a final magnification of approximately $\times$10,000. Actual magnifications were determined by photographing a calibration grid at the same time.

Using a test grid of coarse and fine points in a ratio of 1:8, mesangial volume fraction was estimated from the montages using standard stereologic techniques. Briefly, the volume fractions were obtained by counting fine points falling on mesangial tissue and expressing these as a fraction of the total coarse points falling on the glomerulus, the boundary of the glomerulus being defined by the minimal string polygon enclosing the glomerular tuft (2).

The higher magnification micrographs were used to estimate GBM width (using the orthogonal intercept method (12,13); volume fraction of matrix to glomerulus (by point counting); and foot process width (FPW). For FPW, a grid of intersecting lines was placed over each micrograph. The number of slits between foot processes on the peripheral basement membrane or mesangial-urinary interface were counted along with the number of intercepts of the test line with the

relevant epithelial surface. Mean FPW was then calculated from the ratio of surface density (S_V) to slit length density (L_V) (14):

$$\text{Mean FPW} = S_v/L_v = I \times \text{Line length}/2 \times Q \times \text{Magnification}$$

where I is the number of intercepts, and Q is the number of slits.

Nondiabetic control tissue was obtained from subjects younger than our diabetic patients, but age has a relatively small impact on GBM width and no effect on mesangial volumes (15).

Statistical Analyses

Values for total proteinuria were not normally distributed and were logarithmically transformed. Analysis was carried out using the Statistical Package for the Social Sciences, version 6.1. Comparisons between groups were performed using *t* test. Relationships between parameters were analyzed using Pearson's correlation coefficient. Stepwise linear regression was performed using total protein or creatinine clearance as the outcome variable and adding mesangial volume fraction, GBM width, FPW, and interstitial volume fraction into the model. A two-tailed P value <0.05 was considered statistically significant.

Results

The clinical features of the 21 patients in whom there was sufficient biopsy material for electron microscopy analysis are shown in Table 1, together with the characteristics for the whole group of 47. There were no significant differences between the subgroup of 21 and the total cohort of 47 subjects.

All patients had appearances consistent with diabetic glomerulopathy, with an increase in mesangial and matrix volume fractions together with GBM thickening (Table 2). The percentage of occluded glomeruli ranged from 6 to 68% (mean 32%). The mean values for mesangial volume fraction, matrix volume fraction, GBM width, and FPW are clearly greater in the type 2 patients than in the nondiabetic subjects. The coefficients of variation for these parameters, although greater than in the healthy subjects, are less than previously reported in type 2 patients, and similar to results in type 1 patients (2,16).

Correlations between structural and functional parameters are given in Table 3. The correlations between mesangial volume fraction and total proteinuria (Figure 1); mesangial volume fraction and creatinine clearance (Figure 2); matrix volume fraction and total proteinuria; matrix volume fraction and creatinine clearance; and GBM width and total proteinuria (Figure 3) are consistent with previously published data in type 1 patients (1,3,17). There were no significant correlations between age and any structural parameter in the nondiabetic group.

Mean FPW on the mesangial surface was greatly increased compared with nondiabetic subjects (mean ± SD: 1330 ± 536 *versus* 777 ± 129 nm, $P = 0.001$) and correlated with total proteinuria ($r = 0.60$, $P = 0.004$). There was no correlation between proteinuria and FPW on the peripheral basement membrane. The volume fraction of interstitium was also greatly increased compared with the nondiabetic subjects (mean ± SD: 0.32 ± 0.07 *versus* 0.19 ± 0.06, $P < 0.001$) and correlated with known duration of diabetes and creatinine clearance (Figure 4) but not with proteinuria, the percentage of occluded glomeruli, or age.

Multiple regression analysis revealed mesangial volume fraction only as a structural correlate to proteinuria ($r = 0.66$, $P = 0.002$), and the addition of the other variables did not add to the relationship. However, creatinine clearance was related to both mesangial and interstitial volume fractions ($r = 0.73$, $P = 0.002$), but again, GBM width and FPW did not contribute further.

Biopsies had been classified previously at the light microscopic level as diffuse or nodular glomerulosclerosis (6). In our cohort of 21 out of the original 36 patients, eight were defined as diffuse and 13 as nodular. Patients with nodules had greater mesangial and matrix volume fractions and more proteinuria than those defined as diffuse, although the structural values for the diffuse patients are still very abnormal compared with nondiabetic subjects. Creatinine clearance was lower in the patients with nodules, but this difference was not statistically significant ($P = 0.053$) (Table 4). Examination of the tissue that had been serially sectioned and stained with toluidine blue

Table 1. Clinical characteristics of 47 type 2 diabetic patients with nephropathy and the 21 patients from the cohort who had analyzable biopsy material[a]

Characteristic	Whole Group	Biopsy Group
n [M/F]	47 32/15	21 15/6
Age (yr)	59 (35 to 75)	59 (43 to 75)
Known duration of diabetes (yr)	15 (0.9 to 45)	16 (0.9 to 45)
Total proteinuria (g/24 h)*	2.4 (0.5 to 18.4)	1.2 (0.9 to 12.7)
Creatinine clearance (ml/min)	69 (24 to 198)	65 (26 to 166)
SBP (mmHg)	159 (136 to 188)	157 (136 to 188)
DBP (mmHg)	87 (60 to 109)	85 (60 to 99)
HbA_{1c} (%)	8.6 (6.2 to 10.4)	8.6 (6.2 to 10.3)
Retinopathy status (n/np/p)	14/22/9	6/7/7

[a] Data are expressed as mean (range) or * median (range). SBP, systolic blood pressure; DBP, diastolic blood pressure. Retinopathy status; n, none; np, nonproliferative; p, proliferative. Retinopathy status was not available in two of the 47 patients (one of whom was in the biopsy group).

Table 2. Structural characteristics of 21 type 2 diabetic patients with nephropathy *versus* 14 non-diabetic subjects[a]

Variable	Type 2	Nondiabetic	*P* Value
VvMes/Glom	0.45 (0.29)	0.18 (0.17)	<0.001
VvMat/Glom	0.28 (0.32)	0.10 (0.21)	<0.001
VvInterstitium/tubulointerstitium	0.32 (0.22)	0.19 (0.32)	<0.001
GBM width (nm)	665 (0.21)	361 (0.14)	<0.001
FPWpbm (nm)	892 (0.33)	595 (0.20)	0.001
FPWmes (nm)	1330 (0.40)	777 (0.17)	0.001

[a] Data are expressed as mean values and coefficients of variation (CV = SD/Mean) in parentheses. All structural characteristics are significantly different between the groups. VvMes/Glom, volume fraction of mesangium; VvMat/Glom, volume fraction of matrix; GBM, glomerular basement membrane; VvInterstitium/tubulointerstitium, volume fraction of interstitium; FPWpbm, foot process width on the peripheral basement membrane; FPWmes, foot process width on the mesangial surface.

Table 3. Structural–functional correlations in 21 type 2 diabetic patients with nephropathy[a]

Variable	Total Proteinuria		Creatinine Clearance	
	r	*P* Value	*r*	*P* Value
VvMes/Glom	0.64	0.002	−0.58	0.006
VvMat/Glom	0.65	0.001	−0.58	0.006
GBM width	0.58	0.006	−0.23	0.224
FPWmes	0.60	0.004	−0.25	0.273
VvInterstitium/tubulointerstitium	−0.10	0.689	−0.58	0.008

[a] Mesangial and matrix volume fractions correlate with both total proteinuria and creatinine clearance. GBM width and FPWmes correlate with total proteinuria. Interstitial volume fraction correlates with creatinine clearance. FPWmes, foot process width on mesangial-urinary surface. Other abbreviations as in Table 2.

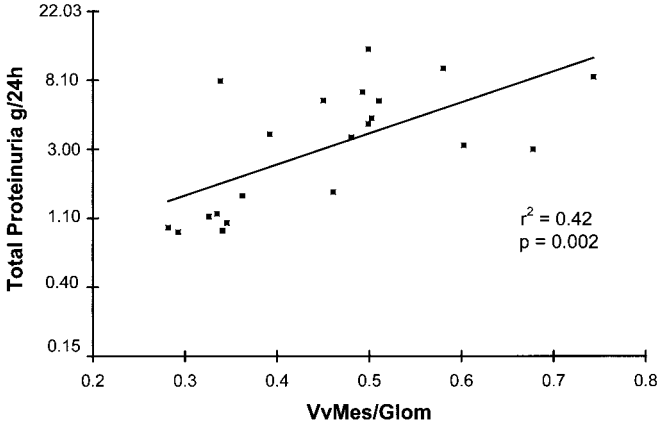

Figure 1. Total proteinuria (g/24 h) (logarithmically transformed, y axis) against mesangial volume fraction (x axis) for 21 type 2 diabetic patients with nephropathy. Pearson's $r^2 = 0.42$, $P = 0.002$.

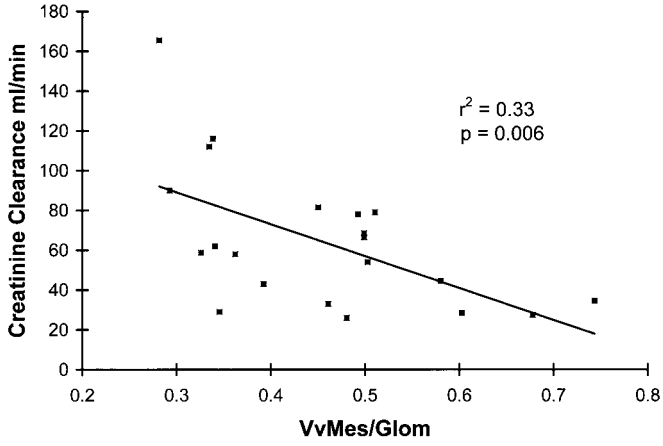

Figure 2. Creatinine clearance (ml/min) (y axis) against mesangial volume fraction (x axis) for 21 type 2 diabetic patients with nephropathy. Pearson's $r^2 = 0.33$, $P = 0.006$.

showed that one out of the eight (12.5%) biopsies that had been classified as having diffuse glomerulosclerosis had nodules in at least one glomerulus.

Only eight patients had >15 glomerular profiles available for the estimate of glomerular volume using the Weibel and Gomez method. The results showed a wide range of values from 1.44 to 6.74 $\times 10^6$ μm^3, which compares with previously published nondiabetic values of $<1.5 \times 10^6$ μm^3 (8).

Discussion

This group of type 2 diabetic patients shows classic changes of diabetic glomerulopathy: an increase in mesangial and matrix volume fractions and GBM width. These are consistent with previously published data (16,18), although our lower coefficients of variation indicate that there may be less heterogeneity than was previously thought. The observed correla-

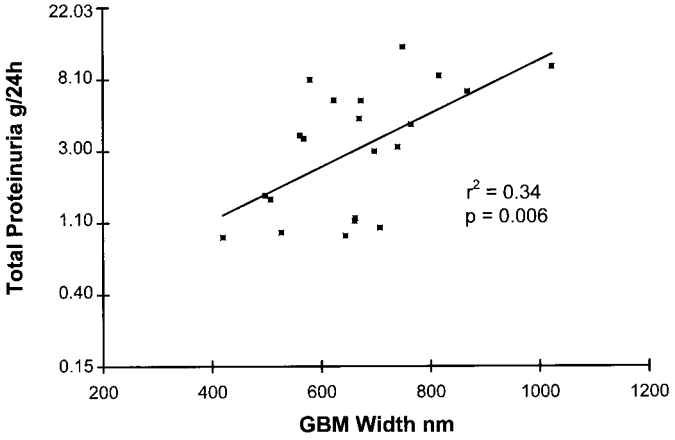

Figure 3. Total proteinuria (g/24 h) (logarithmically transformed, y axis) against glomerular basement membrane width (nm) (x axis) for 21 type 2 diabetic patients with nephropathy. Pearson's $r^2 = 0.34$, $P = 0.006$.

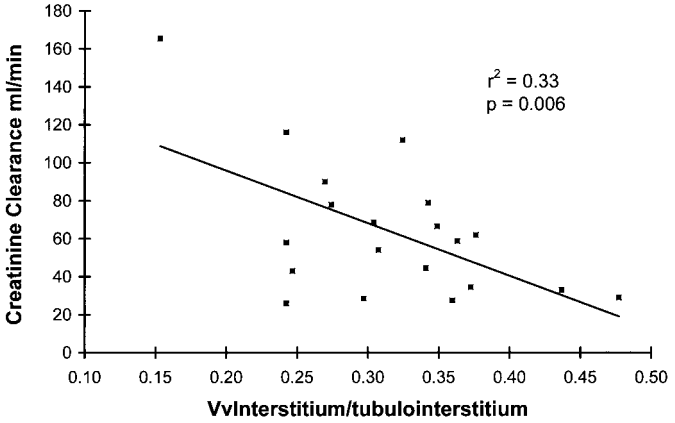

Figure 4. Creatinine clearance (ml/min) (y axis) against interstitial volume fraction (x axis) for 20 type 2 diabetic patients with nephropathy. Pearson's $r^2 = 0.33$, $P = 0.008$. Interstitial volume fraction was unable to be measured in one patient.

tions of the structural parameters with total proteinuria and creatinine clearance are also consistent with earlier findings in type 1 diabetes (7,14,17) (Table 5). The similar strength of the correlations between glomerular and tubular structure and function is consistent with a common pathogenesis of the lesions in the two main types of diabetes.

An increase in renal interstitium is considered an indication of disease progression in diabetic nephropathy and has been shown to correlate with GFR and AER in type 1 diabetes (7). In type 2 diabetes, reported results are inconsistent. Cordonnier *et al.* (19) showed a correlation between the interstitial volume fraction and AER but not creatinine clearance, whereas in our study we found a correlation with creatinine clearance but not proteinuria. In another study, Østerby *et al.* (16) found no significant difference in the volume fraction of interstitium between type 2 patients and control subjects, although there was a correlation between interstitium and percentage of occluded glomeruli. A previous study in type 1 diabetes reported

Table 4. Comparison of 21 type 2 biopsies that have been classified as having either diffuse or nodular glomerulosclerosis[a]

Variable	Diffuse ($n = 8$)	Nodular ($n = 13$)	P Value
VvMes/Glom	0.36 ± 0.08	0.51 ± 0.12	0.007
VvMat/Glom	0.21 ± 0.05	0.32 ± 0.08	0.003
TP g/24 h*	1.1 (0.9 to 12.7)	4.3 (1.1 to 9.6)	0.027
CC ml/min	83 ± 41	53 ± 26	0.053

[a] Data are expressed as mean ± SD or * median (range). Patients with nodules have greater mesangial and matrix volume fractions and more proteinuria than those with diffuse glomerulosclerosis. Creatinine clearance did not differ significantly between the groups. TP, total proteinuria; CC, creatinine clearance.

a correlation between mesangial and interstitial volume fractions (7). We did not find this relationship in our group, suggesting that although both mesangial and interstitial expansion contribute to the decline in renal function, they may be independent factors in the progression of diabetic nephropathy. The result of multiple regression analysis in our patients supports this interpretation and implies an important role of the interstitium in determining creatinine clearance in type 2 patients with nephropathy, whereas the major structural correlate of proteinuria remains mesangial expansion with a negligible contribution from GBM thickening (20).

Other studies have shown correlations between the percentage of occluded glomeruli and both GFR (1) and volume fraction of interstitium (16). Our group of patients does not show these correlations. It is possible that in previous studies these relationships are being driven by a number of biopsies that do not contain any occluded glomeruli at all (1). In our series, there were no biopsies without occluded glomeruli and only one with less than 10% occlusion. Some caution must be applied to our estimate of the percentage of occluded glomeruli, however, as they are generally smaller and therefore less likely to be sampled on a single section. An estimate derived from tissue that has been serially sectioned does not have the same uncertainty, as each glomerulus is sampled independently of size. Our estimate is based on a combination of both sampling strategies, with a minimum sample of 15 glomerular profiles.

The positive correlation between proteinuria and FPW on the mesangial-urinary interface is consistent with a previous study (14). It is possible that mesangial matrix accumulation may lead to an increase in macromolecular leakage into the urinary space across the mesangium and that the relationship with FPW is a result of this process. However, we cannot draw any conclusions about causation from this cross-sectional study.

Although our data on glomerular volume have to be treated with caution, the presence of some very large glomeruli is consistent with previous reports in type 2 patients (16,21). Our data contrast with those reported in a cohort of type 1 patients with nephropathy and nondiabetic control subjects using a

Table 5. Correlation coefficients from structural–functional relationships in 21 type 2 diabetic patients with nephropathy compared with those from published studies in type 1 subjects[a]

Variable	Proteinuria		Creatinine Clearance	
	Type 1	Type 2	Type 1	Type 2
VvMes/Glom	0.63[b]	0.64	−0.51[b]	−0.58
VvMat/Glom	0.63[b]	0.65	−0.53[b]	−0.58
GBM width	0.58[b]	0.58		
FPWmes	0.59[c]	0.60		
VvInterstitium/tubulointerstitium			−0.49[d]	−0.58

[a] Structural parameters show similarly strong correlations with proteinuria and creatinine clearance in both type 1 and type 2 diabetes. FPWmes, foot process width on mesangial-urinary surface. Other abbreviations as in Table 2.
[b] Steffes (17).
[c] Bjørn (14).
[d] Lane (7).

similar methodology. Although nephropathic patients with both type 1 and type 2 diabetes had much larger glomeruli than control subjects, some of our study population had mean volumes three times greater than those reported in type 1 (22). Whether such enlargement is an adaptive response or a deleterious factor is something that has long been debated (23). The increase in size may simply be a consequence of mesangial expansion and be of no benefit to the glomerulus. However, there may also be a compensatory mechanism involved in which the glomerulus grows to try to maintain filtration surface area in the face of an expanding mesangium (22). Glomerular enlargement may also occur as a result of glomerular occlusion—the remnant glomeruli attempting to compensate for a declining filtration surface (16). If this proves to be the case, the capacity for greater glomerular enlargement may explain why some type 2 patients with nephropathy have a slower clinical progression to end-stage renal failure than their type 1 counterparts. These questions require prospective study.

In conclusion, many of the relationships between glomerular structure and function that we have described confirm those previously found in type 1 diabetes. However, the significant correlation between an increase in interstitial volume fraction and lower creatinine clearance is noteworthy, as this part of the kidney is now perceived as playing a major role in determining the rate of progression of nephropathy. Glomerular hypertrophy in diabetic nephropathy remains an area that requires further exploration.

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Appendix

The Collaborative Study Group

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