

Altered Transcapillary Escape of Albumin and Microalbuminuria Reflects Two Different Pathogenetic Mechanisms

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We studied the following in normo- and microalbuminuric hypertensive type 2 diabetic patients: 1) transcapillary escape rate of albumin (TERalb) and 2) expression of mRNA slit diaphragm and podocyte proteins in renal biopsies. Normoalbuminuric subjects had renal cancer, and kidney biopsy was performed during surgery. TERalb was evaluated by clearance of ¹²⁵I-albumin. Real-time PCR of mRNA slit diaphragm was measured in kidney specimens. Albumin excretion rate (AER) was by definition lower in normoalbuminuric subjects than in microalbuminuric subjects with typical diabetic glomerulopathy (group 1), in microalbuminuric subjects with normal or near-normal glomerular structure (group 2), and in microalbuminuric subjects with atypical diabetic nephropathy (group 3). This classification was based on light microscopy analysis of renal tissue. TERalb (%/h) was similar in normoalbuminuric and microalbuminuric group 1, 2, and 3 diabetic patients (medians: 14.1 vs. 14.4 vs. 15.7 vs. 14.9, respectively) (ANOVA, NS). mRNA expression of slit diaphragm proteins CD2AP, FAT, Actn 4, NPHS1, and NPHS2 was higher in normoalbuminuric patients than in microalbuminuric patients (groups 1, 2, and 3) (ANOVA, $P < 0.001$). All diabetic patients had greater carotid artery intimal thickness than normal control subjects using ultrasound technique (ANOVA, $P < 0.01$). In conclusion, the present study suggests that microalbuminuria identifies a subgroup of hypertensive type 2 diabetic patients who have altered mRNA expression of slit diaphragm and podocyte proteins, even before glomerular structure shows abnormalities using light microscopy analysis. On the contrary, altered TERalb and increased carotid artery intimal thickness are shown by

all hypertensive type 2 diabetic patients, both with normal and altered patterns of AER. *Diabetes* 54: 228–233, 2005

Deekert et al. (1) suggested some years ago an interesting view to understanding the pathogenesis of albuminuria in diabetes, which has been usually cited as the Steno hypothesis and implies that albuminuria reflects a widespread vascular damage due to a generalized vascular dysfunction in the overall vascular bed. According to this hypothesis, albuminuria may be a marker of generalized disease in the vascular wall, either at endothelial or glomerular basement membrane (GBM) levels or both, although the precise reasons for this relationship are not completely understood.

If the Steno hypothesis really does account for both the leakage of albumin at kidney level and an increased transport of albumin across microvascular capillaries in the overall body, one should predict that transvascular and urinary leakage of albumin are correlated throughout a gamut of pathological conditions both in type 1 and type 2 diabetic patients.

However, this is not the case in previous reports both in type 2 diabetic patients and in nondiabetic atherosclerotic and hypertensive patients, in whom transvascular escape of albumin was similarly abnormal in normoalbuminuric and micro-/macroalbuminuric patients (2,3).

Major attention has been paid in the last 30 years to the role of the GBM as the primary filter capable of maintaining albumin and large proteins into circulation (4). Because GBM is widespread throughout the entire vascular bed of the organism, as well as at the glomerular level in the kidney, it has been usually thought, at least among diabetologists, that the pathogenetic mechanisms leading to abnormalities of albumin excretion rate (AER) in the urine were similar to those responsible for the transcapillary escape rate of albumin through the GBM in the overall organism. More recently, our understanding of albumin permselectivity at the level of the vascular bed became more complex. In fact, it has been shown that albumin permselectivity at the glomerular level is first determined by a coarse filter, i.e., the GBM, which operates throughout the overall vascular bed but is not very efficient and does not tightly maintain albumin into circulation. A direct

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AER, albumin excretion rate; GBM, glomerular basement membrane; TERalb, transcapillary escape rate of albumin.

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measurement of such a physiological phenomenon is provided by the measurement of the transcapillary escape rate of albumin (TERalb) (1–3). However, a second finer filter has been precisely described at the glomerular level, i.e., the podocyte-slit diaphragm structure, which more efficiently determines the permselectivity of albumin in the kidney and therefore AER and operates above the GBM filter (5–7).

These results raise an important question: Is the meaning of abnormalities of TERalb the same as that of microalbuminuria or can the two phenomena be distinguished? More particularly, one can suggest that abnormalities of TERalb might reflect GBM structural abnormalities and, from a wider point of view, widespread endothelial damage irrespective of abnormalities of AER. On the contrary, microalbuminuria might identify a subgroup of patients with lesions at the podocyte and slit diaphragm level, which may be accompanied, but not necessarily, by abnormalities of TERalb. If these assumptions are true, it is more clear why cardiovascular complications are observed in the majority of type 2 diabetic patients, even without microalbuminuria (8,9), whereas microalbuminuria and diabetic nephropathy do develop in only 20–40% of the patients (10).

The aim of the present study was to investigate the relationship between TERalb, AER, and mRNA expression of some slit diaphragm and podocyte proteins in four groups of hypertensive type 2 diabetic patients with or without microalbuminuria. We decided to study hypertensive normoalbuminuric patients because most microalbuminuric patients are hypertensive, and our aim was to have a comparison between groups different only with regard to one variable, i.e., altered AER.

RESEARCH DESIGN AND METHODS

The criteria we used to recruit four groups of type 2 diabetic patients have been previously thoroughly described (11–15). Briefly, we studied subjects with an age between 40 and 65 years with onset of diabetes after the age of 35 years and no insulin need in the first 3 years after diagnosis. All the subjects were hypertensive ($>130/85$ mmHg) and were treated by ACE inhibitors and thiazides. The definition of microalbuminuria was a median between 30 and 300 $\mu\text{g}/\text{mg}$ albumin over creatinine ratio in three consecutive urine specimens, collected on the spot in the outpatient diabetic clinic in the absence of hematuria. Albumin was measured by an immunoturbidimetric technique as previously described (11); urine creatinine was measured by a modified reaction rate kinetic technique, which overcomes the drawback of pseudo creatinine (12).

Intimal thickness of the carotid artery was measured by an ultrasound technique (Hewlett-Packard Sonos 1000). The normal value in our clinic was <0.9 mm.

AER and TERalb were studied after 15 days of withdrawal from antihypertensive therapy. The clinical characteristics of the patients were as follows: 18 normoalbuminuric hypertensive type 2 diabetic patients with no diabetic retinopathy. These patients with type 2 diabetes and hypertension, without microalbuminuria, were referred to us because of the accidental detection of unilateral renal cancer using the ultrasound technique. This is a routine procedure in diabetic patients in our clinic. The diagnosis was further confirmed using computed tomography scanning and examination during surgical removal of the neoplastic kidney. Kidney cancer is two- to threefold more frequently found in obese hypertensive type 2 diabetic patients than in normal subjects in Sardinia and northeastern Italy. Quite often, these patients are normoalbuminuric. Kidney biopsy was performed during surgical removal of the kidney in the contralateral pole opposite to that where the renal cancer was located. The patterns of glomerular and renal structure in these patients were normal, apart from the renal cancer on the opposite pole. The patients with microalbuminuria were divided in three groups: group 1 consisted of 19 microalbuminuric hypertensive type 2 diabetic patients with retinal lesions with typical diabetic glomerulopathy; group 2 consisted of 12 microalbumin-

uric hypertensive type 2 diabetic patients with normal or near-normal glomerular structure; and group 3 consisted of 11 microalbuminuric hypertensive type 2 diabetic patients with atypical patterns of renal lesions.

Patterns of glomerular structure in microalbuminuric patients. Percutaneous kidney biopsies were performed under ultrasound guidance in microalbuminuric patients. Tissue was examined under a dissecting microscope to make sure there was an adequate number of glomeruli in the kidney specimen. A 1-mm core was immediately snap-frozen in liquid nitrogen for immunofluorescence. A second fraction was placed in Zenker's fixative embedded in paraffin and processed for light microscopy. A third fraction was immediately snap-frozen in liquid nitrogen and used for the evaluation of podocyte and slit diaphragm protein gene expression using RT-PCR. The patterns of renal lesions in the 42 type 2 diabetic patients with hypertension and microalbuminuria were classified into the following groups using the electron microscopy technique.

Group 1: Typical diabetic nephropathy. These biopsies showed established diabetic glomerulopathy with proportionally severe tubulo-interstitial and arteriolar changes. This picture is typical of that seen in type 1 diabetic patients. These patients had proliferative diabetic retinopathy.

Group 2: Normal or near-normal renal structure. These biopsies showed normal glomerular structure or very mild mesangial expansion, tubulo-interstitial changes, or arteriolar hyalinosis. These patients had no diabetic retinopathy (neither proliferative nor background retinopathy).

Group 3: Atypical patterns of renal injury. These patients had absent or only mild glomerular diabetic changes with disproportionately severe renal structural lesions including tubulo-interstitial lesions, advanced glomerular arteriolar hyalinosis, and/or global glomerular sclerosis ($>25\%$) in the presence of absent or mild mesangial expansion (index of mesangial expansion <0.5 according to the score system described by Mauer et al. [16] and Fioretto et al. [13]). The evaluation of the 2- μm -thick sections, stained with hematoxylin and eosin and periodic acid Schiff, was performed by A.S. and R.F. independently and blindly from the other authors. These patients had no diabetic retinopathy (neither proliferative nor background retinopathy) (11,13–15).

In all these patients, we did not find cases of any nondiabetic renal disease, as defined by clinical and biochemical parameters. Immunofluorescence microscopy studies using rabbit or goat antisera, specifically reactive to human IgA, IgG, IgM, fibrinogen, C3, C4, and C1q did not show any abnormality. The study was approved by the Ethical Committee of the University of Sassari, Milan; San Raffaele University; Milan San Carlo Borromeo Hospital; and CNR National Research Center, Rome, Italy.

mRNA expression of slit diaphragm proteins evaluated by RT-PCR. mRNA has been extracted from kidney biopsies using the guanidium thiocyanate phenol-chloroform method with RNA STAT –60. A double extraction was routinely used to eliminate small amounts of DNA contamination. After extraction, the ratio of 18S to 28S RNA was used as a quality indicator, and only samples of high quality were used. From the extracted RNA, single-strand cDNA was obtained using reverse transcriptase (Superscript II) and random hexamer primers. From web-available sequences, we designed PCR amplification primers and probes for each gene, which have been analyzed in an Applied Biosystem analyzer (ABI 7900) in the presence of a standard to calibrate the reactions. The number of samples has been decided on the basis of a cutoff point of 5–10 times the interassay differences between the average value of three different experiments from each intra-assay sample. The RT-PCR contained the following: buffer, 200 $\mu\text{mol}/\text{l}$ dNTP, 5.5 mmol/l MgCl₂, 100 nmol/l forward and reverse primers, 50 nmol/l probe, 0.05 units/ μl AmpliTaqGold, and 5 μl cDNA. The thermocycler protocol consisted of 40 cycles (15' at 95° and 1' at 60° each). 18S RNA was chosen as an internal standard. The internal standard (18S) and the selected genes were analyzed for any single sample in the same batch. The precision of this assay, as assessed by repeat measurements on the same sample, yields a coefficient of variation of 10–15% for all the gene products we have tested. The mean of three independent experiments was used. All PCR products were confirmed to be the expected cDNA by subcloning the PCR product using the PGEM-T vector system (Promega, Madison, WI, and Milan, Italy) and then sequencing the cloned fragments (17–19).

For the selected genes (CD2AP, FAT, Actn 4, NPHS1, and NPHS2), we calculated their relative expression in comparison to the internal standard (18S RNA) in each sample. After correction for the 18S the amount of mRNA was corrected in each single sample also in comparison to the expression of the same gene measured in a pool of RNA obtained from eight nondiabetic subjects (control) undergoing surgical nephrectomy for renal cancer (biopsy obtained from the opposite kidney pole during surgery). From the computer-generated plot of amplification during the RT-PCR, a threshold line was arbitrarily drawn in a position where, in all samples, both internal standard (18S) and the genes under evaluation were in the exponential phase. This

TABLE 1

Age, duration of disease, HbA_{1c}, glomerular filtration rate, blood pressure, cholesterol, and triglyceride circulating levels

	<i>n</i>	Age (years)	Duration of disease (years)	HbA _{1c} (%)	GFR (ml · min ⁻¹ · 1.73 m ⁻²)	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)	Cholesterol (mg/dl)	Triglycerides (mg/dl)
Normoalbuminuric type 2 diabetic subjects	18	58 ± 5	11 ± 2	8.1 ± 0.7	95 ± 7	175 ± 11	97 ± 3	22 ± 23	123 ± 15
Microalbuminuric type 2 diabetic subjects (group 1)	19	60 ± 8	12 ± 3	8.2 ± 0.9	94 ± 9	170 ± 13	98 ± 4	222 ± 28	171 ± 20*
Microalbuminuric type 2 diabetic subjects (group 2)	12	59 ± 4	10 ± 3	8.4 ± 0.8	91 ± 8	165 ± 11	95 ± 3	235 ± 19	145 ± 19
Microalbuminuric type 2 diabetic subjects (group 3)	11	57 ± 6	13 ± 2	7.9 ± 0.6	96 ± 6	173 ± 10	95 ± 4	230 ± 15	180 ± 21†

Data are means ± SE. **P* < 0.05 for micro- vs. normoalbuminuric subjects; †*P* < 0.01.

threshold line identifies in the exponential part of the plot a cycle that is a direct function of the original amount of mRNA in the sample, called cycle threshold (Ct). Correction for the internal standard and control sample is done following the formula: Ct gene - Ct 18S = ΔCt then ΔCt sample - ΔCt control = ΔΔCt. The relative expression of the sample under analysis for each gene is finally calculated as $2^{-\Delta\Delta Ct}$. Data are then expressed for the three groups of type 2 diabetic patients and statistically compared (17–19). A separate set of experiments was repeated with a pool of RNA obtained from the three different groups and compared; these latter data are not shown, but they confirmed the data shown as mean values plus SD reported. Preliminary data on this methodology has been previously presented (20).

Evaluation of glomerular filtration rate, transcapillary escape of albumin from the clearance rate of ⁵¹Cr-EDTA and ¹²⁵I-albumin, and carotid artery thickness using an ultrasound technique. After an overnight fast on two different occasions separated by at least 40–50 days at 8:00 A.M., each patient drank one-half liter of water. A Venflo cannula was inserted into the antecubital vein for tracer injection. For sample collection, a second cannula was placed into the antecubital vein of the opposite arm. At 9:00 A.M., a bolus injection of 1 μgCi/kg of ⁵¹Cr-EDTA was given over 30 s. Seven samples were collected at 5, 12.5, 50, 80, 180, 240, and 300 min, with time zero being the mid-point of the infusion interval. Further details have been provided elsewhere (21).

Patients were treated by iodine orally during the week preceding the bolus administration of radioactive iodine to prevent any uptake of ¹²⁵I-iodine by the thyroid gland. ¹²⁵I-albumin human serum albumin (7–8 μCi, 252–296 kBq, SARI-125 A-2; Sorin Biomedica) was injected as a bolus, and blood was withdrawn from the contralateral arm every 5 min during the hour after the injection. Radiolabeled albumin was separated from free ¹²⁵I, eluting the high-molecular weight components into columns containing Sephadex G-25 mol/l (column PD-10; Pharmacia); free ¹²⁵I content in the injected dose was <1%. Radioactivity was measured (Cobra 5000 gamma counter; Hewlett-Packard) in duplicate in whole blood samples centrifuged for 10 min at 3,000g. Counting time was 90 min with percent error <15%; hematocrit level was determined in each sample by standard methods. Albumin was measured by

immunonephelometry in the serum (Behring Laser Nephelometer System; interassay variation coefficient, 4.7%). The transcapillary escape rate was expressed as the percentage decay per hour (%/h) (TERalb). The plasma radioactivity was expressed as counts per minute per gram total plasma protein to overcome the drawback of small plasma volume changes during the 1-h sampling period. TERalb measurements were accepted only if the coefficient of correlation between the time points for blood collection and the corresponding values of a specific radioactivity exceeded 0.90. Two patients were excluded from the study because they did not meet these criteria. Thus, we recruited 60 patients and reported in the present study the data of 58 patients.

Statistical analysis. Data are reported as means plus SE or SD when normally distributed and as medians with ranges when not normally distributed. Differences among means were tested by ANOVA, correcting the data with the Bonferroni test and then using the unpaired Student's *t* test for parametric parameters and the Wilcoxon's test for nonparametric parameters using the software SPSS analysis approach.

RESULTS

The demographic characteristics of the hypertensive type 2 diabetic patients with normoalbuminuria and microalbuminuria with the typical diabetic nephropathy (group 1), with normal or near-normal glomerular structure (group 2), and with atypical diabetic nephropathy (group 3) are described in Table 1. The four groups were well matched as for age, duration of the disease, HbA_{1c}, blood pressure, glomerular filtration rate, and cholesterol values. Group 1 and 3 microalbuminuric patients had slightly higher triglyceride levels.

AER was by definition significantly higher in microalbu-

TABLE 2

Ratio between albumin and creatinine in the urine of TERalb (%) and carotid artery thickness in hypertensive normoalbuminuric and hypertensive microalbuminuric type 2 diabetic patients

	<i>n</i>	Albumin-to-creatinine ratio (μg/mg)	TERalb (%)	Carotid artery thickness (mm)
Normoalbuminuric type 2 diabetic subjects	18	7 (2–19)	14.1 (9.1–19.2)	1.8 (1.1–3.3)
Microalbuminuric type 2 diabetic subjects (group 1)	19	49 (35–257)*	14.4 (8.0–20.2)	1.7 (1.2–2.5)
Microalbuminuric type 2 diabetic subjects (group 2)	12	53 (41–269)*	15.7 (7.9–21.3)	1.9 (1.3–3.3)
Microalbuminuric type 2 diabetic subjects (group 3)	11	55 (38–241)*	14.9 (7.8–21.4)	2.1 (1.4–3.4)

Data are medians (range). **P* < 0.001 for micro- vs. normoalbuminuric subjects.

TABLE 3

mRNA expression of slit-diaphragm proteins in specimens from kidney biopsy from hypertensive normoalbuminuric and hypertensive and microalbuminuric type 2 diabetic subjects, expressed as the percentage of each protein (CD2AP, FAT, Actn 4, NPHS1, and NPHS2) of the 18S mRNA expression (internal control)

	<i>n</i>	CD2AP	FAT	Actn 4	NPHS1	NPHS2
Normoalbuminuric type 2 diabetic subjects	18	2.34 ± 0.18	1.33 ± 0.11	1.41 ± 0.25	1.13 ± 0.21	1.01 ± 0.13
Microalbuminuric type 2 diabetic subjects (group 1)	19	0.046 ± 0.042*	0.018 ± 0.010*	0.015 ± 0.009*	0.007 ± 0.006*	0.011 ± 0.010*
Microalbuminuric type 2 diabetic subjects (group 2)	12	0.079 ± 0.037†‡	0.019 ± 0.011*	0.021 ± 0.010*	0.049 ± 0.011†‡	0.019 ± 0.010§
Microalbuminuric type 2 diabetic subjects (group 3)	11	0.059 ± 0.033§	0.022 ± 0.009*	0.024 ± 0.01*	0.011 ± 0.007*	0.024 ± 0.009§

Data are means ± SD. Group 1, typical diabetic nephropathy; group 2, normal glomerular structure; and group 3, atypical diabetic nephropathy. * $P < 0.001$ for normo- vs. microalbuminuric group 1; † $P < 0.05$ for normo- vs. microalbuminuric group 3; ‡ $P < 0.05$ for microalbuminuric group 2 vs. microalbuminuric groups 1 and 3; § $P < 0.01$ for normo- vs. microalbuminuric group 2.

minuric patients than in diabetic patients with normoalbuminuria (ANOVA, $P < 0.01$, Table 2). TERalb (%/h) was similar in group 1, 2, and 3 microalbuminuric and normoalbuminuric type 2 diabetic patients (ANOVA, NS).

Table 3 shows the means ± 1 SD of the values of some slit diaphragm mRNA expression in specimens from kidney biopsy of normoalbuminuric and microalbuminuric patients (groups 1, 2, and 3). CD2AP, FAT, Actn 4, NPHS1, and NPHS2 mRNA expression, reported as a percentage of the internal standard mRNS 18S, was all significantly lower in microalbuminuric subjects (groups 1 and 3) compared with normoalbuminuric subjects (Table 3). Also, group 2 microalbuminuric patients showed a significantly lower mRNA expression of CD2AP and NPHS1 than normoalbuminuric patients, albeit with a lower degree of significance (Table 3) compared with group 1 and 3 microalbuminuric patients.

Carotid artery intimal thickness was twice as great in both hypertensive normoalbuminuric patients and group 1, 2, and 3 microalbuminuric patients (Table 2) than in normal nondiabetic and normotensive subjects matched for sex and age in our clinic (0.9 mmHg) (319 subjects, 175 men and 144 women, age 65 ± 4 years [mean ± SE]) (ANOVA, $P < 0.01$).

DISCUSSION

The overall leakage of albumin from circulation to the interstitial fluid and to urine is first determined by hydrostatic pressure in the microcirculation, which strongly depends on blood pressure levels. The present study evaluated four groups of type 2 diabetic patients who had similar levels of blood pressure patterns after a 15-day withdrawal from antihypertensive therapy using ACE inhibitors and thiazides. Thus, it is likely that differences in the hydrostatic pressure among the three groups will not explain our findings.

Second, changes in the structure and function of endothelial cells and in the structure and biochemical characteristics of the GBM may also explain the abnormalities of albumin leakage from the vascular bed. The Steno hypothesis (1) suggests that albuminuria reflects widespread vascular damage (retinopathy, increased AER with nephropathy, and macroangiopathy) secondary to a generalized dysfunction and abnormality of endothelial cells and the GBM. The present study reports that no differences were found with regard to TERalb between hypertensive,

microalbuminuric, and normoalbuminuric type 2 diabetic patients. These observations suggest that further pathogenetic mechanisms need to be advocated to explain microalbuminuria, besides endothelial and GBM abnormalities.

The new finding of the present study is that whenever microalbuminuria occurs in type 2 diabetes with arterial hypertension, evidence of abnormalities of slit diaphragm and podocyte protein expression is observed, irrespective of the patterns of the overall leakage of albumin from circulation. In fact, the leakage of albumin from the vascular bed was observed in both normo- and microalbuminuric patients, as was thickening of the intimal wall of the carotid artery. These findings may suggest that abnormalities of TERalb reflect a widespread vascular damage, whereas microalbuminuria occurs only in the cohort of hypertensive type 2 diabetic patients with abnormalities of mRNA expression of several slit diaphragm and podocyte proteins, even if the morphology of glomerular structure is normal or near-normal using light microscopic analysis of kidney specimens.

On the basis of such results, we then tried to answer the following question: Which is the putative nature of the pathogenesis of glomerulopathy in the patients who had microalbuminuria besides altered TERalb?

Previous studies of our research group had demonstrated heterogeneous patterns of glomerular lesions in microalbuminuric type 2 diabetic patients (11,13–15). About 30–40% of the patients had the typical features of diabetic glomerulopathy, usually shown by type 1 diabetic patients, i.e., GBM thickening, mesangial expansion, and hyalinosis of afferent and efferent arterioles. However, ~20–30% of microalbuminuric patients had normal or near-normal glomerular histological patterns, and a further one-third of these patients had atypical patterns of renal injury, i.e., tubulointerstitial lesions, arteriolar hyalinosis, and global glomerular sclerosis (15).

Glomerular diseases quite often result in podocyte effacement and flattening in Pima Indians with diabetes and altered AER (22). Changes in podocyte structure and density occur from the early stages of diabetic nephropathy and might contribute to increasing albuminuria in Caucasian type 2 diabetic patients. Furthermore, it has been suggested that the density of podocytes may be functionally more relevant than their absolute number (23). Recent findings have demonstrated that the slit

diaphragm complex contains several transmembrane proteins such as nephrin, *p*-cadherin, podocin, alfaactinin 4, CD2AP, and FTA (5–7,24), which may account for its zipper-like structure linking laterally the foot process structure, encompassing an isoporous filter above that of the GBM. A breakthrough that has renewed interest in glomerular biology and pathology was the discovery of a membrane protein nephrin as a major component of the slit diaphragm complex (25). Mutations of the nephrin gene *NPHS1* were identified by positional cloning as the pathogenetic cause of familial Finnish nephropathy (5,6,25,26). The *NPHS1* gene product is nephrin, a 1,241-amino acid protein with a large extracellular domain and a single membrane spanning domain, and a cytoplasmatic portion. Additional components are required to assemble the slit diaphragm. Recent evidence indicates that nephrin is a component of lipids rafts that also contains a podocyte-specific ganglioside (26). Also, mutations in the *NPHS2* gene located on human chromosome 1q25-q31 (27), which encodes a 383-amino acid protein named podocin and the Actn 4 (α actinin 4) gene on human chromosome 19q13.1, have been described in other types of nondiabetic proteinuria in humans (28). A further protein that may determine the function of nephrin is the large cadherin homolog FAT, which has been found in the slit diaphragm domain, but its relation to the other proteins located there remains to be determined (29).

More recently, the group of Langham et al. (30) in France observed lower rates of nephrin expression in the kidney biopsies of type 2 diabetic patients with proteinuria. These findings have been confirmed by Doublier et al. (31), who observed that the expression of nephrin in glomeruli of both patients with type 1 diabetes and patients with type 2 diabetes and nephropathy is markedly reduced and altered in its distribution. Preliminary results from our research group (20) and the present study demonstrate for the first time in hypertensive microalbuminuric type 2 diabetic patients, using the RT-PCR technique in kidney specimens from type 2 diabetic patients, a reduced mRNA expression of nephrin and also of other slit diaphragm proteins such as CD2AP, FAT, Actn 4, and *NPHS2*. These abnormalities were not observed in hypertensive normoalbuminuric patients with type 2 diabetes and renal cancer. These observations suggest that abnormalities of the expression not only of nephrin but also of other slit diaphragm proteins may precede the development of glomerular lesions and be an early event in the progression of diabetic nephropathy, thus conditioning the onset of proteinuria. These abnormalities of mRNA expression of slit diaphragm proteins were observed only in patients with microalbuminuria, irrespective of TERalb values.

It has to be pointed out that the patterns of mRNA expression of slit diaphragm proteins were significantly reduced also in the patients with normal or near-normal glomerular structure, albeit slightly less severely than in those with the typical diabetic nephropathy. This latter finding might be explained by our recent observation that a higher activity of superoxide dismutase 1 partially protects the kidney and particularly nephrin expression in the kidney from the challenge of oxidant factors, such as oxidated LDL, which have been shown to be abnormally

increased in 100% of type 2 diabetes with microalbuminuria at the glomerular level (32). On the contrary, this latter abnormality was found only in 8% of the patients with altered AER because of pathologies other than diabetes (32). Further studies are needed to clarify whether a high superoxide dismutase 1 activity characterizes hypertensive microalbuminuric type 2 diabetic patients with normal glomerular structure in comparison with those with typical and atypical glomerulopathy, partially, albeit not completely, protecting the deterioration of the expression of slit diaphragm and podocyte proteins.

In conclusion, the present study demonstrates that microalbuminuria identifies a subgroup of hypertensive type 2 diabetic patients who have altered mRNA expression of slit diaphragm and podocyte proteins, even if glomerular structure appears normal or near-normal using light microscopy analysis. On the contrary, abnormalities of TERalb are observed in the overall population of hypertensive type 2 diabetic patients, irrespective of the patterns of AER, and are closely related to the thickening of the intimal wall of the carotid artery. These findings support the definition of microalbuminuria, adopted by the European Guidelines for Hypertension Diagnosis and Treatment (33), as a marker of target tissue damage at the renal level, besides being a risk factor for cardiovascular events.

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